Ralf Dürrwald

Swine influenza: Epidemiology and pathogenesis

Effects of high-dose aerosol nebulisation of influenza A viruses on influenza pathogenesis and maternal immunity studies

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FOREWORD

This is the second part of my work on swine influenza. It made sense to split the project because otherwise the scope would have been too large. The first part of the monograph described the dynamics of the circulation of swine influenza viruses in Germany[1]. This work was made possible by funding from the German Federal Ministry of Education and Research. In collaboration with the Institute of Virology and Antiviral Therapy at the Friedrich Schiller University of Jena, this funding has enabled publicly accessible experimental infection studies to begin in the course of the development of swine influenza vaccines in cooperation with Impfstoffwerk Dessau Tornau GmbH.

My work has led to the successful development of three swine influenza vaccines (RESPIPORC® FLU in 2003, RESPIPORC® FLU3 in 2010, RESPIPORC® FLUpanH1N1 in 2017), which could fill a third volume, but are not going to be published. In addition to the development of three vaccines against swine influenza, there have been a number of scientific results.

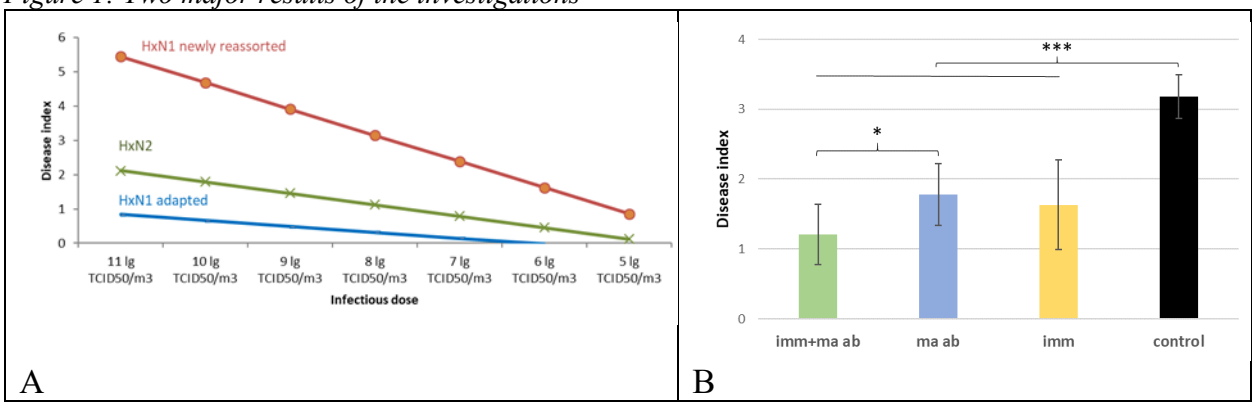
On the one hand, there is the first long-term surveillance of swine influenza in Germany, initiated by me, which was reported in the first part of this monograph[1]. On the other hand, there were so many possibilities for experimental work on swine influenza that they are worth publishing in a second volume.

In this work, animal models for modelling swine influenza are validated. It was shown that the initial exposure dose is a decisive factor for disease induction. Using the established aerosol infection model, the virulence of the influenza viruses can be determined via titrations of the infection dose. The influenza viruses differ in their virulence. Influenza viruses with the N2 neuraminidase show a relatively stable virulence, while freshly reassorted N1 influenza viruses show an extremely high virulence, which they then lose over time (Figure 1 A). Since genetic markers for these virulence differences were not found, morphological peculiarities in the structure of the virus, in particular the cross-linking of haemagglutinin and neuraminidase with host cell membrane proteins, are probably the decisive factor. Neuraminidase activity then determines virulence via the pathways of cytokine induction triggered by its activity in the cell.

The work also includes studies on maternal immunity. For the first time, the interference of maternal immunity with seroconversion after vaccination is investigated in long-term studies. It was demonstrated that this interference can last up to 8 months even in the absence of maternal antibodies. In addition, it was shown in challenge experiments that this interference is not detrimental. Immune priming by vaccination occurs despite the presence of maternal antibodies and the absence of seroconversion after vaccination. In challenge

experiments, maternal immunity and vaccination have been shown to have a synergistic effect (Figure 1 B).

Figure 1: Two major results of the investigations



*A, The data show that influenza viruses differ remarkably in their virulence in pigs when initially exposed to aerosols with high viral load, whereas their virulence is similarly low when pigs are initially exposed to low doses: newly reassorted HxN1 viruses show the highest virulence at high doses, HxN2 viruses show medium virulence and adapted HxN1 viruses show low virulence; B, Vaccination and maternal antibodies have a synergistic effect on infection when piglets are infected with influenza viruses, reducing symptoms to a greater extent than when vaccination or maternal antibodies act alone (shown is the effect on piglets infected at 4 weeks of age, some of which were vaccinated in the presence of maternally-derived antibodies on day 3 of life and 21 days later again - imm+ma ab, some of which were derived from vaccinated sows - ma ab, some of which were derived from unvaccinated sows and vaccinated in the same manner as the other piglets - imm, and some of the piglets were without vaccination and without maternal antibodies - control); *, $p<0.5$; ***, $p<0.001$*

The work was originally planned as a single habilitation thesis. Due to its length, I decided to divide the work into two parts and to declare only the first part as a habilitation thesis.

This volume of the monograph dispenses with an introductory overview of influenza, as this has already been given in part I[1]. Instead, a brief overview of infection models for influenza and maternal immunity is provided. This work is very extensive. The result is the somewhat different structure of this monograph with a long supplement. I could have done without the supplement, but it provides a lot of detail to support the data given in the main text. May one or the other find approaches for his or her academic activity here. The monograph contains the main chapters Introduction, Materials and methods, Results, General discussion, Summary, Acknowledgements, Literature and Supplementary material. Due to the large number of studies, the Results chapter contains short sections on study design (briefly describing the methods of each study) and interpretation of results (discussing the main findings of each study). In addition, the General Discussion chapter contains some figures of major importance that contain results, in order to avoid having to go back to the Results chapter while reading (these figures are not included in the Results chapter and provide additional information). The Results and General discussion chapters contain a summary table at the beginning of each of the three main topics of investigation: Surveillance (volume 1 of this monograph[1]), Pathogenesis, Maternal Immunity, to provide a quick overview of the content. References cited in the supplement are listed in the general

bibliography. Links to information on the Internet were not listed separately in the bibliography and are therefore only included in the text. All links were available on 31.01.2025. The list of abbreviations applies to both parts of the monograph.

Those who want to avoid reading too much can concentrate on the discussion to get the main messages. The special structure of the discussion with supporting figures makes it possible to get the main content of the studies. In the end, there is much that needs further research. Therefore, many hypotheses have been raised in the discussion. May this work give some ideas for further research.

Berlin, 31.01.2025

Ralf Dürrwald

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1. INTRODUCTION

The clinical outcome of influenza A virus infections varies from benign infection without symptoms to lethal disease. The reason for this broad variation in symptoms of mammalian influenza virus infections is not completely known, especially the fatal pandemic of 1918 remains enigmatic[2,3]. Several animal models for influenza have been developed[4-7]. Swine influenza infection models gained new attention with the emergence of the pandemic H1N1 2009 virus. Most experimental infections were done intranasally which is simple to perform but does not reproduce clinical influenza[8-37]. Over the years the intratracheal infection route was also established[38,39]. This enabled the induction of symptoms when higher infection doses were injected into the trachea but is more difficult to execute and the injection can fail[40]. In order to compensate for this a new aerosol-based infection model for swine influenza was established and validated. This model allows for the induction of clinical symptoms and provides new insights into the pathogenesis of swine influenza.

The aerosol infection procedure enables the study of effects of different infection doses on the pathogenesis of influenza. So far no approaches to mimic the 1918 influenza in pigs have been successful. The emergence of a new pandemic virus in 2009 offered the possibility to investigate the effects of such a newly emerged virus in aerosol infection trials.

The model can also be applied for the investigation of maternally-derived immunity. The mechanisms of maternally-derived immunity are not fully understood, especially the interference with antibody induction after immunisation needs further investigation. In order to provide this, long-term investigations were done to investigate this interference and experimental infection trials in piglets were conducted to investigate the influence of maternally-derived immunity on vaccination.

2. OVERVIEW OF INFLUENZA INFECTION MODELS

2.1 EXPERIMENTAL MODELLING OF INFLUENZA

Infection models for influenza contribute significantly to a better understanding of influenza infections. On the one hand, there are infection experiments in humans. These were and are mainly carried out with the aim of better understanding immunological reactions and the efficacy of vaccinations. Since the possibilities of experimental infection in humans are limited, animal models have been and are very important in influenza virus research. Due to the broad infection spectrum of influenza viruses, numerous animal species can be infected[41,42]. Accordingly, there are a wide variety of animal models. Animal models are used to gain a better understanding of the pathogenesis of influenza, to investigate the mechanisms of the immune response, and to test the efficacy of vaccines and antiviral agents[5,6,43-46]. One aim is to draw conclusions from the results for the control and therapy of infections in humans. Another goal is to study pathogenesis and vaccine efficacy in the target animal itself; this applies in particular to equine, porcine, canine and avian influenza. With the emergence of new pandemics and panzootics, such as the $H1_{pdm}N1$ pandemic of 2009 and the H5Nx panzootic starting in 1996, animal models have taken on a new significance. In particular, the use of animal models has shown great advantages in quickly obtaining results that enable the pragmatic implementation of research data to protect humans, especially with the occurrence of H5N1 infections in North American dairy farms and the introduction of the virus into raw milk.

2.1.1 HUMAN AND PRIMATE INFLUENZA

The results of experimental infections of human volunteers have been summerized in several reviews[47-50]. Very interesting is the steep rise in virus shedding in humans within the first 1-3 days after infection which indicates a high replication rate of influenza viruses in humans associated with disease[48]. After a delay of approximately 6 hours infected cells begin to produce influenza viruses; the average life time of infected cells is 11 hours[47]. Dose finding studies in a human challenge model revealed that high doses (10^6-10^7 $TCID_{50}$) of virus are necessary to induce influenza[51,52]. Human infection models and hospital-based human cohort studies were used in order to evaluate antibodies as correlate of infection[53-55]. Influenza A reinfection in human challenge using identical lots of virus revealed sequential infection and clinical evidence in some volunteers raising questions about immune memory responses after infection[56].

Also, infection trials using equine influenza viruses were done in human volunteers reflecting that humans are susceptible to animal influenza viruses[57,58].

Nonhuman primates were used in order to investigate the pathogenesis of severe influenza[4,59,60].

2.1.2 SWINE INFLUENZA MODELLING

Since swine influenza viruses are of great economic importance for pig production but also play a role as zoonotic pathogens, numerous experimental infections with influenza viruses have been carried out in the past. Experimental modelling of swine influenza has been tricky since the times of Richard Shope who isolated swine influenza virus[8] some years before the first influenza virus could be isolated from humans[61] and who performed the first infection trials in pigs[8,11]. The difficulty of imitating swine influenza under experimental conditions was mainly reflected by an absence of prominent clinical symptoms in pigs infected experimentally with the virus alone. Only co-infections with bacteria such as *Haemophilus parainfluenza suis* induced clinical symptoms which led Shope to conclude that "swine influenza is an acute, infectious disease of swine caused by the bacterium *Haemophilus parainfluenza suis* and the swine influenza virus acting in concert" [10].

Due to the air-borne character of transmission of influenza virus most experimental infections were and are done by the intranasal way (direct inoculation into the nostrils or intranasal instillation of sprays by airbrush devices) which is simple to perform but never reproduces prominent clinical influenza[8,30-37,62-67]. Later, the focus was also on other methods of infection. In the 1980s also the intratracheal infection route was established[38,39,68-75]. Here, influenza symptoms could be partially triggered, but in contrast to intranasal infection, the infection is not easy to perform. Intratracheal infection was implemented as the obligatory route for infection for proof of efficacy of swine influenza vaccines into the European Pharmacopoeia[76] (European Directorate of Medicines, 1997, 2005) but it suffers from an unreliability to distribute the virus homogenously in the lungs even in the hands of experienced staff as shown by the work of Kyriakis et al. who reported highly significant differences in viral lung load between right and left side of lungs in the same pigs[40]. This great variation in virus distribution of the lung and the high individual variance in viral lung load are of disadvantage for vaccine development due to the requirement to prove significant differences in viral lung load between vaccinated and unvaccinated pigs. Another route of infection is airborne infection, which is analysed in detail in this monograph, in which a high-dose aerosol-mediated challenge model has been developed. This infection model induces disease with high reliability and ensures a uniform distribution of virus in the lung.

Other ways of becoming infected include contact infection, where pigs are brought into contact with infected pigs (direct contact) or exposed to an environment in which infected pigs are kept (indirect contact).

Experimental infections of pigs were carried out to elucidate pathogenesis and pathology, to test vaccines, to conduct basic research (ANP32, NS1, gene editing), to characterise new porcine influenza viruses, avian influenza viruses and other influenza viruses in pigs, and to analyse zoonotic aspects. Swine are thought to be suited to model human influenza A virus infection[43,77,78].

2.1.2.1 CHARACTERISATION OF PORCINE INFLUENZA VIRUSES

Due to the increasing diversity of European swine influenza viruses in Europe since the 1980s, experimental infections in pigs were carried out at an early stage. Numerous fundamental studies were carried out by the research group of Kristien van Reeth[69,70,79-85]. The increasing heterogeneity of porcine influenza viruses also resulted in the need to develop new vaccines in Europe, which was also the starting point for the development of new aerosol-based infection models for swine influenza[86-88].

With the emergence of the pandemic virus of 2009[89], there were new approaches to test the virulence and transmissibility of the new virus in experimentally infected pigs[36,90-92].

The situation regarding swine influenza in the USA was very stable until 1998 because only classical H1N1 influenza viruses were circulating[93]. This changed significantly in the years that followed[94-98]. It therefore became necessary to analyse the new viruses in the animal model of pigs as well.

Studies of US swine influenza viruses from 1930, 1945, 1968, 1973, 1999, 2001, 2002, 2003, 2004 (H1N1 and H1N2), which were intracheally administered to 4-week-old pigs, showed a high degree of heterogeneity with regard to macroscopic and microscopic lung changes[99]. While the isolates from the years 1930 to 1999 still showed cross-reactivity in the haemagglutination inhibition test, this was reduced compared to the isolates from 2001 onwards[99].

Infection with phylogenetically distinct US H3N2 viruses reflected cross-reactivity between cluster I and III viruses, but not with cluster II viruses[100]. Under experimental conditions, virus replicated in the lungs of 4- and 12-week-old pigs, but clinical signs, gross and microscopic lesions were more pronounced in pigs infected at 4 weeks of age compared with those infected at 12 weeks of age[100]. Microscopically, the epithelial layer was disrupted. Necrotic cells were observed in the lumen of the respiratory tract.

In studies of viruses from a new cluster of US H1N1 and H1N2 porcine influenza viruses in 4-week-old pigs (contact infection and intratracheal infection), it was shown that macroscopic and microscopic lung changes did not differ from those of conventional viruses[101]. Contact animals excreted virus from day 3 after contact and at least until day 7 after contact with infected pigs, while the intratracheally infected pigs no longer excreted virus on day

7 after infection because they had earlier and stronger contact with antigen than the contact-infected pigs and therefore adaptive immunity responses developed earlier[101].

In 2006, an H2N3 influenza virus was isolated from 5 to 6-week-old pigs from 2 farms in Missouri. The pigs had multifocal bronchopneumonia[102]. Since the farms used surface water for cleaning and drinking, it is likely that influenza viruses were introduced from the wild bird population. HA, NA and PA were similar to those of American lineage avian influenza viruses, whereas the other segments were similar to those of American lineage swine influenza viruses, indicating a reassortment event. 4-week-old pigs were experimentally infected with this virus and contact animals were added on day 3 after experimental infection. The infected animals had interstitial pneumonia and excreted virus, and the contact animals seroconverted by day 24 post-contact; however, virus was only detected in 10% of the contact animals on days 5 and 7 post-contact; some of the contact animals showed mild interstitial pneumonia[102]. Overall, the results suggest that the viruses could be transmitted, but had not yet adapted sufficiently to form stable chains of infection.

4-week-old pigs were infected intranasally with a newly reassorted avian H1N1 virus detected in pigs in China (G4 virus)[103]. In experiments, these viruses showed increased replication, longer excretion and caused more severe symptoms and macroscopic and microscopic lung lesions than pigs infected with G1 H1N1 influenza viruses.

2.1.2.2 Experimental infections of pigs with the pandemic virus of 1918

A plasmid-derived 1918 influenza virus was reconstructed by reverse genetics and applied intratracheally to 4-week-old pigs[73]. The pigs showed a transient increase in body temperature on day 1 after infection and mild respiratory symptoms. While the macroscopic lung lesions did not differ from those with a plasmid-derived swine influenza virus from 1930, the lung lesions in pigs infected with the 1918 virus were more pronounced from day 5 onwards. While the pigs infected with the 1918 virus showed severe necrotising inflammatory lesions under the microscope, the lesions in the pigs infected with the 1930 virus had already regressed. Infections with the 1918 virus did not result in lethal outcomes in pigs, in contrast to experimental infections in ferrets[104] and macaques[105].

2.1.2.3 Experimental infections of pigs with avian influenza viruses

No symptoms were observed after intranasal and conjunctival infection of 4-week-old pigs with low-virulence H5 (H5N2, H5N3, H5N9) and H7 (H7N9, H7N2) influenza viruses[106]. Only in H7N9, H7N2 and H5N9 infected pigs was a reduced feed intake observed on day 1 after infection; however, on day 2 after infection, feed intake had returned to normal. All nasal swab samples were negative in the pigs, but virus was detected in bronchoalveolar

lavages in some pigs. Seroconversion was also observed in these animals. Macroscopic lung lesions were either undetectable or mild. In contrast, microscopic lung lesions varied widely.

Infection experiments with various low pathogenic avian influenza viruses (H1N1, H4N1, H4N6, H5N1, H5N6, H7N1) all led to the infection of pigs with virus excretion for 7 days, but the viruses could not be transmitted to other pigs in direct contact, nor to ferrets in indirect contact[107].

In comparative studies of H5N2 virus and porcine avian-like H1N1 virus in 4-week-old pigs that had been infected either intranasally or intranasally, the pigs infected with H5N2 virus, both intranasally and intratracheally, showed a lower excretion rate than the pigs infected with the swine virus[108]. The pigs infected with H5N2 had no symptoms, those infected with H1N1 only mild symptoms, whereas the pigs infected intratracheally showed symptoms in both groups. By means of PCR, H5N2 virus was detected in extraneural tissues of some pigs: mainly in the brainstem after intranasal infection, but also sporadically in the intestine with both routes of infection.

Ten serial passages of an H9N2 avian influenza virus HA-Q226L were carried out in 3-week-old pigs[109]. While the virus was mainly detected only in the upper respiratory tract during the first 3 passages, it spread throughout the lungs from passage 4 onwards. The mutation HA-D225G was discovered here, which could be associated with the increased replication. Nevertheless, the virus was less efficient at transmission than porcine influenza viruses. From passage 7, virus replication decreased and was no longer detectable from passage 10. Investigations using a reassortant H9N2 influenza virus containing the internal protein genes of pandemic (H1N1) 2009 showed increased pig-to-pig transmission after serial passages in pigs[110].

Pigs could be successfully infected with an H7N9 influenza virus isolated from a human[111]. The pigs excreted virus for 5-6 days and showed mild respiratory symptoms on day 1 after infection. However, the virus could not be transmitted to other pigs, either through direct or indirect contact, nor to ferrets through indirect contact.

Infections of 2 to 3-week-old pigs with highly virulent avian influenza viruses of subtype H5N1 (intranasal or feeding of infected poultry meat) led to infection of the animals with no or only mild symptoms[112]. Virus excretion was lower than with porcine viruses. The virus was only detected in the respiratory tract. In contrast to the severe courses in mice and ferrets[112], with spread to extra-respiratory tissues, the pig model differs from the other two animal models.

A highly virulent mink-derived clade 2.3.4.4b H5N1 virus caused interstitial pneumonia with necrotising enteritis in 4-week-old pigs after intratracheal infection[113]. High virus titres were detected in the lower respiratory tract. The infected pigs excreted only small

amounts of virus and there was no transmission to contact pigs. Some critical mammalian-like mutations such as PB2-E627K and HA-Q222L were detected in some of the infected pigs.

The investigations show overall that pigs can be easily infected with avian influenza viruses, but do not become ill or only fall ill slightly. Transmission to in contact animals is difficult and stable infection chains between pigs do not develop. Since avian viruses in the form of avian-like H1N1 viruses were originally transmitted to pigs and established successful infection chains here, other, as yet unknown processes must occur here that favour the introduction of avian influenza viruses into the pig population. In the case of the avian H1N1 influenza virus, it does not appear that there was a direct introduction into the pig population; rather, at least three reassortment events with various avian influenza viruses led to a virus that successfully replicates in pigs[114]. The same may have been the case with the virus of 1918[115]. All other entries then occurred via reassortments with the influenza viruses already circulating in the pig population.

2.1.2.4 ANP32A

The proteins ANP32A and ANP32B are members of the acidic (leucine-rich) nuclear phosphoprotein family of 32 kDa. They are host factors that contribute to influenza A polymerase activity and differ between mammalian and avian species. As a result, the replication of avian influenza viruses is poorly supported by mammalian ANP32. It has been shown that porcine ANP32 is more supportive of avian viral polymerases than other mammalian ANP32[116]. This may explain the high susceptibility of pigs to infection with avian influenza viruses.

2.1.2.5 Experimental infections of pigs with B, C, and D influenza viruses

Influenza B viruses are common in the human population and cause seasonal influenza outbreaks, usually following the influenza A waves, but they can also dominate the flu epidemic, as they did in 2017/2018. In humans, a distinction is made between the B/Yamagata and B/Victoria lineages, with the B/Yamagata viruses thought to have disappeared as a result of the strong 2017/2018 wave (widespread population immunity combined with the contact restrictions during the COVID-19 pandemic). Influenza B viruses are rare in pigs[117]. In infection experiments in pigs, 4-week-old pigs were infected intratracheally or intranasally with B/Victoria or B/Yamagata influenza viruses[118]. Viruses of both lines were able to infect pigs. Some pigs developed fever. The pigs showed slight macroscopic lung changes (mild peribronchiolitis, multifocal alveolitis). Virus was detected in bronchoalveolar lavages. Pigs infected with B/Victoria influenza viruses excreted virus, while no virus

was detected in nasal swabs from pigs infected with B/Yamagata viruses. In contact infection experiments in which pigs were housed with infected pigs, the B/Victoria viruses were partially transmitted, but the B/Yamagata viruses were not.

Influenza C viruses occur in humans and are mainly detected in children[119]. Influenza viruses have also been detected in pigs[120]. In infection experiments in 50 to 60-day-old pigs that were infected intranasally or by contact with infected pigs, virus excretion and seroconversion were observed[121], indicating transmission to and between pigs. No increases in body temperature were observed, but there were slight respiratory symptoms (increased respiratory rate, nasal discharge).

Influenza D viruses are related to influenza C viruses and were first detected in pigs[122]. However, they are more common in cattle[123]. Surveillance activities in Italy indicate an increasing prevalence of influenza D viruses in pigs[124]. In comparative experimental infection trials with B and D influenza viruses following intranasal infection of 5-week-old pigs, mild symptoms in the form of fever and minor macro- and microscopic lung changes were observed for both B and D viruses (mild peribronchiolitis and interstitial pneumonia)[125]. Virus was excreted until day 6 after infection with B and D viruses. Influenza D viruses could be transmitted to in-contact animals.

2.1.2.6 STUDY OF IMMUNOLOGICAL RESPONSES

The porcine model is considered a potential animal model for human influenza[126]. Summaries of the immune response of pigs after experimental infection are available[127-131]. A significant decrease in lymphocytes and an increase in the mean cell count without leukopenia were observed in infected pigs 3-7 days after infection[132]. C-reactive protein, haptoglobin and serum amyloid A increased 1-3 days after infection[133,134]. Cytokines (IL-1β, IFN-γ, TNF-α, IL-6, IL-8, IL-10) also increased after infection[79-82,135-137]. Studies in pigs infected intranasally at 2, 4 and 5 weeks of age have shown innate, proinflammatory cytokines and specific IgA antibodies in the lungs, as well as higher frequencies of cytotoxic T lymphocytes, γδ cells, dendritic cells, activated T cells, and CD4+, CD8+, and immunosuppressive T regulatory cells[126]. Influenza virus infection attracts multifunctional and cross-reactive T cells to the lungs[138-140]. The kinetics of T helper and memory T cells after influenza virus infection have been determined[141,142]. The immune response varies according to the infection model and the intensity of the infection[143]. Heterosubtypic influenza virus infection induces a long-lived increase in $CD8^+$ T cells in the lungs and in the lymphoproliferation response in the blood[144].

2.1.2.7 STUDIES ON COMPARATIVE PATHOLOGY

Comparative pathology studies in pigs have described differences between the subtypes[145]. It was shown that swine H3N2 virus induced more severe gross and histopathological lesions on day 2 post-infection, which progressively decreased, whereas inflammation in lung tissue lasted longer in pigs infected with swine H1N1 virus (at least until day 14 post-infection)[145].

2.1.2.8 GENE EDITING

Using the CRISPR/Cas 9 system, homozygous gene-edited TMPRSS2 knockout pigs were generated[146]. After intratracheal challenge, these pigs showed delayed replication of influenza viruses (swine H3N2 and H1N1), reduced virus shedding, and lower viral load and lung lesions compared to normal pigs. Important for influenza virus infectivity is the proteolytic activation of HA by host cell proteases. The monobasic HA motif is activated by trypsin-like proteases. These include transmembrane serine protease 2 (TMPRSS2).

2.1.2.9 STUDIES ON INFLUENZA VIRUS RECEPTORS IN PIGS

Influenza viruses bind to sialic acids. These receptors differ between species. Avian influenza viruses bind more strongly to sialic acid α2,3-galactose, whereas human influenza A viruses bind more strongly to sialic acid α2,6-galactose. Both receptors are abundant in porcine tissues (trachea, lung, liver, kidney, spleen, muscle, brain, intestine)[147]. Trebbien et al. (2011) investigated the fine distribution of receptors in the respiratory tract of experimentally infected pigs (swine H1N1 and H1N2 influenza viruses, avian H4N6 influenza viruses)[148]. Sialic acid α2,6-galactose was the predominant receptor in all regions of the respiratory tract. Sialic acid α2,3-galactose was found at low levels in bronchioles and alveoli. Compared to non-infected areas, receptor expression was significantly reduced in infected areas. Kristensen et al. (2024) showed that sialic acid α2,3-galactose is expressed in the nasal mucosa of pigs experimentally infected with influenza virus[149].

2.1.2.10 STUDIES ON THE EFFICACY OF VACCINES

Swine influenza has the advantage that the vaccine can be tested directly on the target animal. This provides a deeper insight into the protective mechanisms than is possible with comparative vaccine developments for humans. There are numerous overviews of vaccines for pigs, from which details of the respective experimental studies can be taken[150-154].

2.1.2.11 STUDIES ON ANTIBODY-DEPENDENT ENHANCEMENT OF DISEASE

Mechanisms for the enhancement of disease after previous antigen contact through immunological processes have been little studied in humans. Experimental infection of pigs has the advantage that lungs can be examined directly. In fact, the processes of antibody-dependent enhancement of disease are clinically masked by the rapid onset of immunity in vaccinated animals. However, these processes can be identified by the manifest lung changes, which usually occur when the vaccine strain differs more from the infectious virus, i.e. when the protection provided by hemagglutinin and neuraminidase is not as pronounced as required to protect[155]. Enlarged lung lesions compared to non-immunised control pigs were observed in individual vaccinated pigs in infection experiments if the infection strain was antigenically distant from the vaccine strain[156,157]. This was then referred to as an infection heterologous to the vaccine strain. However, this is a broad term because the opposite term 'homologous' can also range from identical to belonging to the same antigenic group. The mechanisms of this enhancement of pathogenetic reactions are not yet fully understood. This process appears to occur when antibodies against HA and NA are absent or low, but antibodies against the stalk region and M2 are generated[158]. Antibodies against the stalk region of HA promote increased membrane fusion *in vitro*[159]. Antibodies against the M2 protein are not neutralising, but stimulate the activity of natural killer cells even before the appearance of infection-related antibodies[160]. This activity could affect infected cells that have not yet been lysed by the infection and lead to the release of cytokines. Proinflammatory cytokines and cytokine dysregulation are associated with severe lung pathology and neutrophil infiltration. The stronger the vaccine (depending on the ajuvant in the case of inactivated vaccines or when live vaccines are used), the lower the effects on lung pathology in vaccinated animals[161,162]. The results available from infection studies are still contradictory. For example, in heterologous immunisation-infection trials in pigs, no enhancement of disease induction was observed in the presence of antibodies against the stalk region of HA[163]. No increased lung reactions were observed after administration of recombinant vaccines based on the M2 protein[164]. In contrast, in other experiments, significantly increased lung pathology was observed when no antibodies were generated against the two surface proteins of the influenza virus, but antibodies were generated against the M2 or NP protein[165-167]. Effects of increased disease induction after immunisation have also been observed with other pathogens[166]. All these studies were conducted in animals that had been immunised not long before. Taking into account the declining antibody kinetics after vaccination, there comes a point where vaccinated animals become susceptible to antibody-dependent enhancement of disease. Therefore, regardless of the vaccine, it is important to maintain antibodies against HA and NA for as long as possible by means of appropriate vaccination schedules and to vaccinate at least three times.

2.1.2.12 RISK OF ZOONOTIC TRANSMISSION THROUGH EXPERIMENTAL INFECTION OF PIGS

After infection, pigs excrete larger amounts of virus. In particular, on days 1-3 after infection, aerosols containing larger amounts of influenza virus can accumulate in small rooms, posing a potential risk of infection to staff. Therefore, appropriate measures such as the wearing of protective equipment, masks, vaccination of staff with seasonal influenza vaccines and the provision of antiviral agents are important.

Two cases of influenza virus infection after experimental infection of pigs have been reported in the literature[168]. Two days after challenge, two of the four people who had taken nasal swabs from infected pigs fell ill. A 31-year-old woman and a 39-year-old man showed mild respiratory symptoms (sore throat, cough, myalgia). Influenza viruses were isolated from nasal swabs and further characterised.

2.1.3 HORSE INFLUENZA MODELS

Influenza viruses are among the most important pathogens causing respiratory infections in horses[169]. Therefore, immunoprophylaxis of equine influenza plays an important role[170-178]. Infection models in horses have been developed to test the efficacy of vaccines in horses[179,180]. Aerosol infection models in ponies were particularly useful for testing the efficacy of vaccines[181,182]. In these studies, dose-dependent differences were also identified: clinical symptoms only occurred when 10^6 EID_{50}/ml were nebulised[182]. In this case, 20 ml of allantois fluid with a varying virus content (10^2 or 10^4 or 10^6 EID_{50}/ml) was nebulised using a model 65 Devilbiss nebuliser for 30 minutes in a 56 m^2 stable. Only the highest dose induced influenza symptoms in the ponies. Given the stable was 2.5 m high this would be equivalent to lg 5.15 EID_{50}/m^3.

2.1.4 CANINE INFLUENZA MODELS

As early as the 1960s, experimental infections with seasonal influenza A and B viruses from the human population were carried out on dogs[183]. After intranasal and aerogenic infection, dogs develop mild conjunctivitis, lacrimation, nasal discharge and mild depression. No other respiratory symptoms or temperature increases or fever were observed. Virus excretion occurred for 5 days. All dogs developed antibodies against influenza viruses[183].

Although there had been isolated evidence of antibodies against influenza viruses in dogs before, influenza only gained greater importance with the emergence of stable chains of infection of H3N8 and H3N2 influenza viruses in the dog population[184-186]. Subsequently, there were numerous experimental infections of dogs to elucidate the pathogenesis of canine influenza[187-196].

Infections with the $H1_{pdm}N1$ 2009 influenza virus could also be experimentally induced in dogs, but the symptoms were mild[197]. Also, transmission between dogs was low (only 1 of 3 exposed dogs became infected). With avian H5N1 influenza virus, severe symptoms could be induced in dogs after experimental intranasal and intratracheal infection[198].

2.1.5 AVIAN INFLUENZA MODELS

Due to the widespread prevalence of influenza viruses in birds and their high economic significance, as well as their zoonotic potential, numerous infection models exist in birds. However, these are not part of this review, which is more concerned with influenza virus infections in mammals.

2.1.6 OTHER ANIMAL MODELS OF INFLUENZA

There are several other influenza infection models, most frequently used are ferrets[45,199] and mice[200]. The ferret model was comprehensively reviewed by Enkirch and Messling (2015)[201]. Mouse and other animal models were summarised in a review article by Nguyen et al. (2021)[46]. The occurrence of H5N1 clade 2.3.4.4 infections in dairy cows in the USA has sparked new interest in the cattle infection model[202].

2.1.6.1 FERRETS

Domestic ferrets were first used for infection studies with influenza viruses in 1933[61]. In terms of similarity of symptoms to humans, they were superior to hedgehogs, guinea pigs and mice. Infection with human throat swabs induced a diphasic rise in temperature, symptoms of nasal catarrh, and rhinits[61]. The ferret has proven to be an important animal model for influenza because ferrets are easily infected with human influenza A and B viruses and other influenza viruses, and the symptoms in ferrets are similar to those in humans[199,203,204].

Assessing airborne transmissibility in ferrets

One research focus in the ferret model is the study of the transmissibility of influenza viruses. As a result of the H5N1 panzootic in wild birds, it became important to address the extent to which avian influenza viruses have already adapted to circulate in mammals and humans.

On the basis of such infection experiments, mutations could be identified that support a better transmissibility of H5N1 influenza viruses in mammals[205-208].

Such studies could also be carried out for other avian influenza viruses. For example, an H3N8 virus from a patient containing the PB2-E627K mutation could be transmitted airborne in ferrets[209].

For other avian viruses such as H7N9 and H5N8, only limited airborne transmission between ferrets could be demonstrated[210,211].

Infection studies in ferrets using air-sampling devices clearly showed that the number of viruses shed into the air is very important[212]. $H1_{pdm}N1$ virus was efficiently shed into the air by infected ferrets, but not infectious 2005 zoonotic H5N1 and not 2024 bovine H5N1 virus. This study also showed that airborne shedding of infectious virus by a European polecat H5N1 and a dairy worker 2024 H5N1 virus was observed in a few ferrets, suggesting an evolution towards better mammalian transmissibility of recent H5N1 influenza viruses. Further studies in ferrets revealed that the 2024 dairy farm worker H5N1 virus reflects increased virulence, transmissibility and airborne shedding compared to other previously isolated clade 2.3.4.4b H5N1 viruses[213]. Ferrets can also transmit other clade viruses isolated from humans[214].

Investigation of the replication of influenza viruses in the mammary gland

Infection experiments with $H1_{pdm}N1$ 2009 virus showed that influenza virus can also be transmitted to the glandular tissue of the mammary gland[215]. Infectious virus was found in the glandular tissue of the mammary gland of infected mother ferrets and in their milk. The infection led to inflammation of the mammary gland and a decrease in milk secretion. The virus was found in epithelial cells of the glandular tissue. In these studies, influenza virus was also directly applied to the milk duct, causing infection of the mammary gland and transmission of the infection to the pups.

Characterization of the immune response to influenza

The ferret model has also been used to study immune responses to influenza viruses[216]. T-cell populations were shown to increase in the infected lungs, but not in the blood. Disease severity was associated with higher expression of pro-inflammatory cytokines.

Pathogenesis of influenza in ferrets following different forms of exposure

Various forms of exposure were investigated in ferrets, in particular for avian viruses. Both oral[217] and ocular infection proved successful[218-221].

2.1.6.2 MICE

Mice have proven to be an irreplaceable animal model for influenza research. They are susceptible to influenza virus infection and easy to breed and keep. Although their size makes them not quite as easy to handle as larger laboratory animals, these advantages still prevail. There is also a whole range of inbred strains that allow the study of various aspects of immunology. In addition, transgenic mice can be easily established, which allow even more detailed investigation of the effects of individual mutations in the genome on the characteristics of resistance to viral infections. Inbred strains are established by 20 or more consecutive generations of brother-sister matings until on average at least 98.6% of the genome is homozygous in each individual[222-225]. Genetic modifications in mice can be achieved by gene knockout, gene knockin, transgene manipulation, gene trapping, physical or chemical mutagenesis and spontaneous mutations[225].

After the discovery of interferon as a virus resistance factor in 1957[226-228], the mouse model contributed significantly to the discovery of Mx dynamin-like GTPases (Mx1) as an antiviral mechanism of innate immunity and intracellular resistance to influenza viruses[229-235]. Other factors of innate immunity have also been established and investigated in the mouse model[236-238]. Furthermore, numerous studies on the pathogenesis of influenza were carried out in the mouse model[239-246], including the pathogenesis of the 1918 influenza pandemic[247-251]. The mouse model was used to determine the effectiveness of previous antigenic encounters and vaccines[244,252-254]. Antiviral agents were also tested in mice with influenza[255-259].

2.1.6.3 GUINEA PIGS

Guinea pigs are susceptible to influenza virus infections and are therefore also used as laboratory animals for influenza[260-266]. Seasonal influenza viruses can be transmitted between guinea pigs[267].

2.1.6.4 SYRIAN HAMSTER

Syrian hamsters can be infected with influenza viruses and are thus suitable as an infection model[268,269].

2.1.6.5 CATS

Cats have long been considered resistant to influenza[270]. In recent years, however, it has been recognised that cats can also become infected with influenza viruses if they come into contact with other infected species[271]. They can become infected with both seasonal human viruses and avian influenza viruses. In particular, the feeding of infected poultry meat has

led to infections in numerous species of Felidae[272-274]. However, stable chains of infection in cats do not exist. With the increasing number of occasional transmissions of influenza viruses to cats, interest in the cat as an infection model has also increased, as evidenced by some infection experiments in cats[275-280].

2.1.6.6 CATTLE

Although as early as 1953, experimental infections of the udder of a dairy cow, carried out out of scientific interest, led to an efficient replication of infectious virus in the mammary gland[281,282], this was not taken into account for a long time. It was only with the emergence of an artificial infection cycle of H5N1 influenza viruses via milking equipment and the udders of cows in dairy herds in the United States in 2024 that this topic gained new attention. After experimental intranasal infection of calves with H5N1 viruses, only moderate virus replication was observed without induction of conspicuous symptoms and without transmission to other calves[283]. In contrast, direct injection of H5N1 virus into the udders of dairy cows led to high viral replication, mastitis and severe general infection in the cows, without virus being excreted via the respiratory tract, suggesting that there was no systemic spread of the virus in the dairy cows' bodies[202]. The cow's mammary gland is rich in receptors for avian influenza viruses[284]. This explains why influenza viruses replicate well in the udder.

2.1.7 ALTERNATIVES TO ANIMAL MODELS

Liquid-air cell cultures, organ tissue cultures taken from the carcasses of animals, or biopsies can be used to test mechanisms of innate immunity as an alternative to animal models [285-291]. Similarly, immune cells can be cultured *in vitro*. Nevertheless, there are still no alternatives to animal models that can reflect the totality of an organism's reactions to an infectious agent. In particular, the complex mechanisms of adaptive immunity cannot be represented *in vitro*.

2.2 MATERNALLY-DERIVED IMMUNITY

2.2.1 TYPES OF PLACENTA AND CONSEQUENCES FOR MATERNAL-FETAL EXCHANGE

The placenta is an organ that mediates the exchange of nutrients between mother and fetus[292,293]. The placentas of mammals display structural diversity. Based on ultrastructure five principal types of placenta exist: epitheliochorial, syndesmochorial, endotheliochorial, hemochorial, hemoendothelial[294,295] (Table 1).

Table 1: Simplified scheme of placental barriers

Placenta	Layers	Species	Antibody transfer	Receptor
Epithelio-chorial	Fetal blood Fetal capillary endothel Fetal mesenchym Fetal chorionepithel Maternal uterus epithel Maternal Lamia propria of endometrium Maternal capillary endothel Maternal blood	Pigs, Horses*	Colostrum	FcRn in the gut of piglets
Syndesmo-chorial	Fetal blood Fetal capillary endothel Fetal Mesenchym Fetal Chorionepithel Maternal Lamia propria Maternal capillary endothel Maternal blood	Cattle	Partially transplacental + colostrum	unknown
Endothelio-chorial	Fetal blood Fetal capillary endothel Fetal Mesenchym Fetal Chorionepithel Maternal capillary endothel Maternal blood	Dogs, Cats	Partially transplacental + colostrum	unknown
Hemo-cho-rial	Fetal blood Fetal capillary endothel Fetal Mesenchym Fetal Chorionepithel Maternal blood	Hu-mans°	Transplacental + mammary secretions	FcRn
Hemo-endo-thelial	Fetal blood Fetal capillary endothel Maternal blood	Ro-dents°	Transplacental + mammary secretions	FcRn

° placentation in rodents and lagomorphs is slightly different from that of humans and is called haemoendothelial (maternal blood circulates in large sinus-like spaces into the trophoblast) but both permit the transfer of maternal antibodies to the fetal circulation

Hemochorial and hemoendothelial placentation are the most invasive and allows fetal tissues direct contact to maternal blood[296]. Epithelial placentas are the least invasive displaying three layers separating fetal tissues from maternal blood. Pigs possess a noninvasive epitheliochorial form of placenta. Chorionic villi are loosely opposed to the maternal epithelium by microvillus interdigitation[297]. The extent of transmission of antibodies from mother to fetus is related to the number of placental barrier layers[298]. The six layers of the epitheliochorial placenta, the maternal endothelium of the capillaries, the maternal connective tissue (Lamina propria of endothelium), maternal epithelium, and fetal chorionepithel (Trophoblast), the fetal connective tissue (mesenchym) and fetal capillary endothel form a barrier that prevents the contact between maternal and fetal blood and thus the transmission of antibodies[298]. The placenta of sows is of epitheliochorial type and the fetal chorionic epithelium is in contact with intact uterine epithelium. Thus, the transplacental passage of immunoglobulin molecules is totally blocked. Immunoglobulins cannot cross the placenta of sows. Consequently, neonatal pigs are agammaglobulinemic at birth[299]. Where contact between maternal and fetal blood is prevented due to the type of placentation, the transfer of maternal immunity via the colostrum is essential for the newborns.

2.2.2 THE ONTOGENY OF THE PIG IMMUNE SYSTEM

At the end of organogenesis at day 35 of gestation, the lymphatic system is formed[297]. The bone marrow begins to develop its haematopoietic activity at around the 45th day of gestation. At this time, B-cell lymphogenesis begins in the bone marrow. It is also at this time that the early precursors of T cells are derived from stem cells. These precursor cells then migrate to the thymus. After the 70th day of gestation, fetuses become immunocompetent.

Piglets have no protection against pathogenic organisms in their environment at birth. Neonates lack maternal antibodies. They have little capacity to produce antibodies. The B cell pool is immature. It consists of a uniform batch of unprimed B cells. Although the fetal piglet is able to mount an immune response to an antigen, the neonate is immunologically underdeveloped at birth. The immune system of piglets is developed but cannot function at adult levels for several weeks. The intestinal lymphoid tissues lack T cells at birth which appear in intestine between 2 (CD4+ T cells) and 4 (CD8+ cells) weeks after birth (summarized by Tizard, 2004[300]). Natural killer cell activities need time to develop. Newborn piglets display a relatively limited B cell diversity. The antigen-binding repertoire of B cells does not expand until 1 month of age. The fetal immunoglobulin repertoire is not yet diversified at birth and needs stimuli such as bacterial colonisation of the gastrointestinal tract[300]. In order to support newborn piglets the mother sows provide them with maternally-derived antibodies and other components. Therefore, the survival of newborn piglets depends on their intake of colostrum during the first hours of life. Neonates are continuously exposed to microbes. These microbes enter the gastrointestinal tract with food. The

delivery of maternal immunity through the colostrum and the interaction with environmental antigens leads to the appearance of primed T and B cells[297].

2.2.3 TRANSMISSION OF MATERNAL ANTIBODIES VIA COLOSTRUM

In 1954, it was shown that gamma globulins were not formed until the sixth week of life in piglets of Ceasarian origin that did not receive colostrum[301]. In 1957, studies showed that piglets born by caesarean section did not have antibodies in their system unless they had been fed colostrum[302].

From day 80 of gestation, the number of leukocytes and lymphocytes in the mammary parenchyma tissue increases in sows[303]. 70% of IgG and more than 90% of IgM and IgA are synthesized locally in the mammary gland of sows[304]. Vaccinating sows before farrowing can result in high levels of antibodies, which can be passed on to piglets via the colostrum[305]. Immunoglobulins of all classes can protect the gut[306]. The piglets are dependent on antibodies received through the first milk, the colostrum[300]. Colostrum contains the accumulated secretions of the mammary gland over the last few weeks of pregnancy together with proteins actively transferred from bloodstream under the influence of estrogens and progesterone. It is rich in IgG which is predominant but also contains some IgA, IgM and IgE[300]. IgA is dominant in pig milk[304]. Colostrum contains also lymphocytes[300]. These colostral lymphocytes survive up to 36 hours and can enter the blood of piglets[300]. Lymphoid cells of maternal origin are also absorbed from the digestive tract of the piglets after the ingestion of colostrum and are transported to the peripheral blood and to various tissues[307]. Transfer of cell-mediated immunity is possible in this way.

In suckling piglets protein absorption is selective, IgG and IgM are preferentially absorbed, whereas IgA mainly remains in the intestine[300]. The duration of intestinal permeability for immunoglobulines declines after about 6 hours after birth and absorption ceases after approximately one day[300]. Gut closure for macromolecule absorption in piglets occurs 24-36 hours after birth[308], but the presence of the MHC class I related molecule FcRn on intestinal epithelial cells allows IgG import[309]. IgG and IgA transudate into the lungs of piglets after the colostrum has been absorbed and introduced into the circulatory system, peaking at 3 days after birth[310].

2.2.4 THE NEONATAL FC RECEPTOR

The neonatal Fc receptor (FcRn) enables the transfer of passive humoral immunity from mother to offspring[311]. Antibodies have two functional domains, the Fab (fragment of antigen binding) and the Fc region (the crystalline fragment is the carboxyterminal part of an immunoglobulin). Fab is responsible for antigen recognition, whereas Fc couples the immunoglobulin to effector pathways. B cells can vary the expression of the heavy chain constant region of immunoglobulins and thus the Fc region[311]. This results in antibodies with different effector functions. There are five classes of antibodies - IgA, IgD, IgE, IgG

and IgM[312]. In serum and non-mucosal tissues, IgG is the most abundant class. It is transferred from the mother to the offspring and is the source of short-term passive immunity. In mammals with an epitheliochorial placenta, mothers concentrate antibodies in their colostrum. After absorption in the intestine, the antibodies pass through the stomach into the duodenum. Here IgG can bind to FcRn on epithelial cells. Specific IgG transport is mediated by the neonatal Fc receptor FcRn. The Fc portion of IgG binds to FcRn at an acidic pH (<6.5). Fc-bound IgG transcytosed. The villous epithelium of the ileum may play an important role in the absorption of colostral IgG and probably IgA and IgM into the neonatal circulation, and the crypt epithelium in the adsorption of colostral IgA and IgM, possibly by complexation with mucin-bound secretory components[313].

2.2.5 PROTECTION BY MATERNALLY-DERIVED IMMUNITY

The efficacy of maternal immunity can best be investigated in infection experiments in which piglets are challenged. Numerous such infection experiments have been carried out in the past. Keay et al. (2023) analysed all studies conducted on this topic from 1990 to 2021 in a systematic review and meta-analysis[314]. They focused on studies in which the infectious virus matched the vaccine strain administered to the mother sows. In Figure 1 of their paper, they present a scheme of the timing of the infection experiments in relation to the age of the piglets used. Overall, the data suggest that, depending on the time of infection, maternal immunity provides some protection against clinical disease but not against infection. In addition, virus excretion is prolonged under maternal immunity[314].

In the following, some results of studies conducted before the 1990s will be analysed, but also some selective studies from the period thereafter.

Comparative experimental intranasal infection with porcine influenza viruses of 3-day-old piglets from vaccinated and unvaccinated sows showed a lower viral load in the lungs, less extensive lung lesions and a milder disease progression in piglets from vaccinated sows[35]. Furthermore, replication of the influenza virus in the piglets of vaccinated sows was restricted to the respiratory tract, whereas infectious influenza virus was detected in lymph nodes, blood, lungs, liver and pericardium in 30% of the piglets of the unvaccinated sow[35]. Even in comparative studies of piglets from previously infected sows, which had been infected intranasally with influenza virus on day 7 of life, infection in the piglets from the sow with previous exposure to influenza virus could not be prevented[63]. However, the virus titer in the lungs was lower and virus could only be detected until day 5 after infection, while in piglets from the sow that had not been previously exposed, it could be detected until day 12 after infection. In addition, more piglets from the unexposed sow had infectious virus in tissues outside the respiratory tract[63].

Overall, the earlier the piglets were infected after ingesting colostrum, the better the protective effect of maternally-derived immunity. If they were infected before receiving

colostrum (2 hours after birth), they became ill[315]. In another study, pigs with maternal immunity were infected at three weeks of age. These piglets did not develop clinical disease, but were not protected from infection and virus shedding[316]. In experiments by Loeffen et al. (2003), piglets still showed clinical protection if they were infected 7 weeks after birth, but no longer showed protection if they were infected 15 weeks after birth[65]. In trials by Kitikoon et al (2006), piglets infected at 7 weeks of age with maternal immunity showed fewer clinical symptoms than unvaccinated pigs, but these were not statistically significant[317]. In contrast, in a group of pigs with maternal immunity that had been vaccinated in the 3rd and 5th week of life, the clinical score was increased, and even significant, at the second peak on day 4 after infection. This could be an indication that the protective effect of maternal immunity decreases towards the end of the first month of life and that in this case the maternal immunity was neutralised by the vaccination and the more severe symptoms are an effect of the antibody-dependent enhancement of disease (see below). In another study, pigs with maternal immunity were well protected at 5 weeks of age, whereas some pigs infected at 7 weeks and all pigs infected at 11 weeks developed symptoms similar to those of pigs without maternal immunity[318]. These results are consistent with estimates that the time to waning of maternal antibodies was an average of 71.3 days from colostrum ingestion[319].

In field studies on the effectiveness of maternal immunity in piglets of vaccinated sows in different sow herds in France, it was shown that in piglets born to vaccinated sows, the first outbreaks of influenza virus infections can occur around day 35 of life[320].

2.2.6 INFLUENZA VIRUS TRANSMISSION UNDER MATERNAL IMMUNITY

Transmission of influenza viruses is not inhibited under maternal immunity[321-323]. As a result, piglets born to immunised sows can be a source of infection[324]. Longitudinal field studies have shown persistence of influenza A virus in pig herds, mainly due to virus circulation in piglets[325]. Influenza virus was found in the majority of litters. Consequently, the virus was circulating in the farrowing unit but also at the beginning of the nursery period. Antibody studies showed that piglets received maternal antibodies from their sows in this study[325]. The data show the major impact of piglets with maternal immunity on the persistence of influenza A virus in pig herds and the maintenance of infection cycles within these herds. Addressing this source of infection is of great importance when using a combination of vaccination and biosecurity practices to reduce endemic circulation of influenza viruses in pig herds. Mass vaccination of sows in a sow herd resulted in a high number of piglets with maternal immunity, reflecting prolonged virus shedding compared to the period before sow vaccination was introduced[326]. Single vaccination of piglets at week 1 of life with 1/4 of the recommended dose of a commercially available vaccine resulted in a lower number of influenza virus-positive pigs in the nursery compared to unvaccinated pigs, but had no effect on symptoms, weight gain and virus shedding in the preceding period[327] (the vaccine is authorised for use at 8 weeks of age and two vaccinations).

2.2.7 INTERFERENCE OF MATERNAL IMMUNITY WITH VACCINATION

In the 1960s, colostral antibodies were shown to inhibit the active antibody response to influenza virus antigens in piglets[34]. This inhibition was still present 15 and 22 weeks after birth, when colostral antibodies were no longer detectable in the serum of piglets[34]. Maternal immunity inhibits the ability of piglets to mount own serological responses. As a result, active immunisation using vaccines does not induce antibodies. This inhibition is B cell-specific and T cell responses are largely unaffected[300]. Several different mechanisms have been suggested as mediating this suppression: i) rapid neutralisation of the antigen by maternal antibody, ii) antibodies bind to B cell Fc receptors and block signalling, iii) maternal antibodies mask the epitopes on vaccine antigens and so prevent their recognition by the B cells[300]. In the absence of maternal immunity piglets are able to produce antibodies soon after birth[34].

In pigs, maternally derived antibodies have been shown to inhibit active IgA, IgM, Ig,G, haemagglutination inhibition antibody responses and proliferative T-cell responses following exposure to influenza virus[65,328-331].

Maternal antibodies inhibit the production of antibodies, while the T-cell response is less affected[332]. B cell inhibition is mainly mediated by cross-linking of the B cell receptor with the Fcγ receptor IIB by an antigen-antibody complex[332]. In animal experiments, this inhibition has been partially overcome by injection of IgM antibody, which directly stimulates the B cell complex by cross-linking the B cell receptor with a complement protein of the complement receptor signalling complex[332].

2.2.8 VACCINE-INDUCED ENHANCED DISEASE UNDER MATERNAL IMMUNITY

Effects of antibodies enhancing disease are observed in various infections (ADE, antibody-dependent enhancement of disease, VAERD vaccine-associated enhancement of respiratory disease)[333]. In pigs, they have mainly been noticed in the form of enlarged lung lesions[155,334,335]. The causes of such effects may be that antibodies increase the ability of viruses to bind to cells, but there may also be effects at the cell level that lead to increased cytokine release. Since enlarged lung lesions have mainly been observed in pigs, the latter is suspected to be the cause. These effects do not show clinically to such a strong extent because the immunity that is built up more quickly in vaccinated animals, with its protective effect, overlaps the effects of the ADE.

Infectivity experiments in 3-week-old piglets from vaccinated sows showed that heterologous challenge under maternal immunity can exacerbate symptoms and pathology[336]. This could be caused by antibodies against other antigens than hemagglutinin and neuramidase of the influenza virus[336]. A mismatched HA between vaccine and challenge virus was necessary to induce VAERD[158]. However, vaccines containing a matched NA abolished the

VAERD phenomenon induced by the mismatched HA, and this was correlated with the presence of NA-inhibiting (NI) antibodies[158].

In contrast to vaccination with inactivated vaccines, vaccination of sows with live attenuated vaccines did not result in a vaccine-induced increase in disease during heterologous challenge[328].

3. AIM OF THE WORK

3.1 VALIDATION OF INFLUENZA INFECTION MODELS AND INVESTIGATIONS ON PATHOGENESIS

There are no infection models that allow for the reliable induction of disease and the study of the virulence of influenza A viruses in swine. Disease induction is necessary to characterise the virulence of influenza A viruses, to monitor the efficacy of vaccines and antivirals against influenza A viruses circulating in the field, and to study the safety of immunosuppressive compounds.

The aim of the second part of this work was the establishment of an aerosol-mediated infection model for swine influenza and its validation (the first part of the monograph analyses data on the circulation and evolution of influenza viruses in the German pig population including their genetic and antigenic characterisation as well as the analysis of zoonotic transmissions of influenza viruses from pigs to humans[1]). The study covers following topics:

- establishment and validation of a method of aerosol generation for influenza A viruses,

- comparative analysis of different infection models for swine influenza in pigs,

- titration of the infectious dose,

- investigation of the virulence of swine influenza A viruses,

- investigation of the distribution of influenzaviral antigens and infectivity within the respiratory tract and other organs,

- investigation of the effect of simultaneous infections (this has already been reported in the first volume of this monograph and is therefore not repeated in this volume),

- investigation of the protective effect of different proteins of influenza viruses,

- investigation of the effects of maternally-derived immunity under high-dose aerosol infection conditions.

3.2 INVESTIGATION OF MATERNAL IMMUNITY

In addition, the nature of maternally derived immunity is not well understood due to the lack of reliable infection models capable of inducing disease. Little is known about maternally derived immunity transferred to suckling piglets via colostral milk from sows. The aerosol infection model was used to investigate the protective capacity of maternal immunity in piglets and its interaction with vaccination.

The aim of the third part of this work was to investigate:

- the protective capacity of maternally-derived antibodies,

- the interference of maternally-derived immunity with vaccination.

4. MATERIALS AND METHODS

4.1 INVESTIGATIONS ON SWINE INFLUENZA PATHOGENESIS

3131 pigs were infected experimentally with 50 different strains of influenza A viruses (swine avian-like H1N1 virus [$H1_{av}N1$], swine human-like H1N2 virus [$H1_{hu}N2$], swine human-like H3N2 virus [H3N2], pandemic H1N1 virus [$H1_{pdm}N1$], classical swine influenza A H1N1 virus [$H1_{cl}N1$], pandemic H1N2 virus [$H1_{pdm}N2$], H3N1 virus, duck H5N6 virus, and turkey H9N2 virus) in 102 trials over a period of 19 years using a newly developed aerosol infection model and, for comparative analysis, using different routes of infection such as intratracheal, intranasal, intramuscular and direct and indirect contact (Table 2). Topics covered by this part of this monograph are i) comparative analyses of different infection routes (infection of pigs in one comparative trial with the same batch of strain FLUAV/Jena/VI2688/2009 [$H1_{pdm}N1$], ii) analysis of effects of different infection doses (titration of aerosol infection by nebulizing dilutions of the same batch of strains FLUAV/Hamburg/NY1580/2009 [$H1_{pdm}N1$], FLUAV/sw/England/117316/1986 [$H1_{cl}N1$], FLUAV/sw/Papenburg/12653/2010 [$H1_{pdm}N2$], FLUAV/sw/Schallern/19989/2014 [$H1_{pdm}N1$]; further dose titration experiments with $H1_{av}N1$ and H3N2 virus), and iii) evaluation of virulence of influenza virus strains (based on the clinical parameters dyspnoea score and rectal temperatures of more than 1096 unvaccinated, untreated control pigs from defined high-dose aerosol infection experiments). The parameters recorded were rectal temperature, dyspnoea score, respiratory frequency, and viral load in the respiratory tract. Lungs of pigs were investigated macroscopically and microscopically (histology, immunohistochemistry, *in situ* hybridization).

Lung virus titres were determined. Daily kinetics of virus shedding and antibody induction (HI, NT, NI antibodies) were recorded for at least 1 week after infection. Furthermore, interleukins (IFN-γ, TNFα, IL6), fine distribution of virus in the respiratory tract and other organs, stability of virus after aerosol dispersion, and suitability of the aerosol infection model for testing the efficacy of vaccines and antivirals and for the investigation of the effects of immunosuppressive treatment were investigated.

Infection models. The investigations were done during the development stages of inactivated swine influenza vaccines. The local authorities were notified of the animal trials (Landesverwaltungsamt Sachsen-Anhalt, reference no. AZ 42502-3-401, AZ 42502-3-642Ä, AZ 42502-3-743, AZ 45502-3-579) which were conducted under BSL-2 conditions in infection units with HEPA H13 filters. Staff were vaccinated and had access to antivirals.

Ethics Statement. All trial procedures and animal care activities were conducted in accordance with the guidelines and under approval of Good Clinical Practice (VICH GL9, CVMP/VICH/595/98), the Directive 2001/82/EC on the Community code relating to veterinary medicinal products and German Animal Protection Law. The protocols were approved by the Landesverwaltungsamt Sachsen-Anhalt. The trials were supervised by an animal welfare officer. Challenge trials and blood samplings were granted by the local ethics committee TO (TO 12/97, TO15/09, TO 21/15). Investigations of zoonotic cases were approved by Charité Universitätsmedizin Berlin Ethical Board (reference EA2/126/11 and EA2/218/19).

Table 2: Overview of influenza A virus infection trials in pigs

No. of trials	No. of pigs	Strains	Infection modus	Scientific topics investigated°
102	3131 in total including	50	82x aerosol	proof of efficacy of vaccination
	1728 vaccinated	14 $H1_{av}N1$ (1981-2009)	7x intratracheal (2x low dose, 5x high dose)	proof of efficacy of treatment with oseltamivir
	1346 control*	1 reassorted H1N1 (2006)	4x direct contact	simultaneous infection with 3 subtypes
	41 Tamiflu®-treated	11 $H1_{hu}N2$ (1994-2008)	5x indirect contact (3x in the same infection unit, 1x in neighbouring infection unit)	titration of infection dose
	16 immuno-compromised	1 reassorted H1N2 (2003)	2x intranasal	protection of maternally-derived antibodies
		11 H3N2 (1984-2007)	1x intramuscular	investigations of immunosuppressive compounds
		7 $H1_{pdm}N1$ (2009-2015)		comparison of virulence of different subtypes
		1 $H1_{cl}N1$ (1986)		
		1 H3N1 (2014)		
		1 $H1_{pdm}N2$ (2010)		
		1 duck H5N6		
		1 turkey H9N2		

The 102 infection trials were performed in 70 studies in the years 1997-2016. The trials were carried out in 10 various infection units in 4 buildings at different locations. The majority of the studies were set around onset of immunity (OOI) in which the pigs had an age of 12 weeks; 21 trials addressed duration of immunity (DOI) using pigs 9-12 months old; in 3 trials the efficacy of maternally-derived antibodies was investigated (here challenge infection was performed on 12 and 30 and 33 days old piglets. Some trials covered different scientific topics; for viruses investigated see Supplementary Table 1, page LIX , * among these 1096 with aerosol infection

Viruses used in the trials. The viruses investigated are listed in the supplementary material (Supplementary Table 1, page LIX)

Aerosol infection. Stable aerosols were created through SAG-1 aerosol generators[337,338]. An air pressure of 3.5 bar was set on the generator after the containers had been filled with cell culture supernatant from virus-infected cells and the generator had been fixed on the

ceiling of the infection unit. The generators produce droplets in the size range 0.5 to 20 µm under atmospheric pressure. Droplets that are 2-5 µm reach the alveoli. Aerosol generation was carried out in an air volume of 27.60 m^3 to 187.91 m^3. The highest titres of virus which could be produced in cell culture were nebulised. Sometimes virus harvests were concentrated in order to yield higher virus titres. In infection dose titration studies the viruses were diluted. Pigs were exposed to the aerosol for at least 1 hour.

Proof of virus stability during spraying. The aerosol was collected in roller bottles positioned directly before the generator jets during the nebulisation procedure and the virus content was determined after condensation.

Titration of infection dose. Pigs were exposed to aerosols containing different doses of the corresponding infection strain in different nebulisation runs. The infection dose was titrated using a H3N2 strain from 2003 as representative of the virulent H3N2, $H1_{hu}N2$ and 1980s+1990s $H1_{av}N1$ influenza A viruses, an $H1_{av}N1$ strain from 2003 representing the low virulent $H1_{av}N1$ strains of the 2000s, an $H1_{pdm}N1$ April 2009 virus isolated from a human patient standing for the very highly virulent $H1_{pdm}N1$ viruses, an $H1_{cl}N1$ 1986 virus and an $H1_{pdm}N1$ 2014 virus as representative of the low virulent $H1_{cl}N1$, $H1_{pdm}N1$ 2014/15 and $H1_{av}N1$ 2000s viruses, and an $H1_{pdm}N2$ strain of the newly emerged $H1_{pdm}N2$ viruses representing high virulent viruses such as the $H1_{pdm}N2$ and newly reassorted H3N1 viruses.

Comparative analysis of virulence of strains. Based on the parameters dyspnoea, body temperature and macroscopic lung lesions, a comparative analysis of virulence was carried out selecting those trials in which pigs were at an age of 12 weeks at infection.

Investigation of viral distribution in organ samples. The i) fine distribution of virus in the respiratory tract, ii) viral lung load in lung lesion in comparison to the lung at next location without lesion, and iii) existence of virus in other organs were investigated. The localisation of the genome of the viral NP was investigated by *in situ* hybridisation. The distribution of viral proteins was investigated by IHC using a polyclonal anti influenza virus serum.

Details of the aerosol generation procedure. The aerosol generator SAG-1 consists of a 27 cm x 29.5 cm h x w metal bracket and two cylindrical containers (Supplement 1 A, B, page LX). Centred at the top of the metal bracket there is a hook to fix the equipment. Just below this is a connector for the compressed air infeed (Supplement 1 B, page LX). The pipes for the compressed air lead through the metal bracket from the infeed connector to both the end pieces that can be fixed to the containers. The end pieces are circular with an external diameter of 9.8 cm and an internal diameter of 8 cm (Supplement 1 C, page LX). The compressed air pipes have a diameter of 0.8 cm and are connected to a 4.5 cm long connector in the end piece (Supplement 1 D, page LX). A flexible tube runs from the connector through the middle of the container (Supplement 1 B- E, page LX). This tube reaches to the foot of the container and has a notched piece of metal at the end which enables

uninhibited fluid intake. The jets are opposite the connector (Supplement 1 F, page LX). These are 4.5 cm long. The jet intake point has a diameter of 0.8 cm and lies directly opposite and 3 cm from the opening of the connector. The jet nozzle is 0.2 cm diameter and the jets lie directly opposite each other 0.3 cm apart (Supplement 1 F, page LX).

The generator works by discharging the jets against each other, each jet being connected to the same compressed air supply. The low pressure generated sucks the liquid through the flexible tubes and into the compressed air stream and is directed to the jets through the open space of the container (Supplement 1 G, page LX). On exiting the jets both currents impinge on each other and create a highly dispersed aerosol which is distributed throughout the entire room within 5 minutes (Supplement 1 F-H, page LX).

Prior to nebulisation, both generator containers were each filled with cell culture supernatant and screwed onto the end pieces (Supplement 2 A-G, page LXI). The generators were then attached to the top of the infection units and the compressed air pipes attached (Supplement 2 H, page LXI). In large rooms several generators were used to achieve a sufficient infection density in the aerosol (Supplement 3, page LXII). Before every nebulisation procedure walls and equipment were besprinkled with water to reduce condensation. The infection units were closed, cracks on the doors were taped up, the ventilation was switched on and then compressed air was fed at 3.5 bar to the generators.

During nebulisation the ventilation in the infection units was switched off to keep a stable aerosol in the rooms. The ventilation in the units was switched on again after one to two hours. The aerosol then dispersed within half an hour.

The infection units were air-conditioned, the temperature for pigs set at 22-24°C and the toxic gases hydrogen sulphide, ammonia, carbon dioxide kept under constant control. The infection units conformed to Bio Safety Level 2 and were fitted with HEPA H13 filters. They had a three-part personnel lock with shower facility and a separate lock for animals and materials. The infection unit was run by low pressure (-20 Pa). Within the three-part personnel lock were following pressures: -10 Pa in the part close to infection unit, 0 Pa in the medium part, 10 Pa in the entry part. A complete change of clothes took place upon entering and leaving the locks. During the trial showering was compulsory. Staff were instructed about the study plan and the safety measures prior to each trial. The infection buildings were GLP certificated and regularly audited by the local authorities.

All personnel were vaccinated with the seasonal human influenza vaccine and their serological status was regularly examined. Tamiflu™ was available for the duration of the trial. Personnel did not enter the infection units during nebulisation or for the rest of that day. Once the aerosol has been evacuated it poses no higher risk of infection than at farm level when visiting a farm with influenza. Validation of the infection dose after nebulisation was

carried out by me at my own risk. I had high antibody titres against all strains and wore a face mask for the collection of the aerosol (Supplement 4, page LXIII).

The advantage of the aerosol nebulisation is that large groups of animals can be infected at the same time with no animal manipulation. This method is particularly suited for comparative studies, vaccine testing and the testing of antiviral agents. A prerequisite for successful implementation is the determination of the dose / m^3. This is based on the titration of the infection dose that induces clinical symptoms. The induction of clinical symptoms is essential for the testing of vaccines, antiviral substances and for the evaluation of the virulence of strains.

Influenza infections in healthy individuals are only dangerous in the case of high infection doses and certain virulent strains. With few exceptions, pigs recovered quickly from the infection. Animals that became more seriously ill were removed from the experiment if they suffered.

Intratracheal infection. The pigs were fixed; the position of the cartilage was palpated; a cannula with stylet was punctured through the distal part of the cartilage; the stylet was removed; then the position of the needle was again checked by aspiration of air; finally the virus suspension was slowly injected.

Intranasal infection. Pigs were infected via a nasal sprayer (Dr. Niedermeyer Pharma, Art. No. 32-085).

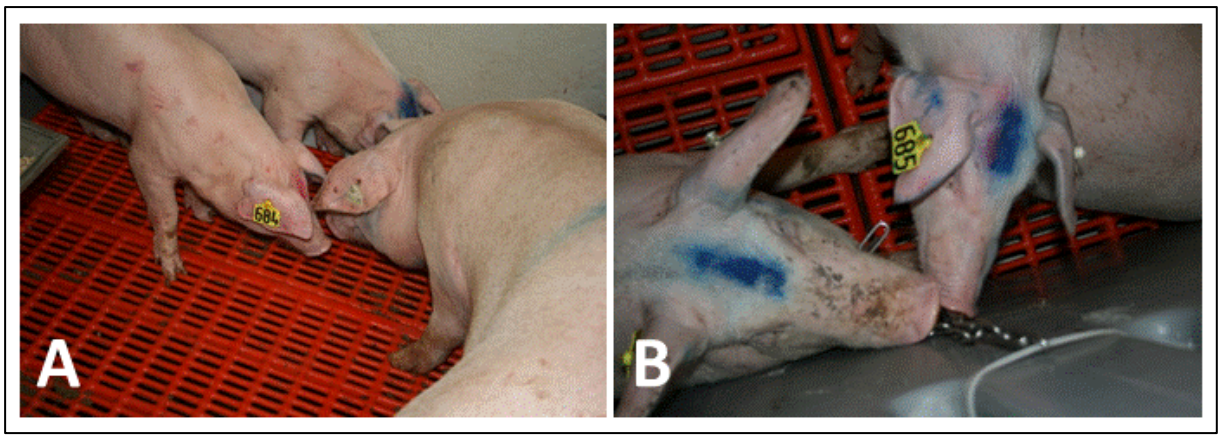


Figure 2: Contact infection
in contrast to indirect contact infection here pigs have contact to each other and can easily transmit virus (A, B)

Contact infection.

Direct contact infection. Two days after infection by aerosol the pigs were held together with uninfected pigs in the same compartment of the infection unit (Figure 2 A, B).

Indirect contact infection. Uninfected pigs were kept in a separate compartment 5 metres distant to the infected pigs in the same infection unit.

Comparative analysis of infection models. The same virus suspension (containing $10^{5.75}$ $TCID_{50}$/ml of strain A/Jena/VI2688/2010 $H1_{pdm}N1$) was used for intratracheal (low dose), intranasal and aerosol infection. Furthermore, direct and indirect contact infection groups were involved. Each group comprised at least 5 pigs.

Study procedures in general. Rectal temperatures and signs of respiratory disease and body weights were recorded before challenge. After infection, rectal temperatures and signs of respiratory disease, dyspnoea, and cough were recorded twice daily 1-3 days p.i. and daily from 4 to 9-14 dpi. Dyspnoea was assessed as follows: 1, increased respiratory frequency and moderate flank movement; 2, marked breathing difficulty and severe flank movement; 3, laboured breathing affecting the entire body, pronounced flank movement and substantial movements of the snout, 4, extreme breathing difficulty reflecting substantial lack of oxygen. Body weights were recorded daily. Nasal swab samples were collected daily in 2.0 ml stabilisation medium containing 60 ml Dextran-Sucrose-Glutamate solution (DSG 72: 126 g dextran 40, 1,5 kg sucrose, 3,6 g potassium-L-glutamate-monohydrate, 5 g potassium-dihydrogen-phosphate, 12,5 g potassium-monohydrogen-phosphate, made up to 10 l with water ad injectionem), 0.2 ml gentamycin (Fagron GmbH, Barsbüttel, Germany), 2 ml amphotericin B (Sigma-Aldrich, Taufkirchen, Germany), made up to 200 ml with cell culture medium (MEM). The animals were removed from the experiment when lungs and tissue samples had to be taken according to monograph EP0963 and at the end of the experiment[76]. This was done according to modern methods, taking into account animal welfare (for details see Dürrwald et al. (2013)[88]). Lung samples were evaluated macroscopically (1, 3 dpi and at the end of the trial). Photographs of the lungs were taken and observed lesions were recorded onto a lung diagram. The percentage of affected lung surface area was assessed for each lobe at dorsal and ventral view. Lung tissue samples were taken from each lobe for virus detection (Figure 3 A). The lung of pigs consists of 2 lobes of the left side (of wich the cranial lobe has two parts) and four lobes of the right side due to morphological characteristics and separation of bronchi; for reasons of convienience the morphological nomenclature was used here and the caudal part of the left cranial lobe was considered as a separate lobe (Figure 3 B). Samples of the right and left halves of the lungs were pooled, ground with sterile sea sand, and diluted 1:10 in dilution medium (1.0 ml Amphotericin B and 0.1 ml Gentamycin, made up to 100 ml with phosphate buffered saline solution). Additionally, lung tissue was collected and fixed in 10% neutral buffered formalin for histopathological evaluation. In some trials lung samples were taken from different parts of the lung, trachea, nose and other organs for fine analysis (Figure 3 B). Nasal swabs and blood samples for immunological analysis were taken before challenge and daily thereafter.

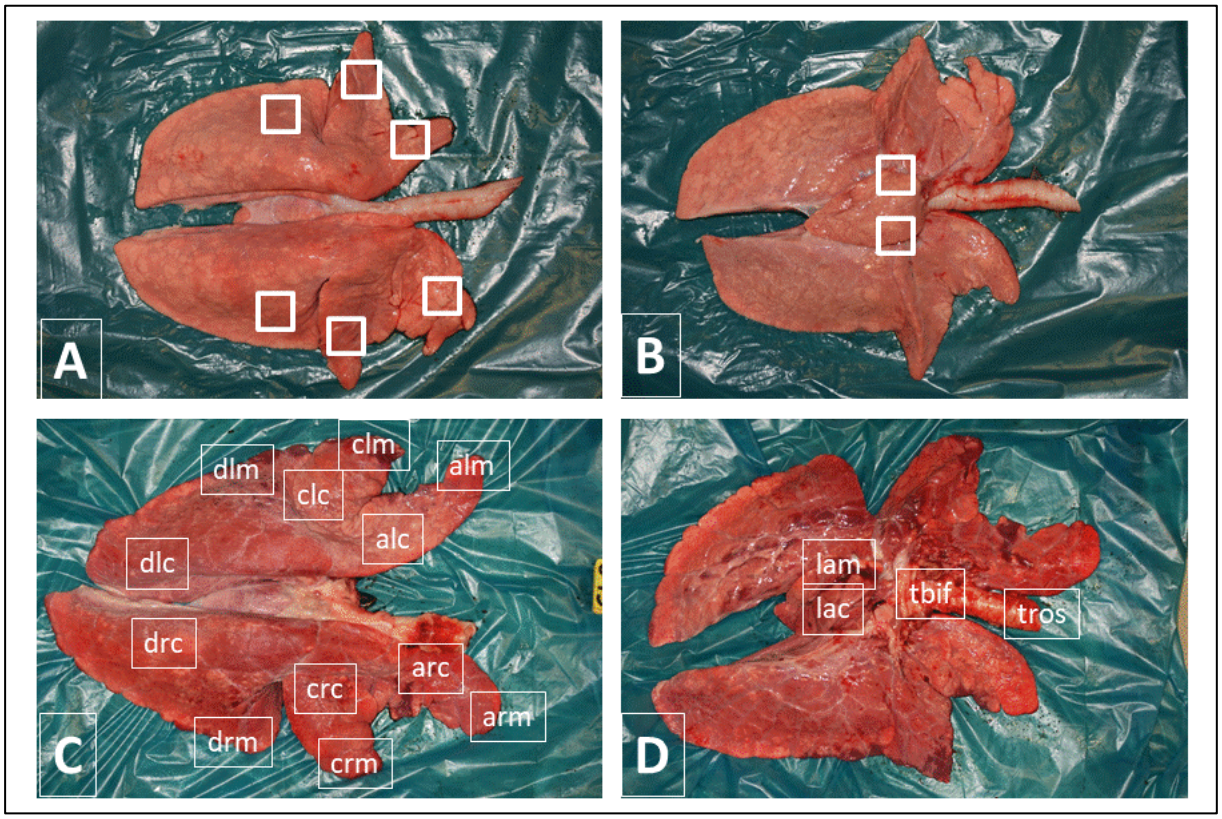


Figure 3: Sampling of pieces of the lung
A, B, uninfected pig without lung lesions; C, D, infected pig with lesions; A, dorsal view, samples taken for lung pool for general investigations: from every lung lobe of every side one piece was taken; B, ventral view; additionally the left and right side of the accessorial lung lobe were sampled; all 4 pieces of every side of lung were pooled; C, dorsal view; for fine analyses more samples were taken: alm, apical lobe left margin; alc, apical lobe left central; clm, cardiac lobe left margin; clc, cardiac lobe left central; dlm, diaphragmatic lobe left margin; dlc, diaphragmatic lobe left central; drc, diaphragmatic lobe right central; drm, diaphragmatic lobe right margin; crc, cardiac lobe right central; crm, cardiac lobe right margin; arc, apical lobe right central; arm, apical lobe right margin; D, ventral view: lam, lobus accessorius margin; lac, lobus accessorius central; tbif, trachea close to bifurcation; tros, trachea rostral

Pigs. The cross-bred swine (Piétrain x LargeWhite in the 1990s or Large White x German Landrace in the 2000s, Figure 4) originated from a pig herd which was monitored regularly for the absence of influenza virus infections. Most trials were conducted on pigs at an age of 12 weeks, but also younger pigs (piglets 12 days old and 30 and 33 days old) and older pigs (9-12 months old) were involved.

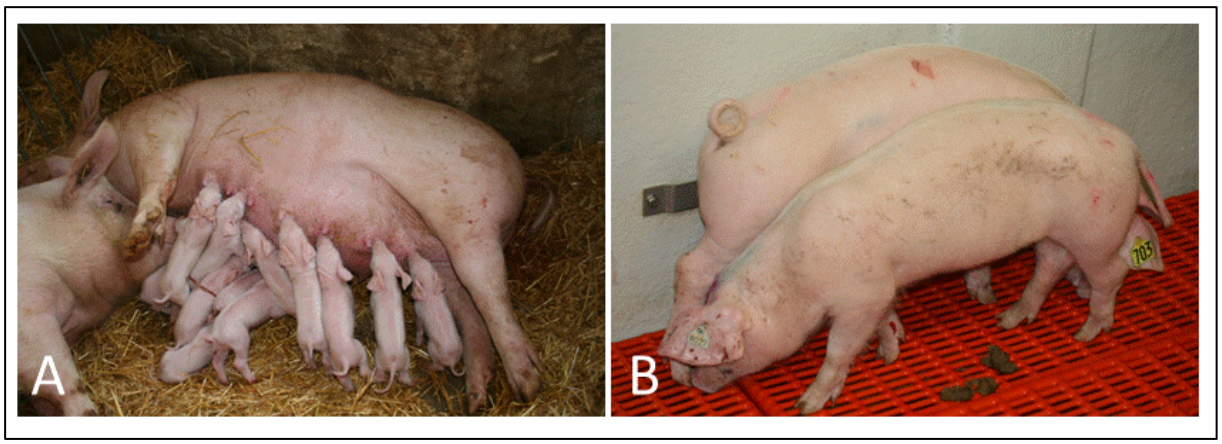


Figure 4: Cross-bred swine were investigated
(Piétrain x LargeWhite in the 1990s or Large White x German Landrace in the 2000s)
A, sow with piglets; B, pigs of an age of 12 weeks were infected in the majority of trials

Treatments. All pigs were treated with antibiotics prior to challenge in order to reduce the influence of bacterial co-infection (Tulathromycin – Draxxin^{TM} 10% ad us. Vet., ZOETIS GmbH – 1 ml per pig in the first week after birth and then every 14 days). This was important for the macroscopic investigation of lungs. In order to verify the freedom of bacteria in the lungs Gram-staining was performed in the histological investigations.

Viruses. European swine influenza A viruses of subtypes $H1_{av}N1$, $H1_{av}N2$, H3N2, H3N1, $H1_{hu}N2$, $H1_{av}N2$, $H1_{pdm}N1$, and $H1_{pdm}N2$ isolated from pig herds during the surveillance of this study in Germany from 2002-2015, $H1_{pdm}N1$ viruses originally isolated from human patients in Hamburg and Jena and Kiel and two avian viruses were investigated (Supplementary Table 1, page LIX). For infection trials, the viruses were grown in MDBK or MDCK cells. A few strains were provided by Jochen Süss, Jena, Germany, Klaus-Peter Behr, Garrel, Germany, Sigrid Baumgarte, Hamburg, Germany, Andi Krumbholz, Jena and Kiel, Germany, Lars Larsen, Copenhagen. Gaëlle Simon, Ploufragan, and Kristin Van Reeth, Ghent, Belgium.

Serological studies. (i) **Hemagglutination inhibition (HI) test** and **(ii) Neutralisation test (NT)** were carried out as previously described (Zell et al., 2008[339]). **(iii) Neuraminidase inhibition assay (NI)** was based on the protocol of Sandbulte *et al*. (2009)[340] and modified (Dürrwald et al., 2013[88]). The HI, NT and NI test were also described in volume 1 of this monograph[1]. **(iv) M antibody-ELISA**. Polystyrene microtiter formate Maxisorp Immuno Modules (Nunc, Roskilde, Denmark) were coated with recombinant influenza A M2 protein (Creative Biomart, Shirley, NY, USA, RFL2949IF, reconstituted to 0.1 mg/ml) in 10 mM sodium phosphate and 250 mM sodium chloride, pH 7.6, for 1 h at 37°C (or overnight at 4°C). After washing (three times in 0.9% sodium chloride + 0.05% Tween 20, Ultrawash Plus, Dynatech Labs, Chantilly, VA, USA) log_2 dilutions of swine sera in PBS (pH 7.2) + 0.05% Tween 20 were put onto the microtiter modules (1:10 to 1:1280) and were incubated for 1 h at 37°C (or overnight at 4°C). After washing Alkaline Phosphatase-conjugated AffiniPure Goat Anti-Swine IgG, Fc Fragment-specific (Jackson Immuno Research Labs, West Grove, PA), diluted 1:3000 in 20 mM Tris- buffered saline pH 8.0 + 0.05% Tween 20 (TBS-T), was added and incubated for 1 h at 37°C. After washing, freshly prepared substrate p-nitrophenylphosphate (pNPP) (1 mg/ml) in 1 M diethanolamin buffer (pH 9.8) + 0.5 mM magnesium chloride was put onto the modules and incubated for 5 min at room temperature. The enzymatic reaction was stopped by the addition of 50 ml of 3 M sodium hydroxide, and read at 405 nm in Dynatech Microplate Reader MRX. An extinction value of ≤0.2 was scored negative.

Virological studies. Pulmonary tissue samples were weighed, ground with sterile sea sand and diluted. Nasal swab samples were introduced into stabilisation medium containing DSG 72, gentamycin and amphotericin B. **(i) Embryonated chicken eggs** were infected by injection into the allantois cavity and incubated for 11 days at 37 °C. Four days after

inoculation allantois fluid was removed. The 50% egg infectious dose (EID_{50}) was established from the hemagglutinating activity by the Spearman and Kaerber method[341,342]. **(ii) Cell cultures.** MDBK or MDCK cells were infected. Trypsin (2 BAEE units/ml) was added to the medium. $TCID_{50}$ was calculated by the Spearman and Kaerber method[341,342]. All titrations of infectious material were done in MDBK cells. Investigation of organ samples was done using MDBK or MDCK cells. The usage of MDCK cells is indicated in the corresponding figures. If not indicated MDBK cells had been used. **(iii) Hemagglutination test.** 0.5% chicken erythrocyte suspensions were used.

PCR, sequencing and phylogenetic analysis. *RT-PCR:* PCR was performed according to Fouchier *et al.* (2000)[343] and as reported recently (Zell et al., 2008[344]). Sequencing and phylogenetic analysis was done by Roland Zell and co-workers at the Universitätsklinikum Jena as desscribed previously (Zell et al., 2008[339]; Krumbholz et al., 2009[345]; Zell et al., 2020[346-348]). The methods used for the phylogenetic analyses in the supplement are listed there. *qPCR:* Viral RNA was extracted using the MagAttract RNA Mini M48 Kit (Qiagen, Hilden, Germany) on the KingFisher Flex Magnetic Particle Processors (Thermo Fisher Scientific, Waltham, USA). Real-time reverse transcriptase qPCR was performed using a pan-Influenza A-M1.2 assay (Hoffmann et al., 2010[349]) and an *in vitro*-transcribed RNA standard.

***In situ* hybridisation.** The *in situ* hybridisation was done by Karin Klingel and co-workers at the Universitätsklinikum Tübingen. Influenza viral RNA in tissues was detected as reported before (Gabriel et al., 2011[350]) using single stranded 35S-Labelled viral RNA probes, which were synthesized from a pBluescript II KS + vector containing a fragment of the NP gene (nt 1,077-1,442) of A/Hamburg/NY1580/2009 ($H1_{pdm}N1$). Linearization of this plasmid with *Hind*III or *Kpn*I, respectively, and subsequent T7 RNA polymerase transcription produced an antisense RNA probe suited to detect NP-specific viral RNA. Control RNA probes were obtained from the vector containing the dual-promotor plasmid of coxsackievirus B3 (pCVB3-R1 (see Klingel et al., 1992[351]). Pretreatment, hybridisation and washing conditions of dewaxed 5-mm paraffin tissue sections were done as described previously (Klingel et al., 1992[351]). Slide preparations were subjected to autoradiography, exposed for 3 weeks at 4°C and counterstained with haematoxylin and eosin.

Immunohistological and immunohistochemical investigations. The pathology of the lungs was evaluated macroscopically, photographs were taken, and observed lesions were recorded onto a lung diagram. Percentage of affected lung surface area was assessed for each lobe at dorsal and ventral view.

The immunohistological and immunohistochemical investigations were done by Dr. Theophilé Vissiennon, Institut für Tierpathologie Leipzig. Sera of not infected rabbits were used to evaluate specificity of the staining (Supplement 5, page LXIV). Formalin-fixed lung tissue samples were embedded in paraffin. 5 µm-thick sections were stained with

haematoxylin and eosin for light microscopy. Inflammation was scored on a semi quantitative scale from 0-7: 0, no inflammation; 1, discreet interstitial alveolar macrophages; 2, slight interstitial bronchial associated lymphoid tissue hyperplasia; 3, distinct interstitial alveolar macrophages; 4, distinct interstitial and massive broncholuminal alveolar macrophages; 5, large areas of interstitial and broncholuminal macrophages and eosinophil granulocytes, 6, large areas of macrophages and massive interstitial neutrophil granulocytes; 7, massive areas of inflammation with fibrin exsudation (see also Supplement 78, page CLXVI).

In order to stain viral antigens specifically the StreptAvidin-Biotin Peroxidase Complex (SABC) method was applied using Dako Autostainer Plus (Dako GmbH, Hamburg). Samples were treated with xylol and ethanol in decreasing concentrations to remove paraffine and heated to 37°C. Endogenous peroxidases were inactivated by Flex Peroxidase-Blocking Reagent over 5 min. The sample was adjusted at pH-value of 6. A polyclonal monospecific rabbit immune (corresponding to the subtype used in the trial) serum was diluted 1:100 and used as primary antibody. Samples were incubated at 37°C 30 min. Flex/HRP (HRP, Dako GmbH, Hamburg) was added to the substrate and incubated over 30 min. The substrate-chromogen-reaction was performed twice, each 5 min. Then the samples were treated with Flex Substrate Buffer. Preparations were stained with Haematoxylin in order to make the nucleoli visible. Gram-staining was performed in order to exclude bacterial co-infection.

Investigation of clinical chemical parameters and interleukins. The clinical chemical parameters haptoglobin, creatinine kinase, aspartate transaminase, alanine transaminase, gamma-glutamyl transferase, glutamate dehydrogenase, creatinine, urea were investigated under contract by Synlab, Leipzig, Germany. IFNγ, TNFα and IL6 were determined by commercially available ELISAs (Quantikine porcine IFNγ, TNFα and IL6 Kits, R&D Systems, Wiesbaden, Germany).

Statistical evaluation. The Mann-Whitney U-test was performed. Data of statistical calculations were either provided in tables shown below the figures or shown in the figures by asterisks (*, $p<0.05$ = significant; **, $p<0.01$ = highly significant; ***, $p<0.001$ = very highly significant) or mentioned in the legends to the figures (if inclusion into the figures would have led to an overload of the figure). With the exception of a few figures (in which the not significant result was very important) not significant results were not marked in the figures (which means if significances were not marked in the figures or mentioned in the legend of the figure the differences shown were not significant).

Investigation of virulence of swine influenza viruses. The virulence of selected influenza A viruses was investigated in animal experiments. For this purpose, an aerosol-mediated challenge model was developed and validated. In order to match the different doses used

in the studies to the clinical score (dyspnoea score), an index of the severity of the disease (short disease index) was developed. This index is based on the observation that there is a linear relationship between the dose of infection and the degree of dyspnoea. The disease index is the deduced dyspnoea score for the corresponding infectious dose calculated by regression analysis. The equation for this disease index (DI) is as follows:

DI = m ID + b

DI = arithmetic mean of the dyspnoea score of the first three days after infection (= most important period after infection during which the strongest degree of dyspnoea is expressed), ID = infectious dose (lg $TCID_{50}/m^3$)

i) for low virulent viruses (like $H1_{cl}N1$ 1986, $H1_{pdm}N1$ 2014/15, $H1_{av}N1$ 2000s viruses):

m=0.1636; b=0.4897 ($H1_{cl}N1$) (titration of infectious dose of strain A/sw/England/117316/1986 $H1_{cl}N1$)

m=0.1869; b=0.9995 ($H1_{av}N1$ 2000s)

m=0.1736; b=0.8995 ($H1_{pdm}N1$ 2014) (titration of infectious dose of strain A/sw/Schallern/19989/2014 $H1_{pdm}N1$)

ii) for common influenza A viruses circulating in pigs (like H3N2, $H1_{hu}N2$, $H1_{av}N1$ 1980s viruses):

m=0.2454; b=0.0563 ($H1_{av}N1$ 1980s)

m=0.5493; b=3.1783 ($H1_{hu}N2$)

m=0.3343; b=1,2186 (H3N2)

iii) for more virulent influenza A viruses (like $H1_{pdm}N2$ and H3N1 virus):

m=0.5008; b=1.8263 (titration of infectious dose of strain A/sw/Papenburg/12653/2010 $H1_{pdm}N2$)

iv) for highly virulent influenza A viruses (like $H1_{pdm}N1$ April 2009 virus):

m=0.7662; b=1.5190 (titration of infectious dose of strain A/Hamburg/NY1580/2009 $H1_{pdm}N1$).

The equations provided in bold result from infection trials in which the infectious dose was titrated. All other equations are approximations resulting from calculations with selected strains of the corresponding subtype that had been nebulised at different doses.

The data obtained by regression analysis were used for the calculation of the corresponding values for single dose infection trials by introducing a correction factor (Cf):

Cf = DI of the corresponding virus type – arithmetic mean of dyspnoea 1-3 dpi measured for the special strain at the infection dose used in the trial.

These data were used for comparative analysis of different influenza A viruses.

4.2 INVESTIGATION OF MATERNAL IMMUNITY

The main subjects studied are listed in Table 3. Essentially, the following aspects were investigated: i) How long do maternal antibodies persist and how long does the immunity that cannot be measured via maternal antibodies last? The latter was determined indirectly by immunising pigs from vaccinated or convalescent sows at different times after birth and determining antibody induction. If they did not react serologically to the vaccination, this was a sign that maternal immunity still existed. ii) What happens after vaccination in maternal immunity? Are there priming effects? Are there synergistic effects between vaccination and maternal immunity? iii) How strong is the protection of maternal antibodies against challenge infections and how long does it last? iv) What are the shedding kinetics of pigs with maternal immunity compared to those without?

Sows were vaccinated at different stages of pregnancy and serum and colostrum antibodies were tested after farrowing. To investigate the interference of maternal immunity, sows were vaccinated before farrowing. Their piglets were monitored for the kinetics of maternally derived antibodies. The piglets were then divided into groups and vaccinated at different times. Blood samples were taken at intervals and analysed for antibodies. To investigate the protection provided by maternal immunity, piglets from vaccinated sows were challenged at different times after birth and compared with piglets from unvaccinated sows. The laboratory methods used to study maternally derived immunity and the procedures in infection experiments are described in the previous chapter (4.1).

Table 3: Overview of investigations on maternal immunity

Topic of investigation	Procedure	Animals*
Maternal antibodies in serum and colostrum	Sows were vaccinated in the last trimester and serum and colostrum antibodies measured at birth	13 sows
Post-vaccination seroconversion affected by maternal immunity	Pigs from one farm with previous $H1_{av}N1$ infection and one farm without infection were tested for the presence of antibodies over 61 weeks and vaccinated 4 times during this period (bivalent $H1_{av}N1$+H3N2 mineral oil-adjuvanted vaccine)	20 pigs
Duration of interference of maternal immunity with seroconversion after vaccination	10 sows were vaccinated (trivalent $H1_{av}N1$, $H1_{hu}N2$, H3N2 Carbopol-adjuvanted vaccine) and the antibody kinetics of their piglets were determined; the piglets were vaccinated at different times to study the serological response to vaccination (with the same vaccine), at 33 weeks of age a challenge infection was performed (simultaneous challenge with $H1_{av}N1$, $H1_{hu}N2$, H3N2)	10 sows, 18 piglets

Table continued on next page

Topic of investigation	Procedure	Animals*
Investigation of vaccination priming effects in piglets with maternal immunity	Pigs from sows with and without previous $H1_{pdm}N1$ infection were vaccinated at 8 and 12 weeks of age, and a challenge infection was carried out on these pigs and an unvaccinated control group at 13 weeks of age	11 pigs
Protection of maternal immunity two weeks after birth	Five sows were immunised 5 and 2 weeks before farrowing ($H1_{av}N1$+H3N2 bivalent mineral oil adjuvanted vaccine) and two sows remained unvaccinated; one sow from each group and her piglets were challenged with $H1_{av}N1$ two weeks after farrowing	7 sows, 24 piglets
Protection of maternal immunity four weeks after birth	Sows were vaccinated 5 and 2 weeks before farrowing (monovalent $H1_{pdm}N1$ Carbopol-adjuvanted vaccine), other sows remained unvaccinated, half of the piglets of both groups were vaccinated with the same vaccine 3 and 24 days after farrowing, antibodies were measured, 7 days after the second vaccination a challenge was performed with a different $H1_{pdm}N1$ virus than the vaccine formulation	20 sows, 75 piglets
Protection of maternal immunity five weeks after birth against simultaneous challenge	6 sows were vaccinated 4 and 1 week before farrowing (trivalent $H1_{av}N1$, $H1_{hu}N2$, H3N2 Carbopol-adjuvanted vaccine), other sows remained unvaccinated; piglets from vaccinated and unvaccinated sows were challenged at 33 days of age with a different $H1_{av}N1$, $H1_{hu}N2$ and H3N2 virus mixture from the vaccine	12 sows, 36 piglets

* Piglets used in challenge experiments are also included in Table 2

5. RESULTS

5.1 PATHOGENESIS OF SWINE INFLUENZA

Key findings on swine influenza pathogenesis are summarised in Table 4. Only the most important data is presented in the evaluations in this chapter. Detailed analyses were carried out for all tests. Two examples of the analysis of individual data in the animal experiments can be found in the Supplement (page CLX, page CLXXI).

Table 4: Overview of key results on pathogenesis

Topic	Subtopic	Major results
Investigation of swine influenza infection models	Respiratory infection models	All models are linked by infectious dose, with only high doses producing marked disease (page 42 - 43)
	High dose infection models	Aerosol infection is superior in terms of ease of use and validity of results (pages 42 - 43)
	Contact infection	Contact infection does not induce pronounced disease despite high viral replication (pages 42 -43)
Validation of high-dose aerosol infection	Nebulization procedure	Nebulisation does not reduce the infectivity of the virus being sprayed (Supplement chapter 9 – Stability of nebulized viruses, page XCVII)
	Virus distribution in respiratory tract	The viruses are spread through the whole of the respiratory tract (pages 61 - 68)
	Lung gross lesion	Expansion of the lungs gross lesions follow a regular pattern (pages 69 - 70)
	Parameters of infection	Disease can be induced by rapidly delivering high doses to the lungs; disease is best reflected by the dyspnoea score parameter; fever occurs at 1 and sometimes 3 dpi; virus shedding peaks 1-3 dpi; first antibodies appear at 5-7 dpi, but mechanisms of immunity may act earlier, reflected by reduced virus shedding (pages 42 - 43)
Titration of infectious dose by aerosol	Investigation of different FLUAVs infecting pigs	Influenza A viruses show different degrees of virulence, which is expressed in the steepness of the regression line of dyspnoea; viruses from virulence groups with steep regression lines can lead to fatal disease progression at high exposure doses (pages 44 -54)
Investigation of virulence	Characterisation of the pattern of virulence of FLUAV at high-dose aerosol infection	Different patterns of virulence have been observed for the subtypes, with the highest virulence being induced by the April 2009 $H1_{pdm}N1$ viruses, leading to mortality rates of around 20% (page 47 - 54)
Organ distribution	Immunostaining	There is a wide distribution of viral antigens in all organs after infection, despite a lack of lytic infection outside the respiratory tract (pages 61 - 65)
Lung lesions	Expansion	The expansion of lung lesions follows a regular pattern (pages 69 -70)
	Comparison to neighbouring regions	The comparison of lesions and neighbouring regions without lesion indicate a higher cytokine release in lesions (Figure 40, page 75)

Table continued on next page

Topic	Subtopic	Major results
Application of high dose aerosol infection for scientific investigation	Investigation of immunogenic effects of HA, NA, and M genes	HA and NA protect against disease, whereas M does not protect sufficiently and may worsen lung pathology, possibly by induction of necroptotic pathways leading to cytokine secretion (pages 76 - 82)
	Investigation of the efficacy of oseltamivir	Treatment with oseltamivir resulted in less severe respiratory distress and fewer lung lesions compared with pigs that were not treated (pages 83 - 89)
	Investigation of the effects of immunosuppression	Immunosuppression prolonged viral shedding (pages 89 - 94)

5.1.1 INVESTIGATION OF EXPERIMENTAL INFECTION MODELS

5.1.1.1 COMPARATIVE ANALYSIS OF SWINE INFLUENZA INFECTION MODELS

Background. In terms of disease induction, the results of different infection models vary. To identify the reasons for this variation in clinical outcome, a comparative analysis of infection models using identical material for infection may be useful.

Study design. The same viral suspension (containing $10^{5.75}$ $TCID_{50}$/ml of strain A/Jena/VI2688/2010 $H1_{pdm}N1$) was used for intratracheal, intranasal and aerosol infection. Direct and indirect contact infection groups were also included in the study (Table 5). Each group consisted of at least 5 pigs. For intranasal and intratracheal infection, 2 ml of this suspension was used per pig, corresponding to $10^{6.05}$ MDBK $TCID_{50}$ per pig. For aerosol infection, 2000 ml of suspension was nebulised, corresponding to a dose of $10^{7.48}/m^3$. Direct contacts were housed with aerosol-infected pigs. Indirect contacts had no contact with pigs but were housed in the same room in a distant compartment. Dyspnoea was scored as follows: 0 = breathing unaffected; 1 = increased respiratory rate and moderate flank movement; 2 = marked dyspnoea and severe flank movement; 3 = dyspnoea affecting the whole body together with marked flank movement and severe snout movement; 4 = severe dyspnoea reflecting substantial oxygen deprivation. Dyspnoea was scored independently by two veterinarians. One veterinarian (RD) has participated in all trials since 1997 to ensure consistency of assessment.

Table 5: Overview of the experimental design of the trial

Infection route	Infection dose	Number of pigs
aerosol	$10^{5.75}$ MDBK $TCID_{50}$ x 2000 in air (37,5 m^3)	5
intratracheal	$10^{5.75}$ MDBK $TCID_{50}$ x 2	5
intranasal	$10^{5.75}$ MDBK $TCID_{50}$ x 2	5
Direct contact	unknown	5
Indirect contact	unknown	6

Results. The data show similar antibody and shedding profiles for all infection groups, with the exception of a delay of 2 days in the indirect contact group. Despite this similarity, viral shedding peaked earlier and antibodies appeared earlier in the aerosol-infected pigs compared to the other groups (Figure 5).

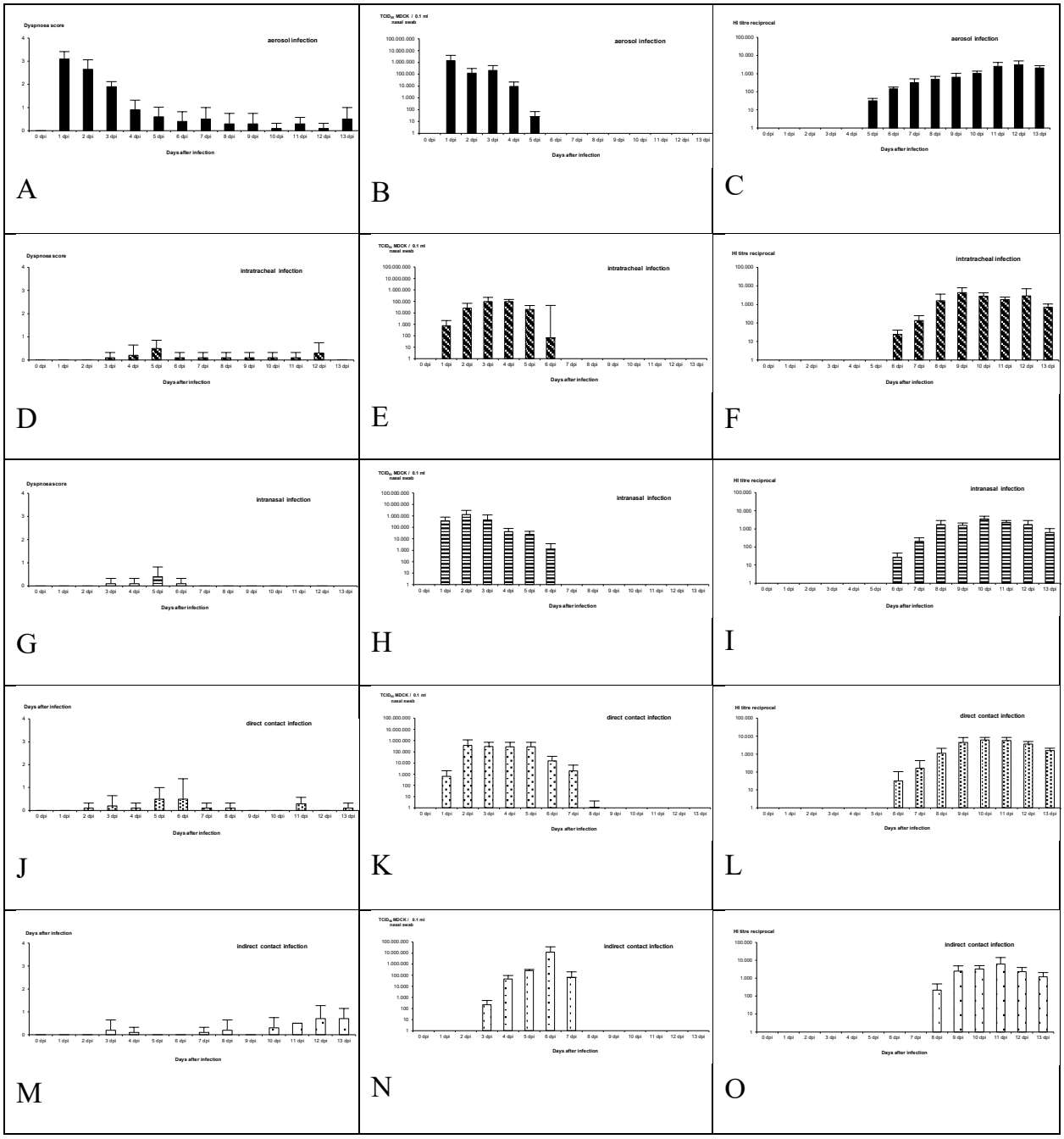


Figure 5: Kinetics of key parameters after different routes of experimental infection

with strain A/Jena/VI2688/2010 (H1$_{pdm}$N1), A-O: (A) dyspnoea, (B) virus shedding, (C) HI antibody kinetics after aerosol infection, (D) dyspnoea, (E) virus shedding, (F) HI antibody kinetics after intratracheal infection, (G) dyspnoea, (H) virus shedding, (I) HI antibody kinetics after intranasal infection, (J) dyspnoea, (K) virus shedding, (L) HI antibody kinetics after direct contact infection, (M) dyspnoea, (N) virus shedding, (O) HI antibody kinetics after indirect contact infection. For the full printout of this comparative study see Supplement 6, page LXVII; dpi, day post infectionem; A-C aerosol infection, D-F intratracheal infection, G-I intranasal infection, J-L direct contact infection, M-O indirect contact infection; further data on indirect contact infection are shown in Supplement chapter 6 – Indirect contact infection, page LXIX, arithmetic means with standard deviation, geometric means for antibodies

This reflects that regardless of the route and dose of infection, pigs respond with an immune response immediately after contact with the virus. The 2-day delay in the indirect infection group reflects the delayed infection due to the lack of direct contact and most likely reflects airborne transmission in the compartment. The rapid infection of the contact groups indicates that the virus is highly infectious. The induction of antibodies was always associated with an elimination of the virus. Notably, shedding profiles and antibody kinetics were similar in all groups, indicating infection in all infection models. Reults of trials on intramuscular infection and comparative analysis of aerosol and high-dose intratracheal infection are shown in the supplement (page LXV, page LXXI).

Short interpretation of results. The infection profile was similar in all groups, with the major discrepancy in disease expression occurring only in the aerosol-infected group in the form of severe dyspnoea (Figure 5). In addition, virus shedding peaked at 1 dpi and antibodies appeared one day earlier in aerosol-infected pigs compared to the other routes of infection (Figure 5). The reason for the difference between aerosol and the other infection groups is the higher dose. Despite the fact that the same viral suspension was used for infection (intratracheal, intranasal), the nebulisation of 2000 ml of this viral suspension confronted the aerosol-infected pigs with a high viral dose that was immediately positioned in the lungs, which is also reflected in the high virus shedding at 1 dpi. This is in contrast to the other infection models. Therefore, dose titrations of the infectious dose in aerosol infection are necessary to further elucidate this phenomenon.

5.1.2 THE INFECTIOUS DOSE - THE MAIN CONTRIBUTOR TO DISEASE SEVERITY

5.1.2.1 DOSE TITRATION OF INFLUENZA VIRUSES BY AEROSOL

Background. As mentioned above, the dose of infection may be critical for disease induction in experimental influenza A virus infection. Nebulisation of different doses of influenza A virus may provide further insight into this issue.

Study design. Infection dose titration was performed using aerosol infection with different viruses (Table 6). Infection was carried out on 3-month-old-pigs in successive runs in a separate room close to the infection unit, starting with the lowest dose. The pigs were exposed to the aerosol for 1 h and then returned to the infection unit.

Results. The studies show a clear dose-dependent linearity in the induction of clinical symptoms by influenza viruses. High infectious doses induce strong symptoms, low infectious doses hardly any symptoms. In addition, there are differences in virulence between the viruses. Highly virulent viruses reflect steeply sloping regression lines, low virulence viruses flat regression lines (Figure 6, Figure 7, Figure 8, Figure 9).

Table 6: Overview on infection trials in which the infectious dose was titrated in pigs

Virus	Infection doses in lg $TCID_{50}/m^3$ or EID_{50}/m^3 *	Number of pigs in each dose group§
FLUAV/Hamburg/NY1580/April 2009 ($H1_{pdm}N1$)	6.3, 4.3, 2.3, 0.3, -1.7 °	6
FLUAV/sw/England/117316/1986 ($H1_{cl}N1$)	7.71, 6.71, 4.71, 2.71, 0.71	6
FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$)	10, 8, 6, 4	13
FLUAV/sw/Schallern/19989/2014 ($H1_{pdm}N1$)	9.46, 8.21, 7.21, 6.21	13
A/sw/Bakum/1769/2003 (H3N2)"	9.06, 7.06, 5.03, 3.05, 1.04, -1.01, -3.64	2
A/sw/Vechta/2623/2003 ($H1_{av}N1$)"	7.43, 6.97, 5.97, 4.97, 3.97, 2.47	2

* determined in MDBK cells (°for MDCK higher: + lg 2), " for H3N2 and $H1_{av}N1$ virus the EID_{50} was determined as shown in this table; § there were additional groups not listed in the table like contact and control groups

A steep regression line was observed in dose titration with $H1_{pdm}N1$ April 2009 virus (Figure 6).

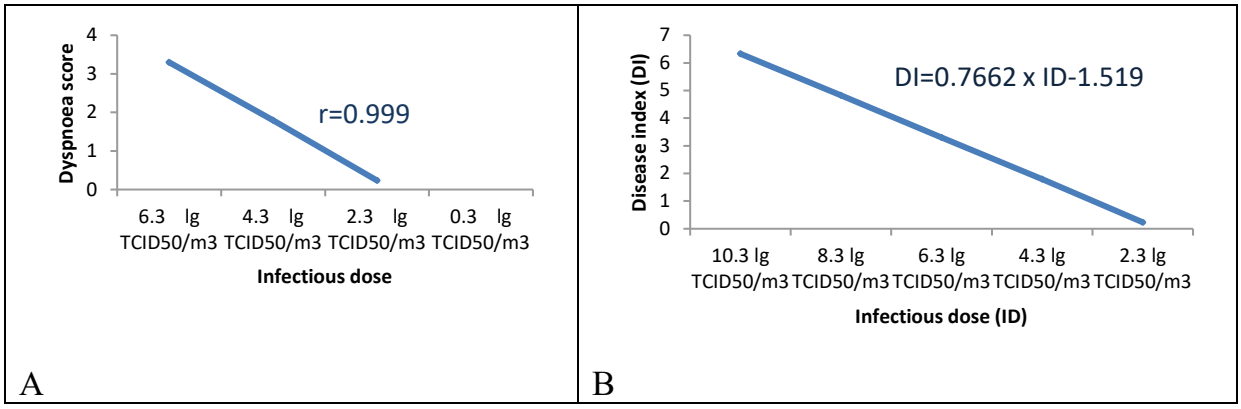


Figure 6: Strong correlation between dyspnoea and infectious dose ($H1_{pdm}N1$)
Infection of pigs with $H1_{pdm}N1$ April 2009 virus (A/Hamburg/NY1580/April2009); A, Pearson correlation coefficient r=0.99994574; B, Regression RGP: 0,7662xID-1.5190 allows for the calculation of disease index (arithmetic mean of dyspnoea 1-3 dpi)

Dyspnoea after infection with FLUAV/sw/England/117316/1986 ($H1_{cl}N1$) followed a linear trend (Figure 7). The slope of the trend line was low, which is an indication of very low virulence of this virus (Figure 7).

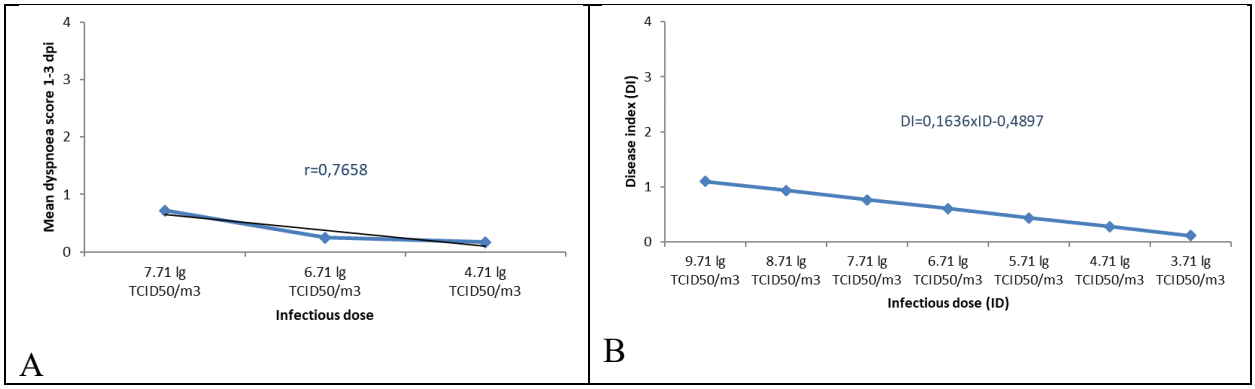


Figure 7: Correlation between infectious dose and induction of disease ($H1_{cl}N1$)
Infection with FLUAV/sw/England/117316/1986 ($H1_{cl}N1$); A, arithmetic mean of the dyspnoea score 1-3 dpi and trend (r=0.7658); B, deduced Disease index (DI); DI= 0,1636xID-0.4897; ID, Infectious dose

Based on data obtained after titration of FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$) in pigs, the disease severity index (short disease index, DI) was derived for newly reassorted and more virulent strains such as $H1_{pdm}N2$ virus. The arithmetic mean of the dyspnoea score of the first 3 dpi was calculated and its correlation with the infectious dose was determined (Figure 8 A). A regression analysis was performed (Figure 8 B).

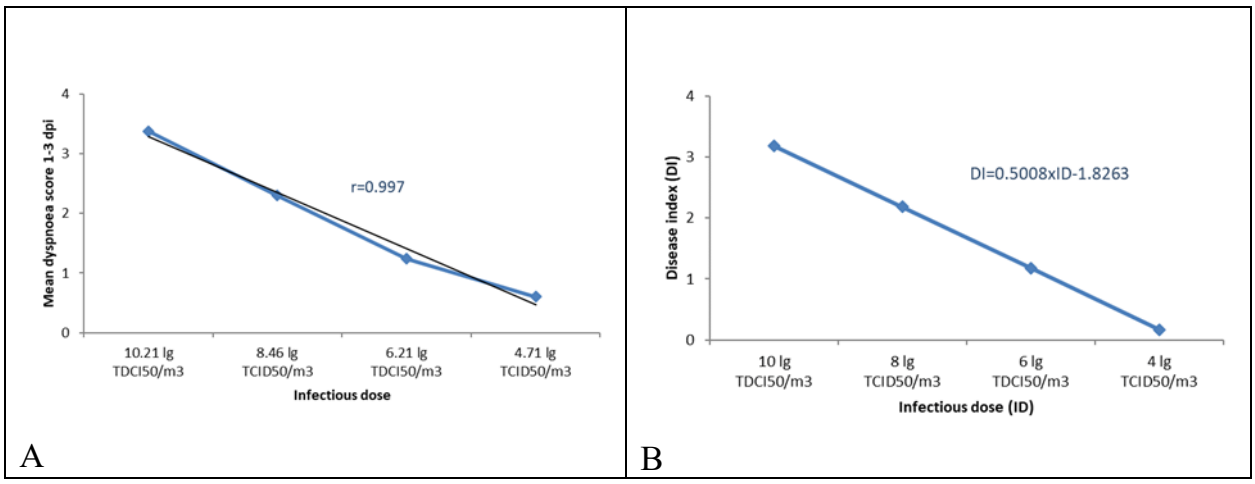


Figure 8: Correlation between infectious dose and dyspnoea score ($H1_{pdm}N2$)
Infection of pigs with A/sw/Papenburg/12653/2010 ($H1_{pdm}N2$) virus; A, Pearson correlation coefficient; B, Deduction of disease index for $H1_{pdm}N2$ virus (DI = 0.5008 x ID – 1.8263); DI, Disease Index; ID, Infectious Dose

The data established after titration of FLUAV/sw/Schallern/19989/2014 ($H1_{pdm}N1$) in pigs showed a strong linear dependence of disease induction on infectious dose (Pearson correlation coefficient r=0.92). The straight line derived by regression analysis was flat, indicating a very low virulence of this virus, similar to that observed for the 1986 $H1_{cl}N1$ virus (Figure 9*)*.

Due to the low number of pigs included in the H3N2 and $H1_{av}N1$ dose titrations no calculations were done.

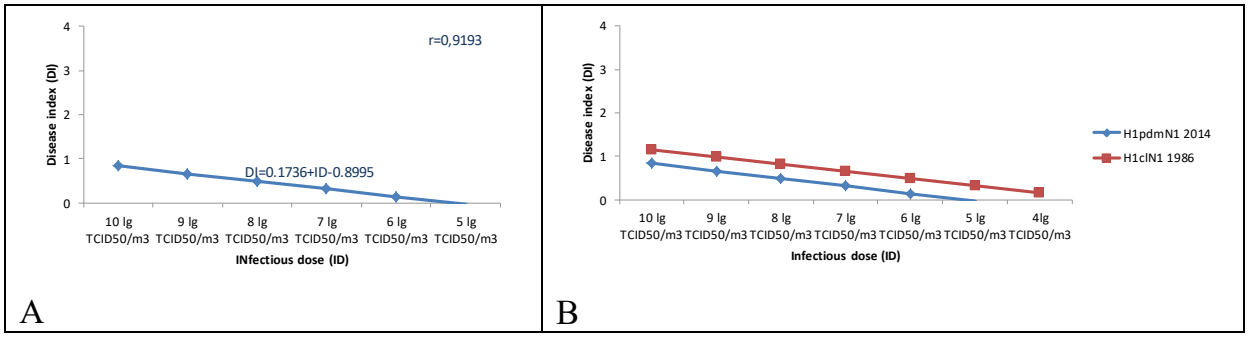


Figure 9: Deduction of disease index for swine pandemic $H1_{pdm}N1$ 2014 viruses
Infection with A/Schallern/19989/2014 ($H1_{pdm}N1$) virus; blue lines, B, in comparsion to classical $H1_{cl}N1$ 1986 virus (A/sw/England/117316/1986), red line

Discussion. The results show that the virulence of influenza viruses can be determined via dose titrations. Influenza viruses differ in their virulence. The linearity of the induction of clinical symptoms allows the establishment of virulence groups and the determination of the corresponding virulence within these groups, even for viruses in which only one infective dose was used experimentally.

5.1.3 INVESTIGATION OF THE VIRULENCE INFLUENZA A VIRUSES AFTER HIGH-DOSE AEROSOL NEBULISATION

5.1.3.1 COMPARATIVE ANALYSIS OF VIRULENCE OF INFLUENZA A VIRUSES IN PIGS

Background. Different disease expression was observed for different virus subtypes, but also within the H1N1 subtype, during the years of experimental infection of pigs with influenza A viruses. H3N2 viruses were reliable strains for infection and showed a constant induction of disease. With the use of $H1_{av}N1$ strains from the early 2000s onwards, it was difficult to see any induction of disease, even when very high doses of these viruses were nebulised. The first infection studies with pandemic H1N1 viruses showed remarkable virulence.

Study design. In order to examine this pattern in detail, pigs in the control groups of all trials that received high-dose aerosol infection were examined. The following parameters were selected for analysis: i) dyspnoea score at 1 dpi (when clinical signs are overt), ii) rectal temperatures at 1 dpi (this is when the highest temperatures are observed), iii) lung pathology. An overview of the analysed data is given in Table 7. For lung pathology, four relevant studies were selected in which $H1_{av}N1$, $H1_{hu}N2$, H3N2 and $H1_{pdm}N1$ 2009 viruses were analysed (Table 8).

Table 7: Number of viruses and control pigs used to analyse clinical parameters

Subtype	Strains investigated	Number of pigs in total	Range of Infection dose ($TCID_{50}/m^3$)
$H1_{av}N1$	12	324	$10^{7.08\text{-}10.0}$
H3N2	8	166	$10^{8.02\text{-}10.26}$
$H1_{hu}N2$	7	258	$10^{6.18\text{-}8.76}$
duckH5N6	1	11	$10^{7.0}$
turkeyH9N2	1	6	$10^{8.58}$
$H1_{pdm}N1$ April 2009	6	331	$10^{5.5\text{-}7.26}$

Table 8: Viruses and pigs used to compare macroscopic lung lesions

Subtype	Strain	Number of pigs	Infection dose ($TCID_{50}/m^3$)
$H1_{av}N1$	Bad Griesbach/5604/2006	6	$10^{8.5}$
$H1_{hu}N2$	Kitzen/6142/2007	8	$10^{8.76}$
H3N2	Damme/5673/2006	8	$10^{9.76}$
$H1_{pdm}N1$	Hamburg/NY1580/2009	5	$10^{5.75}$

These were the pigs included in the analysis shown in Figure 14 D-H, page 51.

Results. The earliest $H1_{pdm}N1$ viruses of 2009 induced the most prominent symptoms. They were associated with severe pneumonia, large lung lesions and the highest mortality (Figure 22, page 59). In contrast to the $H1_{pdm}N1$ viruses of 2009, $H1_{av}N1$ viruses from the beginning of 2000 onwards caused almost no illness. They did not even cause fever (Figure 14 B).

While for the evaluation of the symptoms only those trials were selected in which the daily examination of the symptoms was included, for the calculation of the mortality rate all control pigs were used. One strain of $H1_{pdm}N1$ virus (A/Hamburg/NY1580/2009) repeatedly caused death in a few pigs. 9 of 331 control pigs infected with this $H1_{pdm}N1$ virus died 2-3 dpi due to severe hypoxia (mortality 2.72%: compare case-fatality rate of 1918 influenza in humans 2.5%; the mortality of A/Hamburg/NY1580/2009 in single trials was between 5 and 23%). All fatal cases had strong lung lesions covering more than 30% of the lung. In comparison, of the 324 control pigs infected with $H1_{av}N1$ viruses, only 1 pig died (0.31% mortality; caused by the more virulent strain A/sw/Bakum/3543/1998 ($H1_{av}N1$ 1C.1). 1 of the 166 control pigs infected with H3N2 viruses died during the course of the infection (0.6% mortality, sudden death of a pig with a body weight of 200 kg 1 dpi caused by strain A/sw/Bondelum/5959/2007). None of the 258 unvaccinated control pigs infected with $H1_{hu}N2$ virus died. The duck H5N6 virus did not induce disease in pigs, whereas the turkey H9N2 virus induced symptoms similar to the low virulent $H1_{av}N1$ strains of the 2000s.

The higher virulence of the $H1_{pdm}N1$ viruses and the lower virulence of the $H1_{av}N1$ viruses of the 2000s turned out to be highly significant. Despite the slight variation in the infectious dose, this conclusion is justified because all the infectious doses sprayed were high infectious doses. In addition, the infectious doses used for the 2000s $H1_{av}N1$ viruses were higher in all the trials than the infectious dose used for the 1980s and 1990s $H1_{av}N1$ viruses. Furthermore, infection doses for $H1_{pdm}N1$ viruses were comparable or lower than for $H1_{av}N1$ viruses. Infections with avian influenza A viruses turned out to be mild, causing either no symptoms or mild symptoms comparable to the $H1_{av}N1$ viruses of the 2000s. $H1_{av}N1$ viruses isolated since 2000 (HA cluster 1C.2) were significantly less virulent than $H1_{av}N1$ viruses isolated before 2000 (HA cluster 1C.1) (Figure 10). H3N2 and $H1_{hu}N2$ viruses have not changed their virulence over the decades (Figure 11, Figure 12).

To make the values comparable, a disease index was calculated for a given infectious dose based on data obtained from regression analysis and correction factors calculated for each strain (Figure 10, Figure 11, Figure 12). The data reflect remarkable differences in virulence between $H1_{av}N1$ viruses of the 1980s/1990s and 2000s, with an intermediate virus isolate from 2001 (Figure 10).

In the study of H3N2 viruses, different age groups were analysed. Older pigs (12 months) are more tolerant to infection and show less disease. Despite variations in the virulence of individual strains, H3N2 viruses retain their virulence over years.

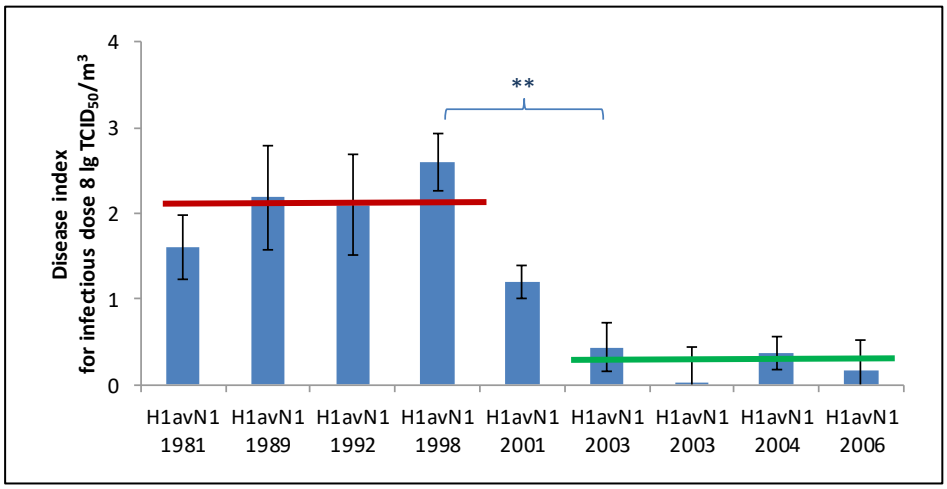


Figure 10: Virulence of German H1$_{av}$N1 swine influenza A viruses isolated 1981-2006
*(in pig infection trials, age of pigs at infection: 12 weeks); the disease index (DI) was calculated for an infectious dose of 8 lg TCID$_{50}$/m^3 (DI = 8 x values of the corresponding regression line – correction factor for the corresponding strain); DI is based on the arithmetic mean of dyspnoea score 1-3 dpi, **, p<0.01 (all H1$_{av}$N1 viruses isolated before 2000 reflected a significantly higher virulence in comparison to those isolated thereafter; the strain isolated in 2001 = FLUAV/sw/Belzig/02/2001 = ranges exactly between both other groups); red bar, arithmetic mean value of H1$_{av}$N1 1980s/90s viruses; green bar, arithmetic mean of H1$_{av}$N1 2000s viruses; the data refer to trials in which 12 weeks old pigs were infected; greater standard deviations display a quicker recovery from disease 1-3 dpi*

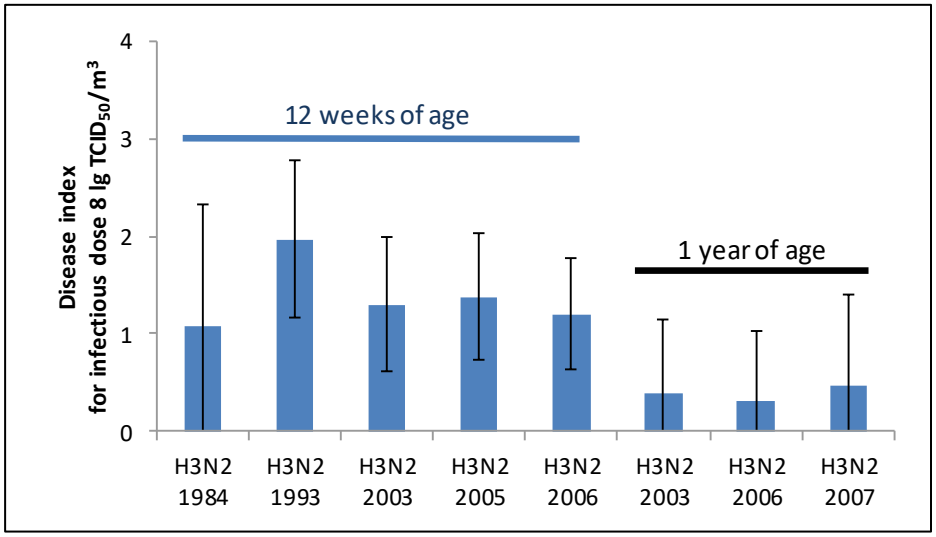


Figure 11: Virulence of German H3N2 swine influenza A viruses isolated 1984-2007
(age of pigs at infection 12 weeks and 1 year); the disease index (DI) was calculated for an infectious dose of 8 lg TCID$_{50}$/m^3 (DI = 8 x values of the corresponding regression line – correction factor for the corresponding strain); DI is based on the arithmetic mean of dyspnoea score 1-3 dpi; note the differences in virulence in different groups of age (12 months old pigs can better cope with infection); greater standard deviations display a quicker recovery from disease 1-3 dpi; further data on age-dependent differences in outcome of infection are shown in Supplement chapter 18 – Infection of pigs of different age, page CLIV

The 1996 H1$_{hu}$N2 isolate was more virulent than the other H1$_{hu}$N2 viruses analysed. However, the virulence of H1$_{hu}$N2 viruses has remained stable since the late 1990s.

A regression analysis of the different subtypes was done (Figure 13). H1$_{av}$N1 viruses were divided into 2 groups: H1$_{av}$N1 1980s (viruses from the 1980s and 1990s) and H1$_{av}$N1 2000s

(viruses from 2003 onwards; one virus of intermediate virulence was excluded from the analysis: FLUAV/sw/Belzig/1/2001) (Figure 13). Further analysis confirmed three types of virulence: low ($H1_{av}N1_{2000s}$), medium ($H1_{av}N1_{1980s}$, $H1_{hu}N2$, H3N2) and high ($H1_{pdm}N1$ April 2009) (Figure 14).

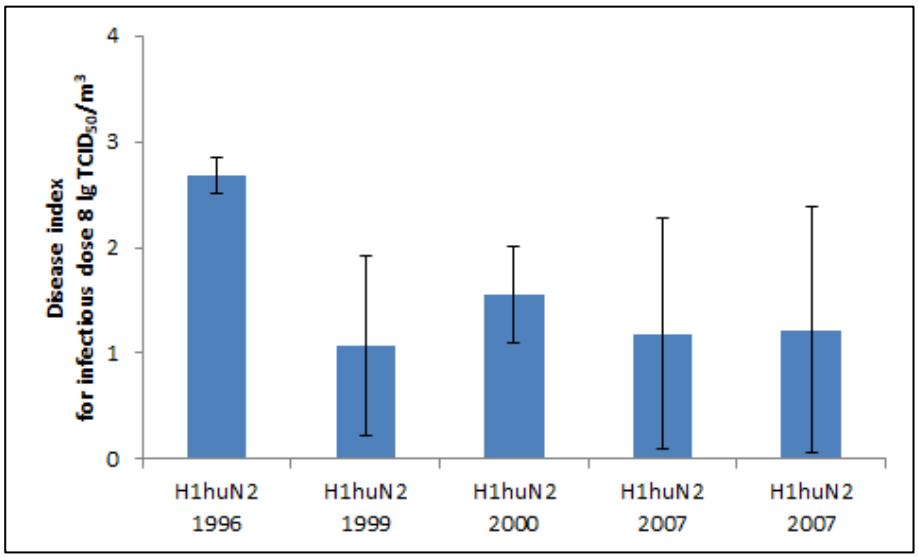


Figure 12: Virulence of $H1_{hu}N2$ swine influenza A viruses isolated 1996-2007
(age of pigs at infection: 12 weeks); the disease index (DI) was calculated for an infectious dose of 8 lg $TCID_{50}/m^3$ (DI = 8 x values of the corresponding regression line – correction factor for the corresponding strain); DI is based on the arithmetic mean of dyspnoea score 1-3 dpi; greater standard deviations display a quicker recovery from disease 1-3 dpi

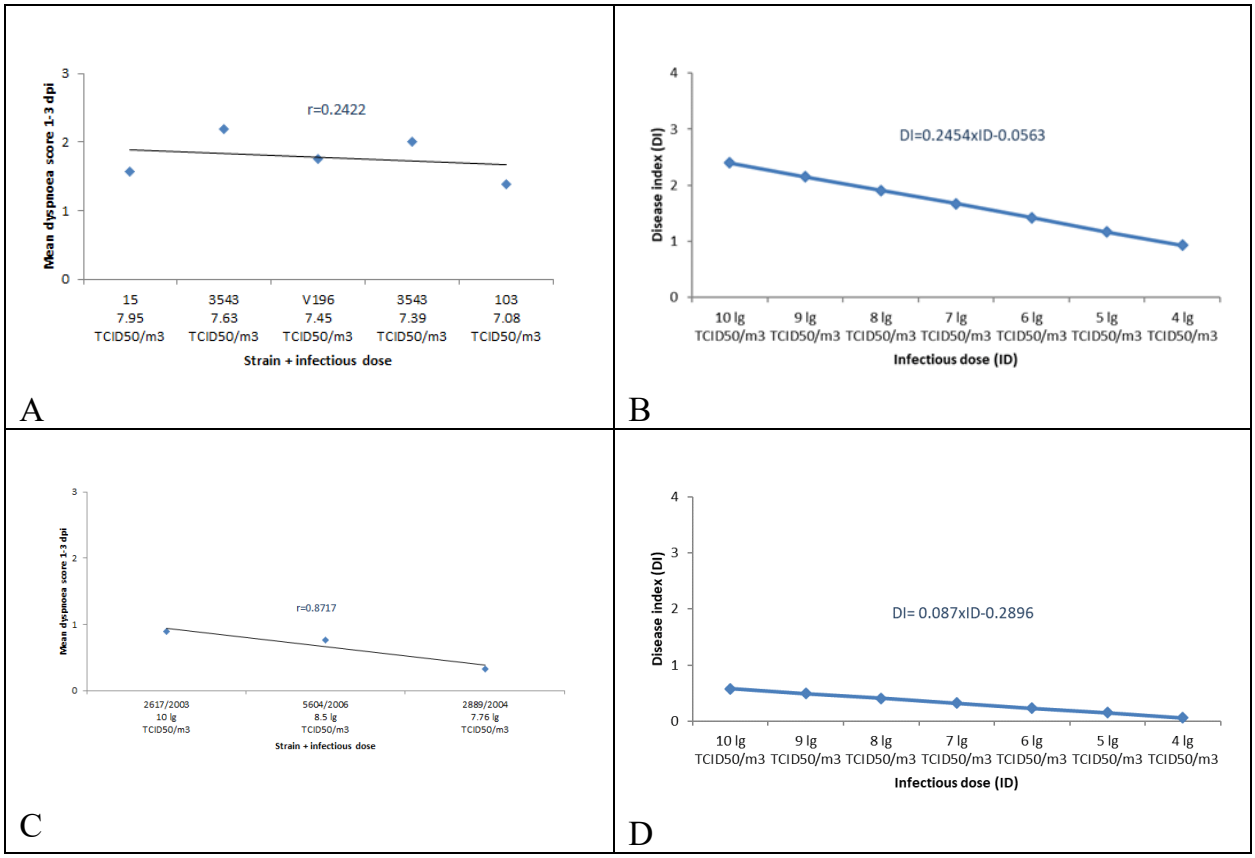


Figure 13: Trend and regression analyses for the virulence of $H1_{av}N1$
A, trends $H1_{av}N1$ 1980s (Pearson correlation coefficient r=0.2422); B, deduced disease index $H1_{av}N1$ 1980s (regression line: DI=0.2454-ID-0.563); C, trends $H1_{av}N1$ 2000s (Pearson correlation coefficient r=0.8717); D, deduced disease index $H1_{av}N1$ 2000s (regression line: DI=0.087-ID-0.2896)

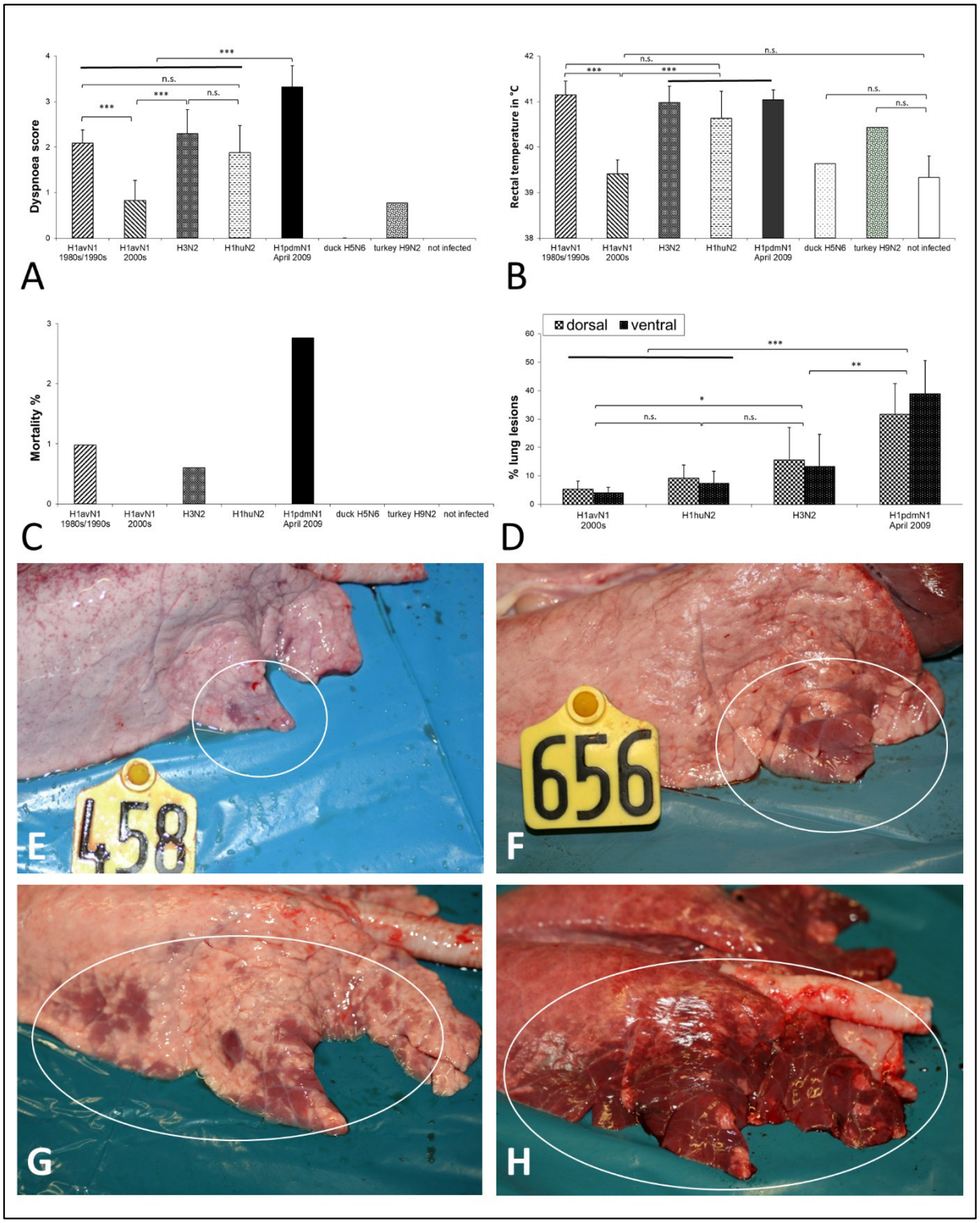


Figure 14: Different virulence between influenza A viruses
after high-dose aerosol infection of pigs
*A-H: (A) Dyspnoea score 1 dpi, $H1_{pdm}N1$ virus is significantly more virulent than the other viruses; (B) Rectal body temperatures 1 dpi, $H1_{av}N1$ viruses of the 2000s barely induced increases in body temperature; (C) Mortality in % calculated on following numbers of pigs: $H1_{av}N1$ 1980s/1990s: 102, $H1_{av}N1$ 2000s: 222, $H1_{hu}N2$: 258, H3N2: 166, $H1_{pdm}N1$: 331; duck H5N6: 11, turkey H9N2: 6; (D) Lung lesions % 3 dpi, $H1_{pdm}N1$ lung lesions were significantly more severe; (E-H) Lung lesions 3 dpi, (E) $H1_{av}N1$ 2006, (F) $H1_{hu}N2$ 2007, (G) H3N2 2006, and (H) $H1_{pdm}N1$ 2009 virus. The statistical analyses were done using Mann-Whithney U test: *** $p<0.001$, ** < 0.01, * $p<0.05$; n.s. not significant*

$H1_{hu}N2$ influenza A viruses were analysed in the same way (Figure 15). An $H1_{hu}N2$ reassortant virus was excluded from the calculations (FLUAV/sw/Cloppenburg/4777/2007). Also excluded were $H1_{hu}N2$ viruses nebulised in pigs at 12 months of age.

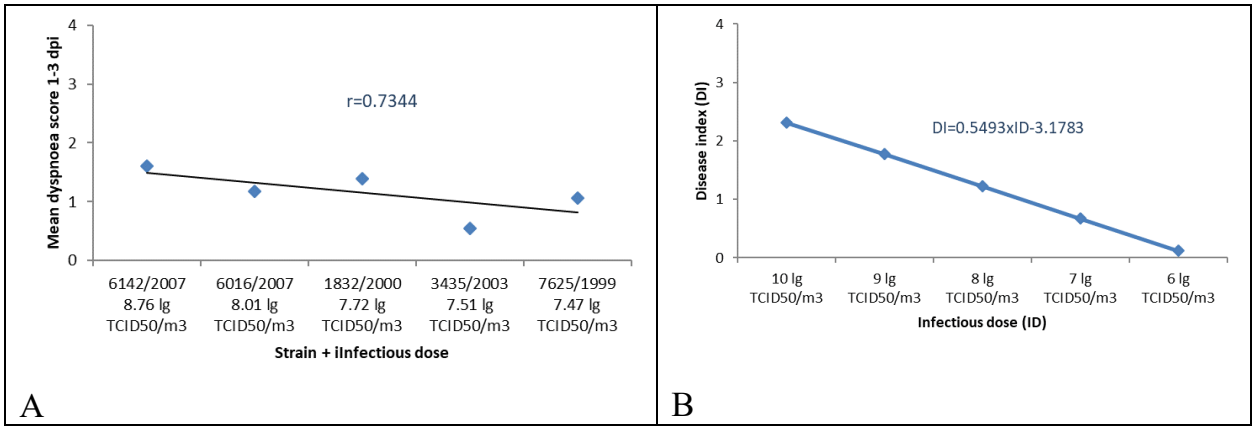


Figure 15: Trend and regression analyses for $H1_{hu}N2$ influenza A viruses
A, trends (Pearson correlation coefficient r=0.7344); B, deduced disease index (regression line: DI=0.5439xID-3.1783)

For H3N2 viruses, no data were available from regular infection studies in which infectious doses (ID) below 8 lg $TCID_{50}/m^3$ were nebulised. Therefore, data from the above reported dose titration of H3N2 virus were combined with selected data from the regular studies for this analysis (arithmetic mean ID of 8 lg $TCID_{50}/m^3$ and highest dyspnoea score obtained with viruses nebulised at doses greater than 9 lg $TCID_{50}/m^3$), see Figure 16).

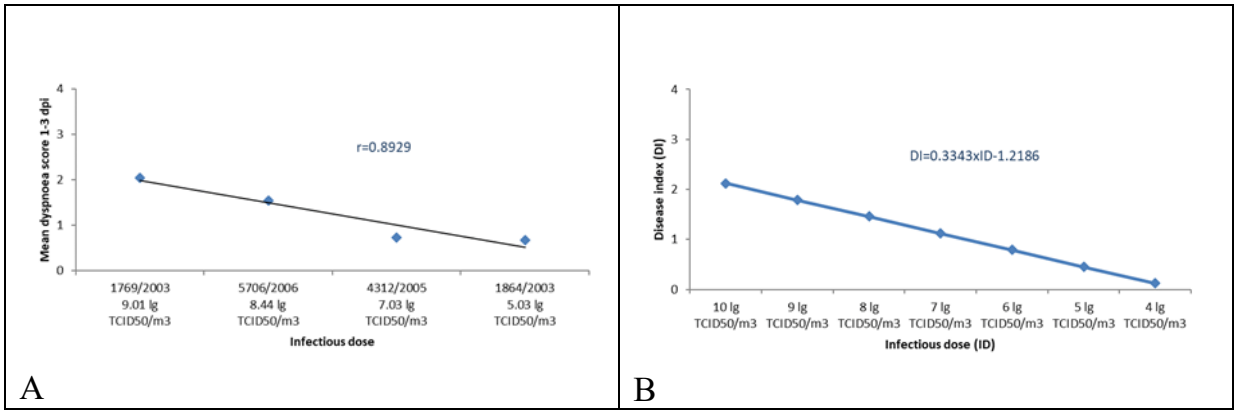


Figure 16: Trend and regression analyses for H3N2 influenza A viruses
A, trends (Pearson correlation coefficient r=0.8928); B, deduced disease index (regression line: DI=0.3343xID-1.2186)

Short interpretation of results. Influenza A viruses induce different degrees of virulence in pigs. Investigating the determinants of this virulence is of interest for future studies. The aerosol infection model may be a valuable tool for further research in this area.

5.1.3.2 Reassortant viruses reveal a unique role of the neuraminidase for virulence

Background. The ESNIP3 framework provided the opportunity to study selected influenza A virus strains from other European countries (France: Franck et al., 2007[352]; Denmark:

Hjulsager et al., 2006[353], Trebbien et al., 2013[354]). In collaboration with the University of Ghent (Kristien Van Reeth), the opportunity arose to investigate a Belgian H1N2 FLUAV (sw/Ghent/102/2007 $H1_{hu}N2$) in a comparative vaccination study after intranasal, intratracheal and aerosol infection in parallel. The results obtained for the aerosol control group are evaluated here.

Study design. Viruses were cultured in the MDBK cell line and nebulised at low passage levels (ps1-ps3) in infection assays. An overview of the infection studies can be found in Table 9. The pigs were free of antibodies against influenza. Pigs were transported to the infection unit at 5 weeks of age and were given 7 weeks to acclimate. Pigs were infected at 12 weeks of age. Recording of clinical parameters and sampling followed the regular pattern. Respiratory rate was measured daily as an additional parameter in the trial with virus FLUAV/sw/Ghent/102/2007. At 1 and 3 dpi, lung samples were collected from half of the pigs each group.

Table 9: Experimental design of infection trials with European FLUAV from European countries other than Germany

Infectious dose lg $TCID_{50}/m^3$	Virus used in trial	Genotype	Number of pigs included
5.04	FLUAV/sw/Ploufragan/0214/2006 ($H1_{hu}N2$)	G1 $H1_{hu}N2$	14
5.81	FLUAV/sw/Ploufragan/0113/2006 ($H1_{hu}N2$))	G2 $H1_{hu}N2$	10
4.85	FLUAV/sw/Ploufragan/0190/2006 ($rH1_{hu}N1$)	$rH1_{hu}N1$	15
4.08	FLUAV/sw/Ploufragan/0070/2005 (H1N1)	H1N1	10
6.05	FLUAV/sw/Denmark/12687/2003 ($rH1_{av}N2$)	$rH1_{av}N2$	20
7	FLUAV/sw/Ghent/102/2007	$H1_{hu}N2$	12

Results. The data show that the viruses differ in virulence. H1N1 viruses from the 2000s reflect low virulence, whereas all viruses containing N2 have higher virulence. Within the H1N2 viruses, the newly reassorted Danish variant ($rH1_{av}N2$) and the $H1_{hu}N2$ virus from Belgium had a similarly high level of virulence, although $rH1_{av}N2$ contained the $H1_{av}$ of the low virulent $H1_{av}N1$ viruses (Figure 17). The data are consistent with the observations made for influenza viruses isolated in Germany. The $H1_{av}N1$ viruses show low virulence. On the other hand, the reassortant $rH1_{hu}N1$ virus A/sw/Ploufragan/0190/2006, which captured the N1 from the $H1_{av}N1$ viruses, lost its stronger virulence. Despite a lower lung viral load, G2 H1N2 caused more dyspnoea but less increase in rectal temperature (data not shown). H1N1 viruses remained almost inconspicuous in terms of symptoms. Danish $rH1_{av}N2$ replicated to similar titres in the lungs as French H1N1 and G2 H1N2 viruses (data not shown). Danish $rH1_{av}N2$ virus induced severe and prolonged dyspnoea and fever. The Danish $rH1_{av}N2$ virus trial was used to study immune genes, proteins and microRNAs

in the lungs and circulating leukocytes of pigs (Skovgaard et al., 2013[355]; Brogaard et al., 2016[356]).

Short interpretation of results. The data reveal a unique role for neuraminidase in virulence. The N2 neuraminidase stabilises the viruses at a higher level of virulence, whereas the N1 derived from $H1_{av}N1$ viruses drives the viruses to a lower level of virulence as they circulate in their hosts.

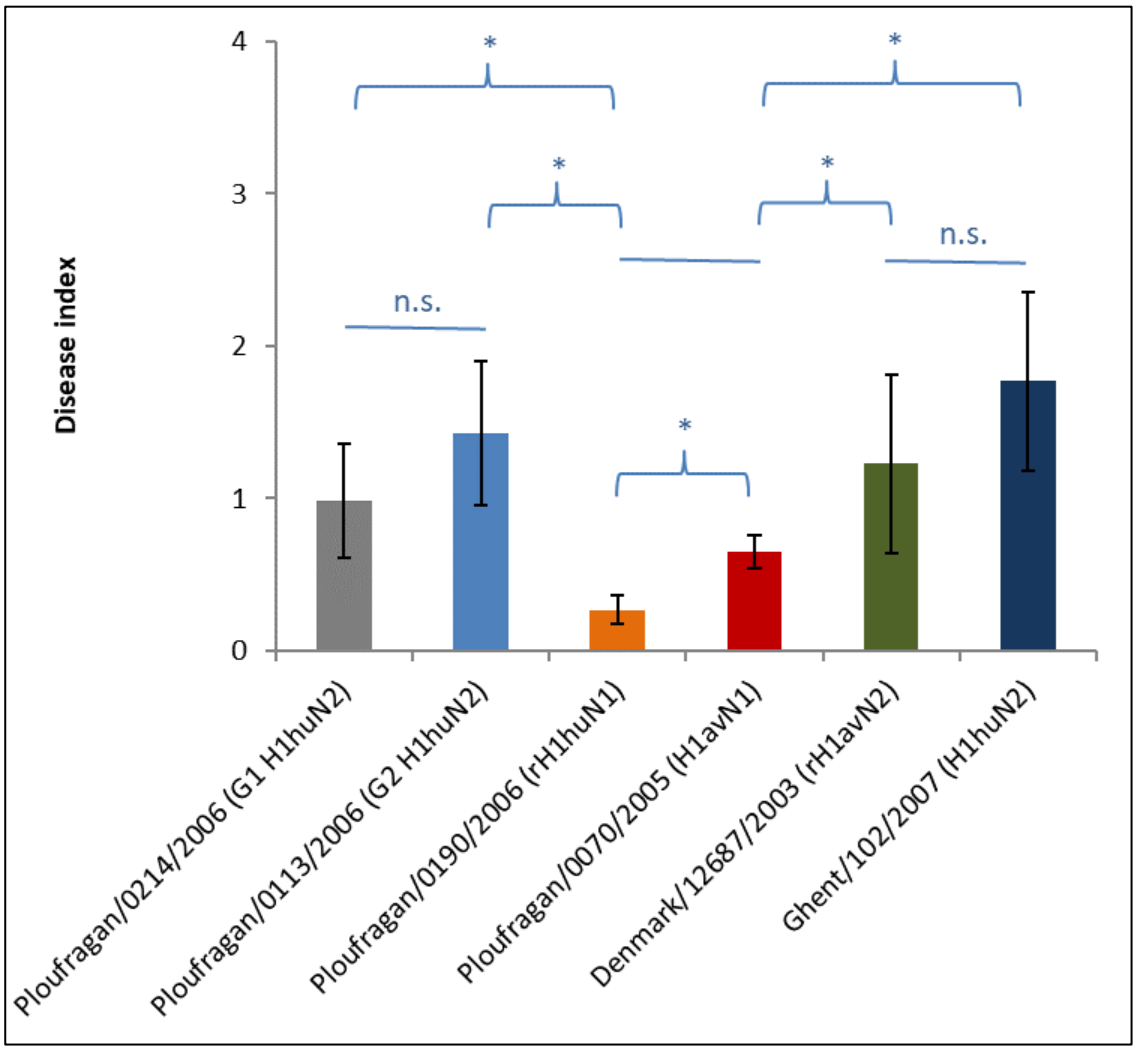


Figure 17: Virulence of European influenza A viruses isolated outside Germany
*(DI, Disease index adjusted to an ID of 7 lg $TCID_{50}/m^3$); for G1+G2 $H1_{hu}N2$ + $rH1_{av}N2$ the equation of $H1_{av}N1$ 1980s fit best, for $H1_{hu}N1$ 2006 that of $H1_{av}N2$ 2000s, for $H1_{av}N1$ 2005 that of $H1_{cl}N1$, *, $p<0.05$; note: acquisition of N2 increases virulence*

5.1.3.3 Evolution of pandemic (H1N1) 2009 viruses towards a lower level of virulence

Background. The $H1_{cl}N1$ virus of 1986 showed only low virulence. This could be due to adaptation over a long period of circulation. The early $H1_{pdm}N1$ viruses showed a slight loss of virulence in the first year after their appearance. To investigate this further, the issue

of virulence was followed up in infection studies with $H1_{pdm}N1$ isolates from 2014/2015 and compared to trials with viruses from 2009 and 2010.

Study design. Six studies were conducted with pandemic (H1N1) 2009 viruses isolated from human patients and pigs in different years. 3-month-old pigs were used for high-dose aerosol infection (passage level of strains 2-3 passages from swab sample); each group contained at least 13 pigs at the start of the study; infection dose $10^{8.21\text{-}8.5}$ $TCID_{50}$ MDCK/m^3; on days 1, 3 and 9 after infection, 5 pigs from each group were sacrificed for lung examination (Table 10).

Table 10: Overview of trials included in the investigation of virulence of $H1_{pdm}N1$
($TCID_{50}$ MDBK is shown in the table; MDCK $TCID_{50}/m^3$ was HA09: 8.5, JE09: 8.5; JE10: 8.5; SC14: 8.21; KI15: 8.25, TE15: 8.5)

Strain	Number of pigs	Age of pigs weeks	Infection dose $TICD_{50}/m^3$	Lethality %
Hamburg/NY1580/2009 HA09	17	11	$10^{5.75}$	17.64
Jena/VI5258/2009 JE09	15	12	$10^{5.5}$	0
Jena/VI2688/2010 JE10	15	11	$10^{7.26}$	0
sw/Schallern/19989/2014 SC14	13	12	$10^{7.75}$	0
Kiel/18909686/2015 KI15	19	16	$10^{6.75}$	0
sw/Tesp/2015 TE15	20	12	$10^{6.75}$	0

Further studies were performed with the most virulent virus (A/Hamburg/NY1580/April 2009 – HA09 –) in order to validate its virulence and investigate its effect on different age groups (Table 11).

Table 11: Overview of lethality induced by an $H1_{pdm}N1$ 2009 virus isolated in April 2009 in different trials (strain 1580)

Trial	No. of pigs	Infection dose lg $TCID_{50}/m^3$	Age of pigs months	Pigs that died number	Time of death dpi	Lethality* %
1	17	5.75	3	3	2x 2 dpi, 1x 3 dpi	17.64
2	13	5.92	3	3	2x 2 dpi, 1x 3 dpi	23.07
3	6	6.3	3	1	2 dpi	16.67
4	6	4.3	3	0	-	0
5	19	6.17	1	1	5 dpi	5.26
6	18	5.92	12	0	-	0
7	13	6.38	12	0	-	0

* number of pigs that died in relation to the pigs which developed disease; see also Supplement chapter 17, page CLI

Results. $H1_{pdm}N1$ viruses of April 2009 reflected severe virulence with high lethality, severe lung pathology and inflammation (Table 11, Figure 18, Figure 19, Figure 20).

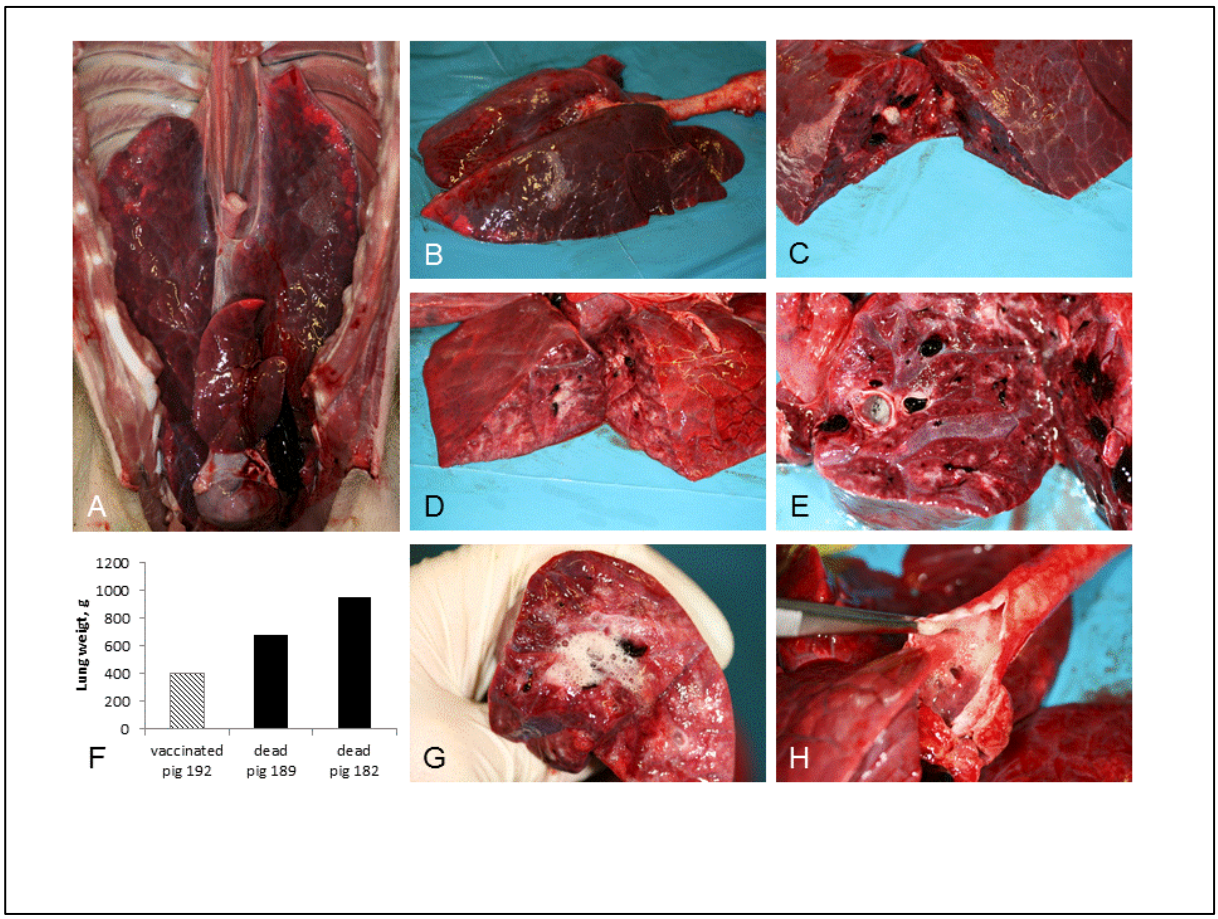


Figure 18: Lung lesions of pigs after lethal infection with $H1_{pdm}N1$ April 2009 virus
(strain A/Hamburg/NY1580/2009 $H1_{pdm}N1$ April 2009); A, B, C, D, E the entire lung tissue is involved and reflects strong oedema, F, the weight of the lung is 1.5 to 2-fold higher in comparison to a healthy vaccinated pig of the same age (3 months), E, G, H, foam is visible in bronchiolae, bronchus and trachea

In an infection trial in which an $H1_{pdm}N1$ virus was nebulised that had been isolated 3 months after the emergence of the $H1_{pdm}N1$ virus (June 2009), two out of five control animals became severely ill and had significant weight loss, but none died. These 2 out of 5 pigs represent 20% of infected pigs. The rate of severe disease induction in this trial (20%) is similar to the mortality rate observed in the April 2009 $H1_{pdm}N1$ trials, suggesting that the virulence of the virus has changed and lost its ability to be lethal (Figure 21). All pigs had very high virus titres in the lungs.

Infection with another early $H1_{pdm}N1$ influenza virus that was isolated in 2010 (FLUAV/Jena/VI2688/2010 ($H1_{pdm}N1$)) was not as severe despite the high dose of nebulised virus (see Supplement 26, page C). The investigation of clinical-chemical parameters haptoglobin, creatinine kinase, aspartate transaminase, alanine transaminase, gamma-glutamyl transferase, glutamate dehydrogenase, creatinine) and cytokines (IFNγ, TNFα and IL6) revealed a high degree of individual variability (Supplement chapter 12 –

Investigation of clinical chemical parameters; page CXXII). This could indicate that individual reaction patterns have a major influence on these parameters and that, consequently, host genetic factors play a role.

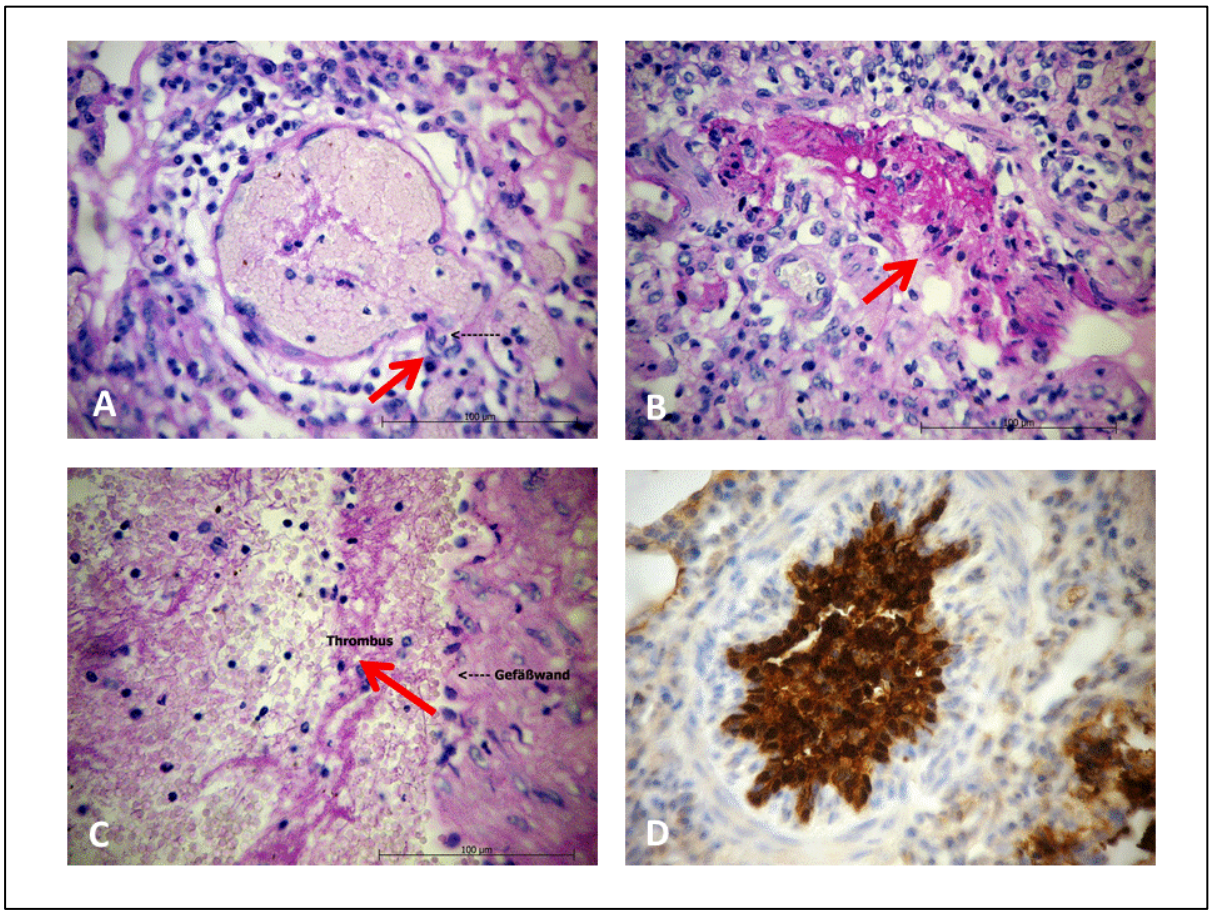


Figure 19: Alterations in lungs of fatal cases of $H1_{pdm}N1$ April 2009 infection
A, PAS staining, Lesions in epithelial cells of vessels (arrow) result in bleeding, B, PAS staining, Accumulation of fibrin, C, PAS staining, Thrombosis (arrow), D, Immunohistological staining (Streptavidin-Biotin-Method, SABC) reflects accumulation of macrophages, virus and debris in bonchioli and obstruction of bronchiole

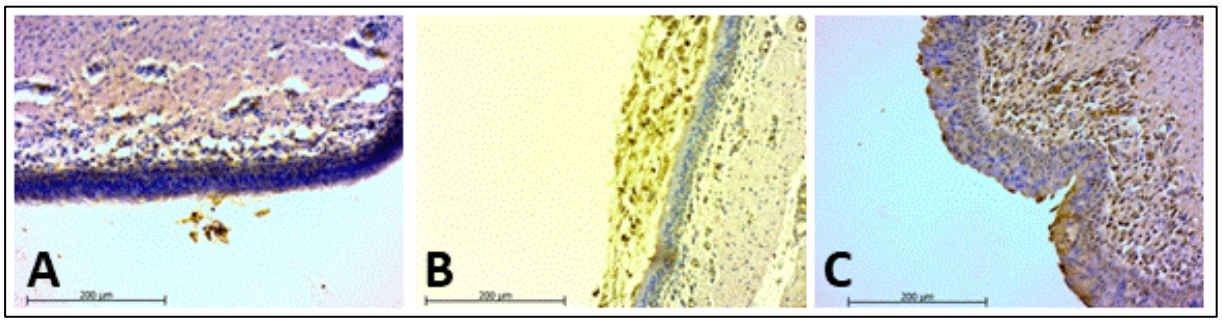


Figure 20: Trachea of pigs after infection with $H1_{pdm}N1$ April 2009 virus
(A/Hamburg/NY1580/April 2009)
A, 1 dpi (SABC staining of epithelial cells and macrophages) ; B, 3 dpi (SABC staining of macrophages, desquamation of epithelial cells, loss of ciliae, fribrin exsudation); C, 9 dpi (SABC staining of submucosal cells and microvilli; mononuclear infiltrates and neutrophil exocytosis)

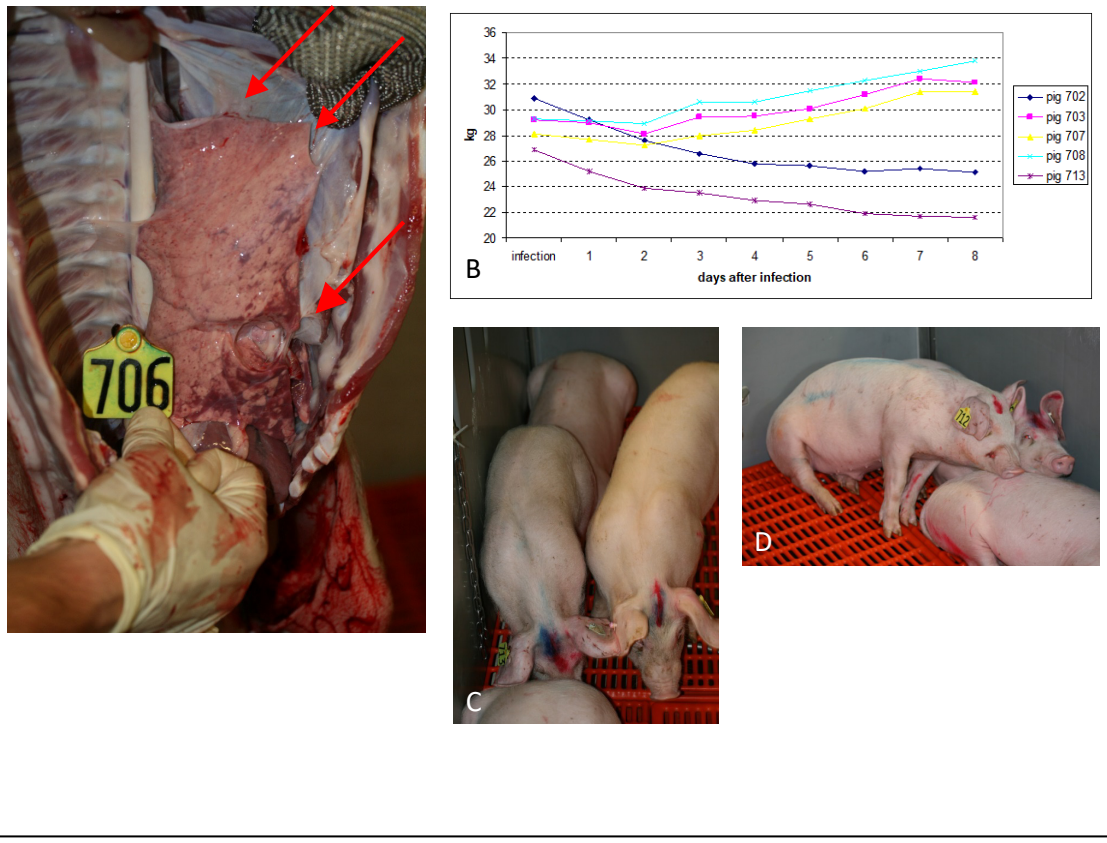


Figure 21: Infection with strain FLUAV/Jena/VI5258/2009 ($H1_{pdm}N1$ June 2009)
caused severe respiratory disease (C,D), severe lung lesions and adhesions of the lung with surrounding tissues (red arrows) as a sign of pleuritis (A), and stagnation or severe loss in body weights in 20% of the pigs (B)

The data show a highly significant decline in the virulence of $H1_{pdm}N1$ within half a decade of its emergence (Figure 22 A, B, C, D). When analysing body weight, pigs infected with 2009/2010 $H1_{pdm}N1$ viruses showed losses, whereas $H1_{pdm}N1$ 2014/2015 viruses showed no effect on body weight gains (Figure 22 C).

To compare the results with other studies, a disease index was calculated for an infectious dose of 7 lg $TCID_{50}/m^3$. The data reflect a dramatic loss of virulence from 2009 to 2015 (Figure 23). The evolution towards a lower level of virulence began as early as 2009 (Figure 23).

Short interpretation of results. The determinants of virulence are still unknown. The D222G mutation in HA has been discussed as a virulence factor. The strain FLUAV/Jena/VI2688/2010 ($H1_{pdm}N1$) had this mutation. This strain was isolated from a pregnant woman who died of influenza.

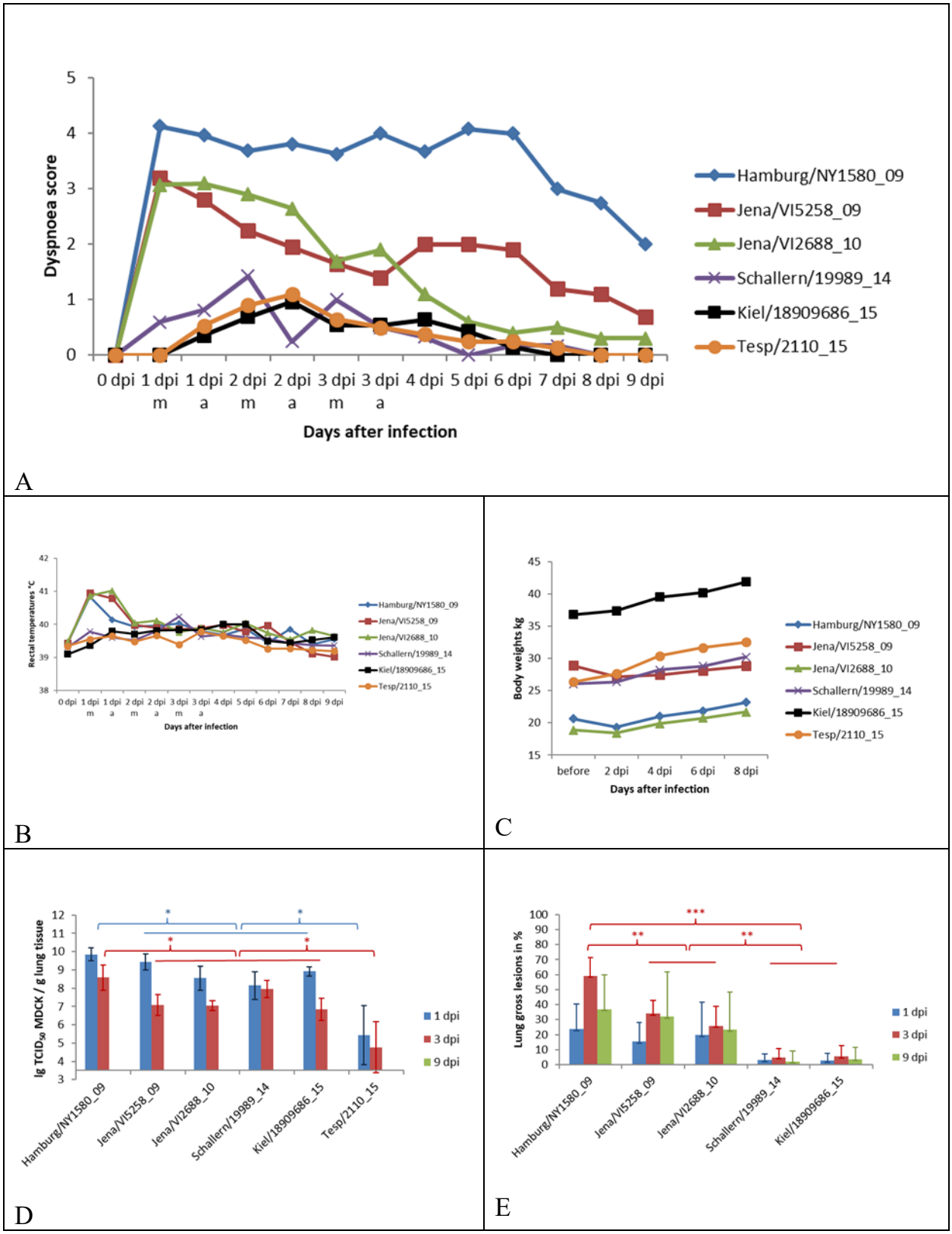


Figure 22: Parameters of infection of 2009 - 2015 $H1_{pdm}N1$ viruses

*A, Respiratory symptoms (dyspnoea score, arithmetic mean); B, Rectal temperatures (°C, arithmetic mean); C, Body weights (kg, arithmetic mean); D, Viral lung load (lg $TCID_{50}$ MDCK/g lung, arithmetic mean), viruses of 2009 replicate to significantly higher titers in lung tissue on 1 dpi (samples taken from 8 different parts of the lung covering all seven lung lobes; 5 pigs in each group included; E, Lung lesions (%), Lung lesions after infection with $H1_{pdm}N1$ viruses: viruses of 2009/2010 induced more lung lesions in comparison to those isolated later; statistics: *** p <0.001, ** p<0,01, * p<0.05*

However, this strain did not cause any mortality in pigs, although it caused severe disease. Compared to the other two strains without the D222G mutation, the disease was not as severe. The less severe disease after infection with the FLUAV/Jena/VI2688/2010 ($H1_{pdm}N1$) strain compared to previous $H1_{pdm}N1$ viruses could be an indication of an initial adaptation of this virus to its new host and an evolution towards less virulence. The most virulent strain was FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$). This strain caused losses in pigs in four high-dose infection trials in 3-month-old-pigs. Single gene reassortants of PB1, HA and NA were identified as critical for the virulence of the 1918 pandemic H1N1 virus (Pappas et al., 2008[357]). The results of this work show that NA is indeed important for the fixation of virulence: N2 neuraminidases are able to fix virulence, whereas N1 neuraminidases do not and allow evolution towards lower virulence. Lethality is most likely due to a combination of the virulence of the influenza virus strain and additional factors provided by the host. All influenza A viruses have been sequenced and analysed, but no specific mutation has been identified that is responsible for the high virulence of $H1_{pdm}N1$ 2009 viruses (Supplementary Tables 16+17, pages CXII, Supplement 36, page CXIII): Minor mutations were found in all segments of the sequenced influenza A viruses but no specific mutation related to virulence could be identified. It is clear from the experimental infections that new reassortant viruses often caused severe disease. The $H1_{pdm}N1$ 2009 and H3N1 viruses were recently reassorted viruses. It is possible that the components of these new reassortants have not yet adapted to each other. The lack of balance in the newly reassorted segments may therefore be a reason for more severe effects on infected cells.

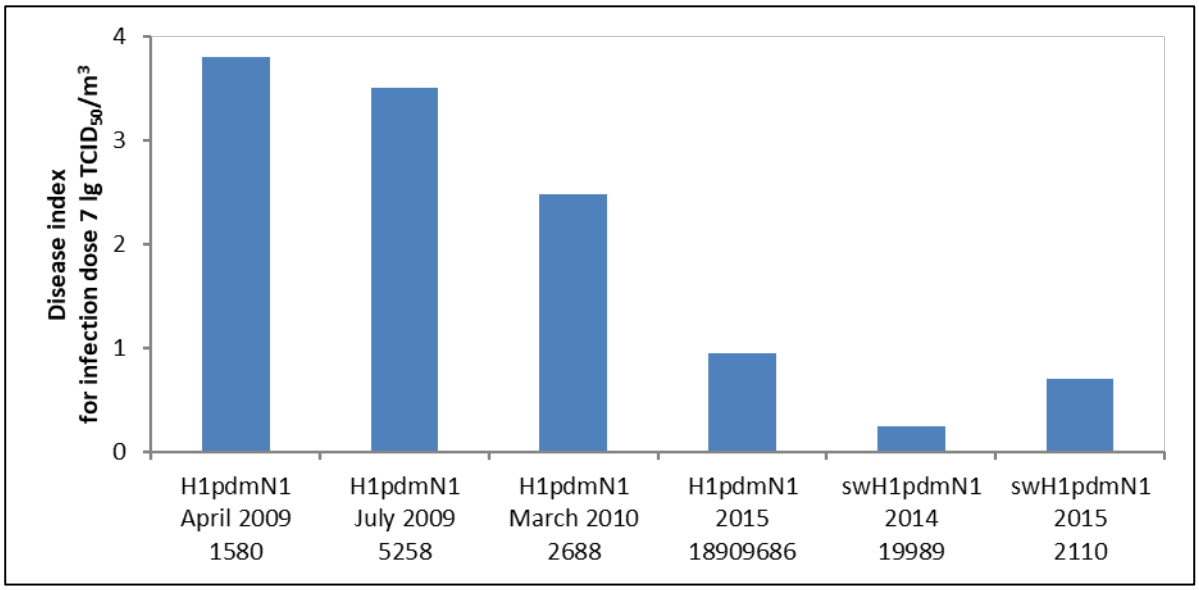


Figure 23: Comparison of virulence of $H1_{pdm}N1$ viruses
Age of pigs 12 ± 4 weeks at infection) of $H1_{pdm}N1$ viruses April 2009 – April 2015 (Disease index calculated for a infectious dose of 7 lg $TCID_{50}/m^3$)

5.1.4 VALIDATION OF AEROSOL NEBULISATION

5.1.4.1 INVESTIGATION OF VIRUS STABILITY DURING NEBULISATION

To investigate the influence of the nebulisation process on virus infectivity, the nebulised material was collected in roller bottles during nebulization (Supplement 4, page LXIII). Virus titres and pH were determined after condensation. No effect of the nebulisation procedure on virus titer was observed (Supplementary Table 13, page XCVII). As a result, all of the infectious material is in the air after nebulisation, which makes it possible to calculate the nebulised viral dose in $TCID_{50}/m^3$ based on the $TCID_{50}$ and the volume of the infection unit.

5.1.4.2 INVESTIGATION OF VIRUS DISTRIBUTION IN PIG TISSUES AFTER INFECTION

Background. Not much is known about the fine distribution of the virus in the respiratory tract and other organs of pigs.

Study design I (Viral signals detected by in situ hybridization and/or immunohistology). To investigate the distribution of the virus in the respiratory tract, *in situ* hybridisations were performed using an influenza A virus-specific probe directed against NP, and histological and immunohistological studies were performed using specific polyclonal rabbit antisera. Lung samples were analysed from infected pigs from one trial in which human influenza A/Hamburg/NY1580/2009 ($H1_{pdm}N1$) (representing viruses of very high virulence) was aerosolized, but also from four trials in which swine influenza A viruses representing viruses of lower virulence were aerosolized (A/sw/Ploufragan/0070/2005 $H1_{av}N1$, A/sw/Ploufragan/0113/2005 G2 H1N2, A/sw/Ploufragan/0190/2006 reH1N1, A/sw/Ploufragan/0214/2006 G1 H1N2).

Results. The *in situ* hybridisation data show that viral signals against NP are distributed throughout the lung. Fine signals are seen in the alveoli. Strong signals are expressed in bronchioli and bronchi, but also in epithelial cells of the trachea. Viral signals do not appear outside the airways (Figure 24). In contrast, the influenzaviral antigen detected by polyclonal antibodies raised against whole virus is widely distributed in all organs and is expressed mainly by macrophages and immune system cells, but also by epithelial cells and cardiomyocytes (Figure 25). Mild mononuclear encephalitis and leptomeningitis were present in the brain (Figure 25). The heart showed multifocal infiltration and onset of cardiomyocyte destruction (Figure 26). There was hyperplasia and hyperemia of the spleen (Figure 25). Liver reflected multifocal infiltrates and activation of Kupffer cells (Figure 27). The kidney showed varying degrees of nephritis (Figure 28). Small and large intestine showed

hyperplasia of mucosa-associated lymphoid tissue and a strong staining pattern (Figure 25). Overall, influenza A virus infections affect the whole body, despite the clinical predominance of respiratory disease.

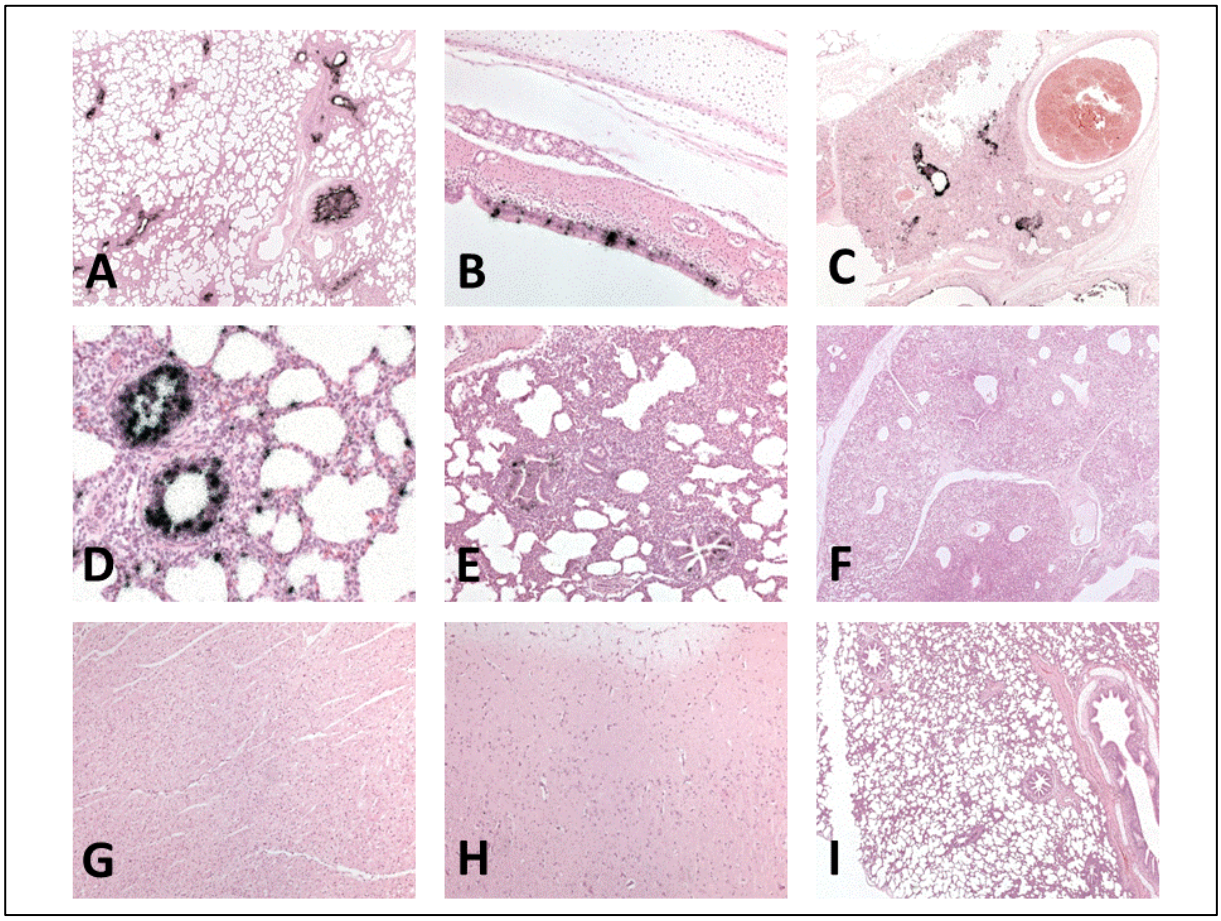


Figure 24: In situ hybridization of organ samples ($H1_{pdm}N1$ April 2009 virus)
A-I: (A) Lung 1 dpi, magnification x25, (B) Trachea 1 dpi, x100, (C) Lung of lethal case, x25, (D) Lung 1 dpi, x 200, (E) Lung 3 dpi, x 100, (F) Lung 14 dpi, x25, (G) Heart 3 dpi, x100, (H) Brain, 3 dpi, x100, (I) Lung, not infected 1 dpi, x25. Strong viral signals were detected in bronchial and alveolar epithelial cells and in some airway macrophages at 1 dpi. The signals were lower at 3 dpi. The virus had completely disappeared by 14 dpi. Inflammation increased from 1 dpi to 14 dpi. All organs outside the respiratory tract were negative for NP staining at all times of infection, as shown in the heart and brain - hippocampus region; investigations were carried out by Dr. M. Sauter and Prof. Dr. K. Klingel, Tübingen, Germany, using samples from pigs from the animal experiments

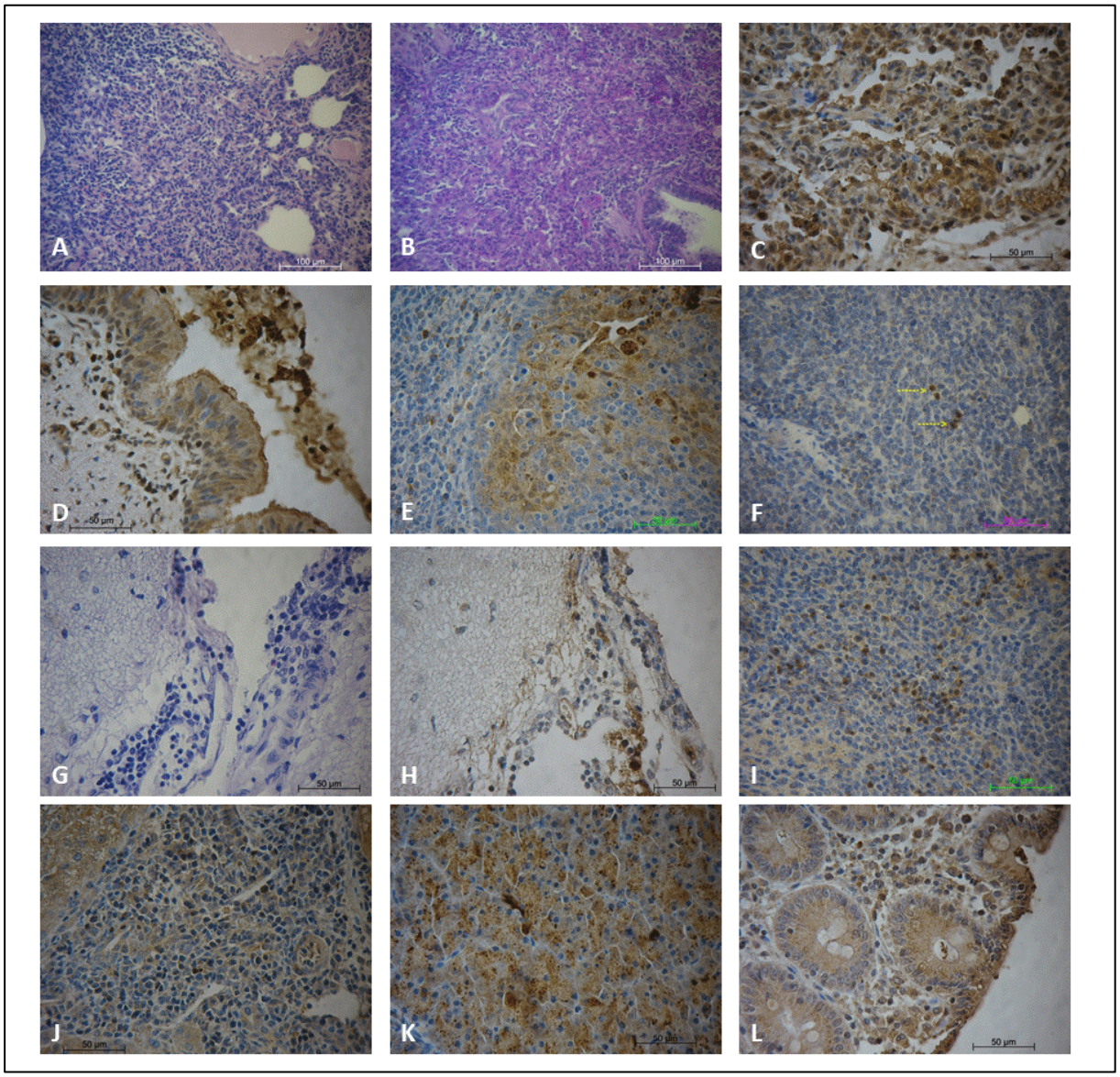


Figure 25: Immunohistological investigation of tissues ($H1_{pdm}N1$ April 2009 virus) of one and the same pig which developed influenza after experimental contact infection with $H1_{pdm}N1$ 2009 virus (A/Hamburg/NY1580/2009)

(A) Large areas of mononuclear infiltration in the lung (HE staining, (B) Mononuclear infiltrates in lung tissue (PAS staining), (C) Virus-specific staining of macrophages in the lung with $H1_{pdm}N1$ antiserum (SABC staining), (D) Specific staining of epithelial cells in the trachea (SABC), (E) Tonsils: hyperplastic germinal centres and secondary follicles with atrophy of the parafollicular space, no necroses, slight mitotic index, starry sky-like occurrence of individual macrophages - clear viral expression, especially in macrophages and in cortex (SABC) = reactive lymphadenopathy, (F) Antigen in macrophages of tonsils, arrows (SABC), (G) Cerebellum expressing slight mononuclear encephalitis and leptomeningitis, (H) Cerebellum with specific staining of mononuclear infiltrates (SABC), (I) Specific staining of macrophages in the spleen (SABC), (J) Liver: activation and virus-specific staining of Kupffer cells (SABC), (K) Pancreas: virus-specific staining of Langerhans cells (SABC), (L) Large intestine: virus-specific staining of Lieberkühn glands

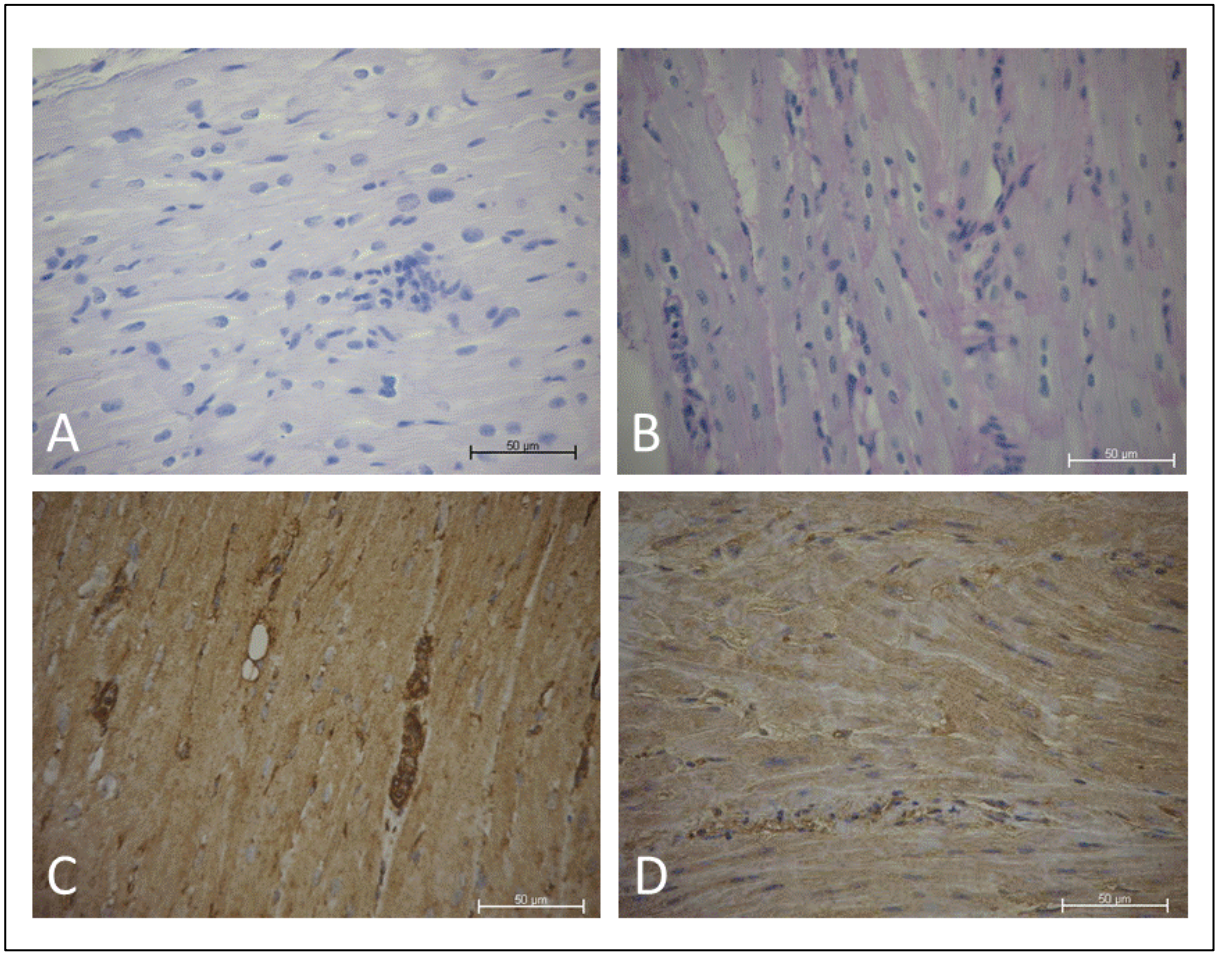


Figure 26: Heart muscle of a pig infected with influenza virus G1H1N2 A/sw/Ploufragan/0214/2006 (G1 H1N2) 3 dpi
showing multifocal infiltrates and beginning destruction of cardiomyocytes (A, B) and influenza-virus specific staining by αH1N2 polyclonal rabbit serum of cardiomyocytes and macrophages (C, D); A, HE staining, B, PAS staining; C+D, SABC staining

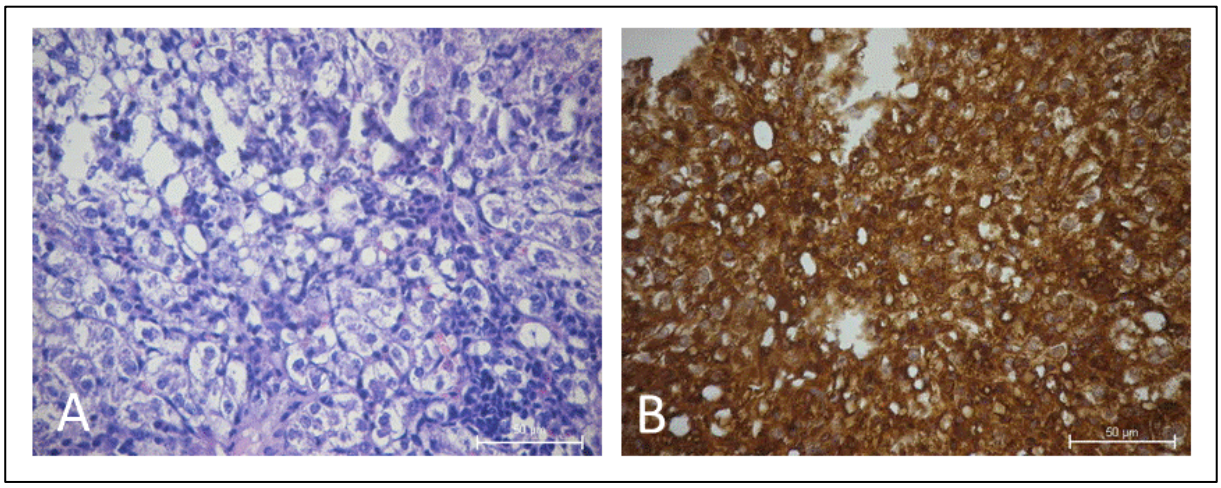


Figure 27: Liver of a pig infected with influenza virus $H1_{av}N1$ A/sw/Ploufragan/0070/2005 ($H1_{av}N1$) 3 dpi
showing multifocal cellular infiltration and activation of Kupffer cells (A, HE), intracytoplasmatic staining of hepatocytes (B, SABC); A, HE staining, B, SABC staining

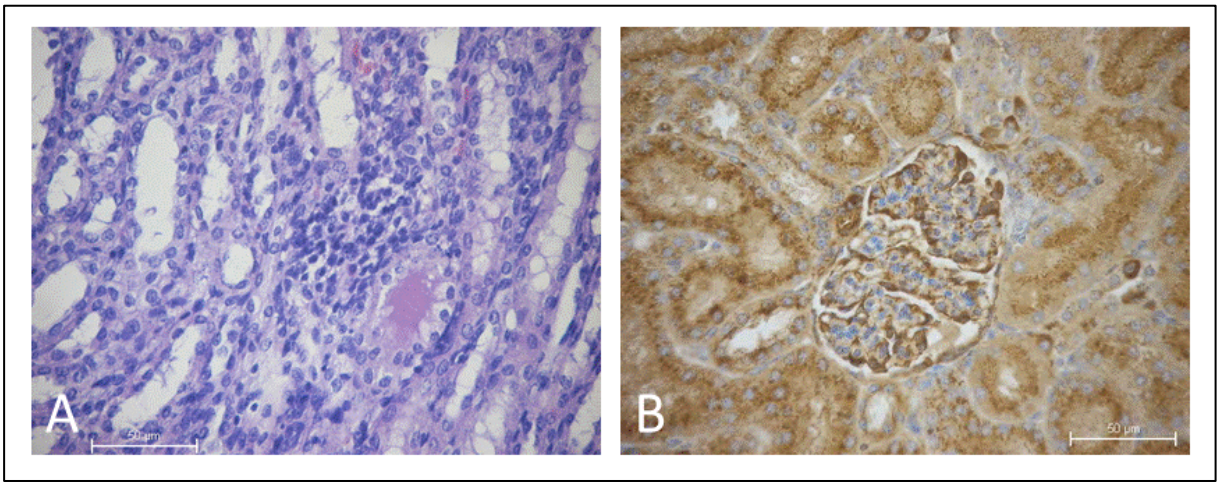


Figure 28: Kidney of a pig infected with influenza virus $H1_{av}N1$ A/sw/Ploufragan/0070/2005 ($H1_{av}N1$) 3 dpi
showing multifocal mononuclear infiltration of zona intermedia and zona basalis (nonpurulent pyelonephritis) (A, HE), intracytoplasmatic virus-specific staining of cells of interstitium, tubuli, glomeruli and macrophages (B, SABC); A, HE staining, B, SABC staining

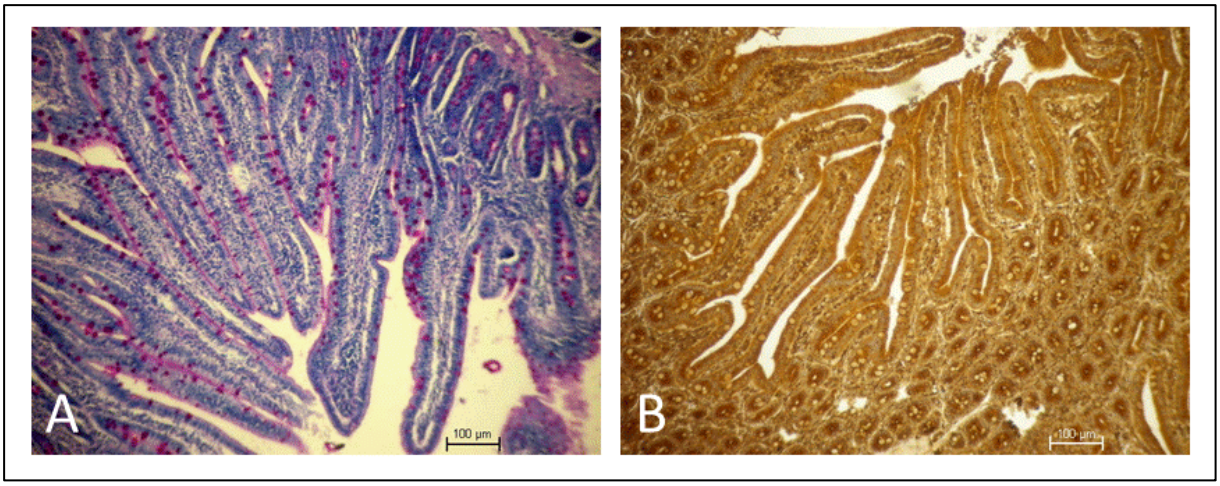


Figure 29: Intestine of a pig infected with influenza virus G2 H1N2 A/sw/Ploufragan/0113/2005 (G2 H1N2) 3 dpi
demonstrating gentle MALT hyperplasia (A, PAS staining), virus-specific intracytoplasmatic staining of cells of mucosa, muscularis, epithelial cells, villi and luminal chymus (B, SABC); A, HE staining, B, SABC staining

Study design II (Infectious virus). To verify the viral lung load after nebulisation, several tissue samples were taken and investigated. This was done in a number of trials in which the swine influenza A $H1_{hu}N2$ virus, a human-derived pandemic $H1_{pdm}N1$ virus, a classical swine A H1N1 virus, different infection doses of a swine $H1_{pdm}N2$ virus, an H3N1 virus, and human-derived pandemic $H1_{pdm}N1$ virus of 2015 and a swine-derived $H1_{pdm}N1$ virus of 2014 had been nebulised. For H3N1, aerosol infection was compared with contact infection.

Results II. The data show that infectious influenza A viruses are distributed throughout the respiratory tract. However, infectious viruses were not found in other tissues.

High dose-aerosol nebulisation of FLUAV/Jena/VI2688/2010 ($H1_{pdm}N1$) induced rapid distribution of virus throughout the respiratory tract (Figure 30).

After nebulisation of an $H1_{pdm}N2$ virus isolated from pigs, similar patterns of virus distribution in the respiratory tract were measured: the virus replicated at high titres throughout the respiratory tract, regardless of the infectious dose; there was no infectious virus outside the respiratory tract (Figure 31).

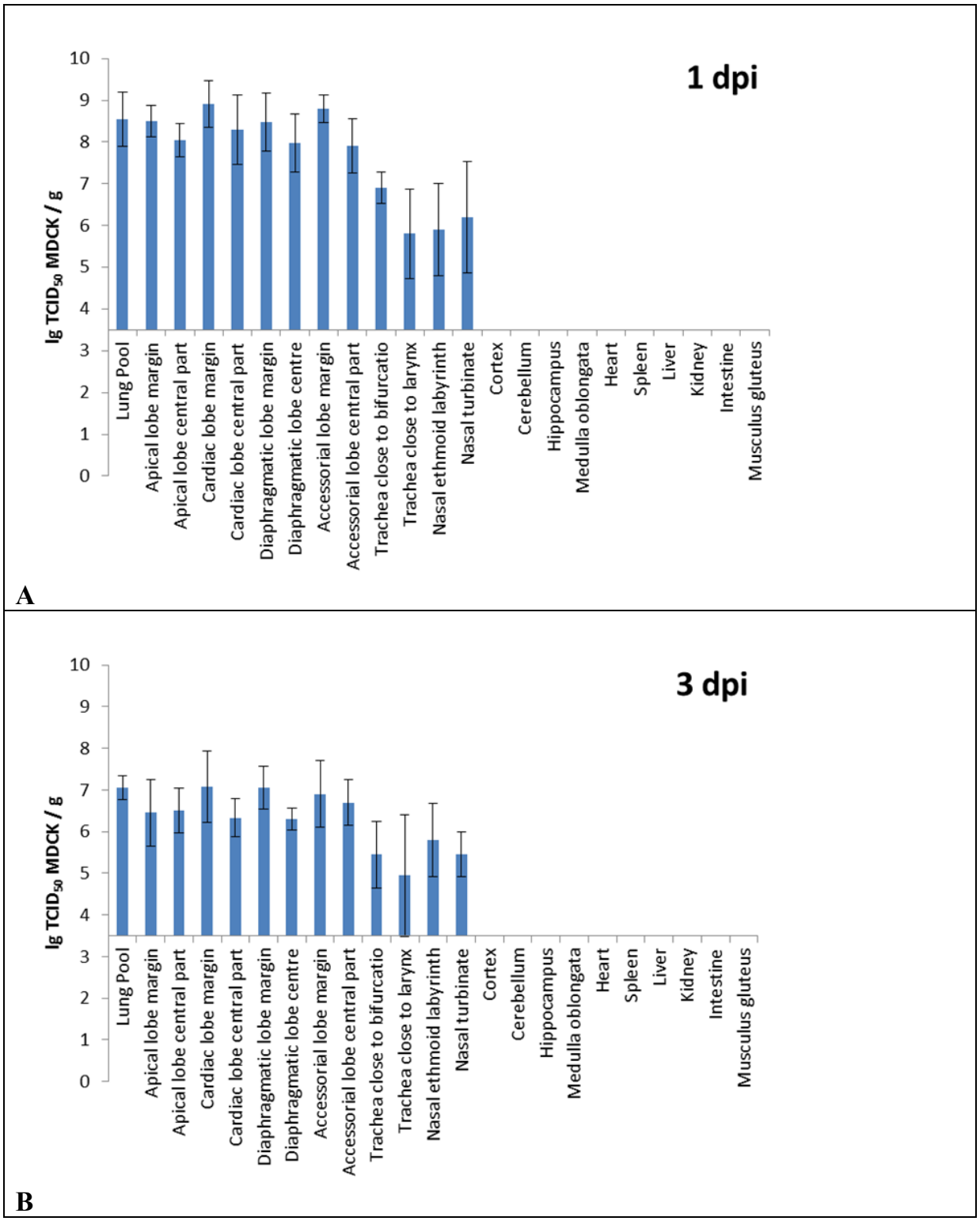


Figure 30: Virus distribution in the respiratory tract ($H1_{pdm}N1$ 2010 virus) after infection with 7.26 lg $TCID_{50}/m^3$ of FLUAV/Jena/VI2688/2010 $H1_{pdm}N1$ 1 day and 3 days after infection
(lg $TCID_{50}$ / g); A, 1 dpi; B, 3 dpi; arithmetic mean values with standard deviation

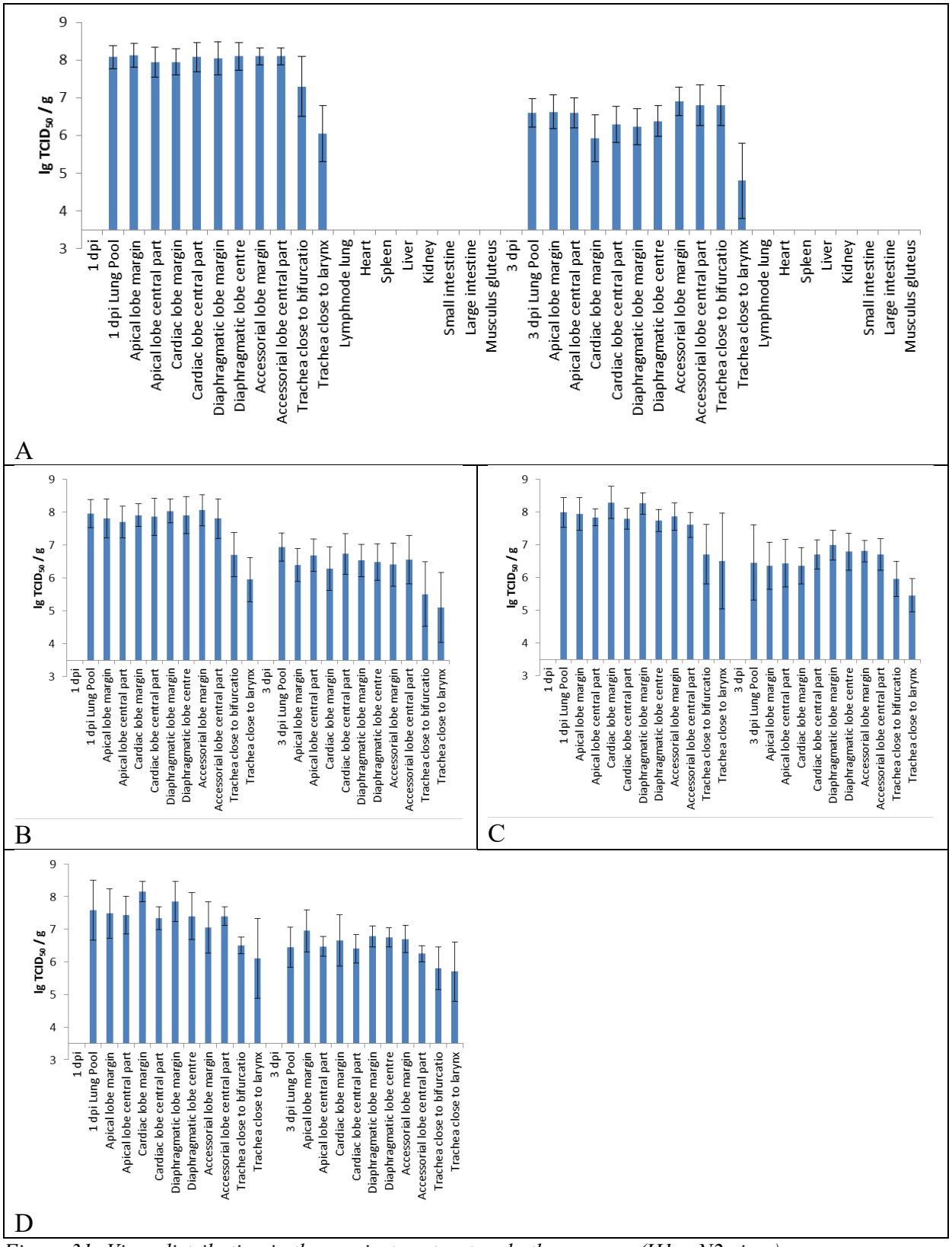


Figure 31: Virus distribution in the respiratory tract and other organs ($H1_{pdm}N2$ virus) in pigs after high dose-aerosol infection with FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$) on 1 and 3 dpi
A, lg $TCID_{50}$ 10.21 / m^3, B, lg $TCID_{50}$ 8.46 / m^3, C, lg $TCID_{50}$ 6.21 / m^3; D, lg $TCID_{50}$ 4.71 / m^3; note the differences in viral lung load on 1 dpi depending on the infectious dose; arithmetic means with standard deviation

After nebulisation of an H3N1 virus isolated from pigs, similar patterns of virus distribution in the respiratory tract were measured as for other influenza A viruses. In this study, an additional direct contact group was followed. On day 3 post-infection, lung viral loads

were still low and reflected a large inter-pig variability. On day 6 post-infection, high levels of virus were observed throughout the airways, similar to those observed in the high-dose aerosol-infected group on day 3 post-infection.

A similar pattern of virus distribution was observed when a 2014 $H1_{pdm}N1$ virus, which was less virulent than the 2010 $H1_{pdm}N1$ virus, was nebulized; see below (Figure 35, page 71).

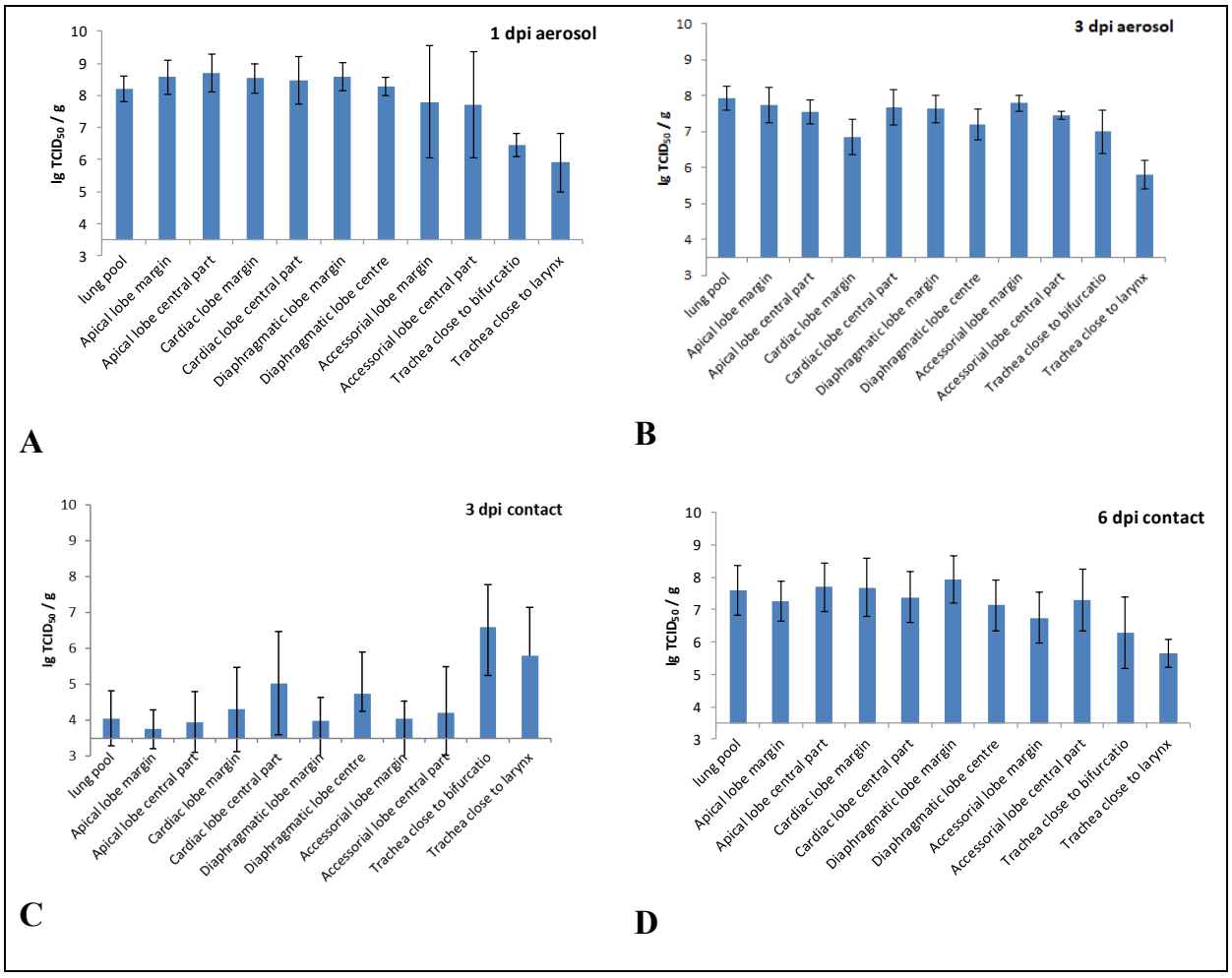


Figure 32: Virus distribution in the respiratory tract (H3N1 virus) infection with FLUAV/sw/Coesfeld/19949/2014 H3N1 (lg $TCID_{50}$ / g)
A, high-dose aerosol nebulisation 1 dpi; B, high-dose aerosol nebulisation 3 dpi; C, direct contact infection 3 dpi; D, direct contact infection 6 dpi; arithmetic mean values with standard deviation

Short interpretation of results. Influenza A viruses are highly adapted to cells of the respiratory tract and spread rapidly throughout the respiratory tract where they replicate to high titres. *In situ* hybridisation with NP probes indicates that expression of the nucleoprotein is also restricted to respiratory tissues. Influenza A viruses also infect other tissues in the body, but do not replicate efficiently in tissues of other organs despite the expression of antigens. No infectious viruses were found in tissues outside the respiratory tract.

5.1.5 MACROSCOPIC LUNG GROSS LESIONS

Background. Macroscopic lung lesions are common after influenza A virus infection, but a comprehensive description of the pattern of lung changes is still lacking.

Study design. The lungs of more than 3000 pigs were analysed after infection with 50 strains of influenza A virus. Lesions were mapped and photographs of the lungs were taken.

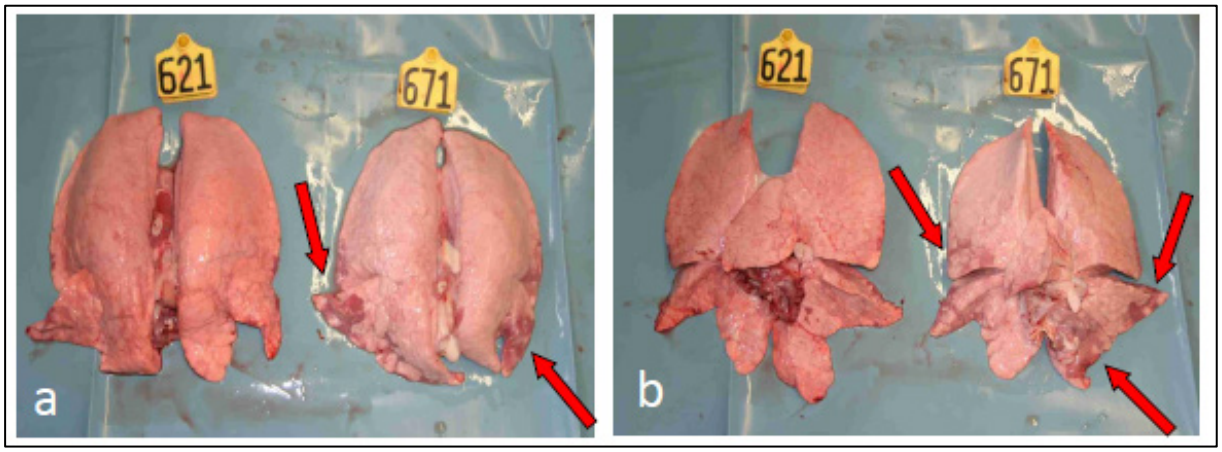


Figure 33: Macroscopic lung lesions 3 dpi after aerosol infection with $H1_{av}N1$ virus
a) dorsal view, b) ventral view; 621, vaccinated pig; 671, unvaccinated pig; arrows indicate lung lesions mainly at the apical part of the cardiac lung lobe

Results. Lesions always started at the margins of the cardiac lobes, which was the most affected lobe, followed by lesions at the edges of the apical lobes. The margins of the diaphragmatic lobes were affected in areas close to the heart lobe (Figure 33). Lesions caused by $H1_{av}N1$ strains were mild, while those caused by $H1_{hu}N2$ and H3N2 strains were much more severe (Figure 14, page 51). The $H1_{pdm}N1$ viruses of April 2009 have caused the greatest expansion of lesions (Figure 18, page 56). Lesions from the dorsal view were larger after infection with European swine influenza A viruses, whereas $H1_{pdm}N1$ caused more lesions from the ventral view. Lesions increased from 1 dpi to 3 dpi. At 10/14 dpi, lesions were clear in most studies, but in some studies they had worsened.

The regular pattern is always: gross lesions start at the margins of the heart lobe, followed by the margins of the rostral part of the diaphragmatic lobe and the margins of the cranial lobes. From the margins, gross lesions extend into the more central parts of the lobes, depending on the virulence of the virus. In more severe cases, gross lesions cover the entire cardiac and cranial lobes and the rostral parts of the diaphragmatic lobes. In fatal cases, the entire lung is affected by gross lesions (Figure 34).

Evidence for the cause of the first appearance of gross lung lesions at the margins of the heart lobes was obtained from nebulisation experiments with $H1_{pdm}N1$ in pigs vaccinated with low antigen content vaccine batches at the limit of protection. The data obtained show the highest levels of virus in the margins of the heart lobes despite vaccination (Figure 35). This effect will be more pronounced in individual pigs and will be stronger in pigs with a higher degree of protection achieved by the vaccination (Figure 36). Even in well-protected

immunised pigs, a small part of the edge of the pulmonary lobe may have small gross lesions. The high virus titres in this region of the lung in immunised pigs suggest that this high virus replication is due to lower protection in this region. Perhaps the most distantly blood flow in this region is an explanation for this lower level of protection. The blood circulation in these peripheral regions may provide fewer antibodies for protection, but also fewer nutrients, leading to stress in the infected cells.

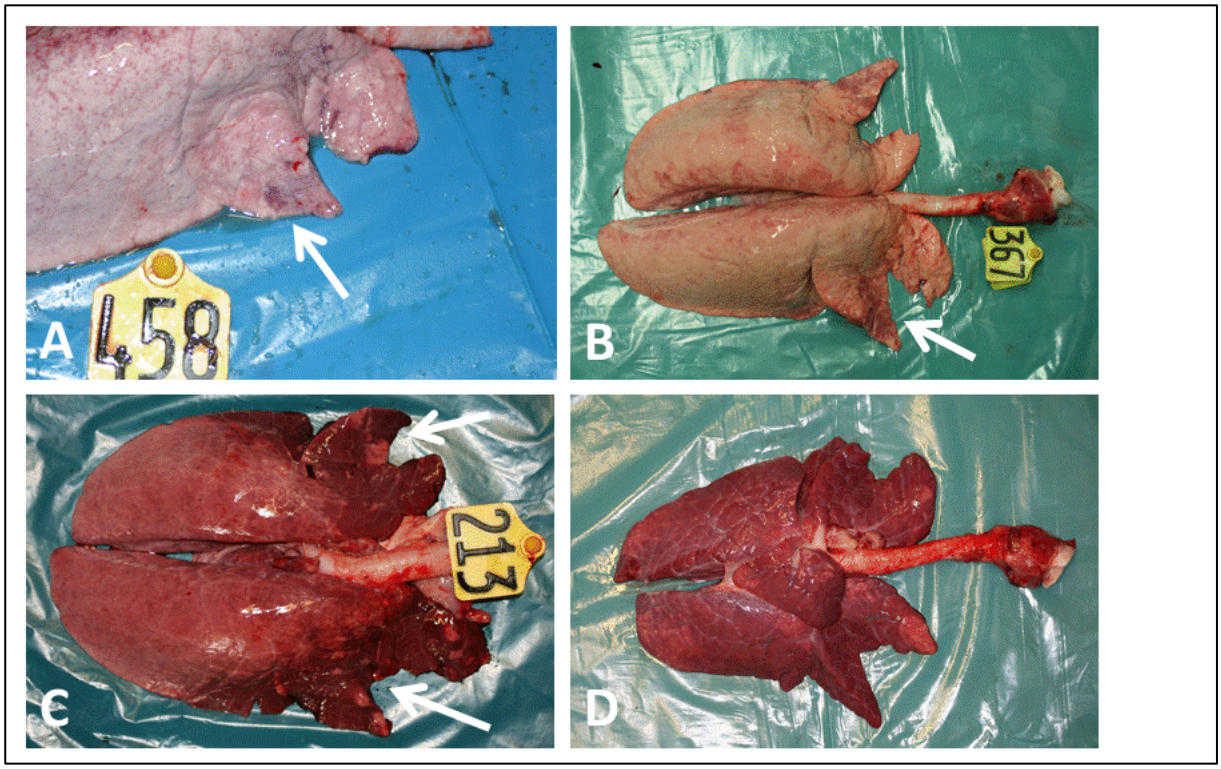


Figure 34: Extension of gross lung lesions

A; infection begins with faint lung lesions at the margins of the cardiac lobe (lesions do not extend further in infections with low virulent viruses and in immunised pigs); B, further progression of lesions; C, lung lesions after infection with highly virulent FLUAVs: the entire apical and cardiac lobes and the rostral parts of the diaphragmatic lobes are affected; D, infection with very highly virulent strains of FLUAVs: the entire lung is affected: lung weight is double that of uninfected lungs

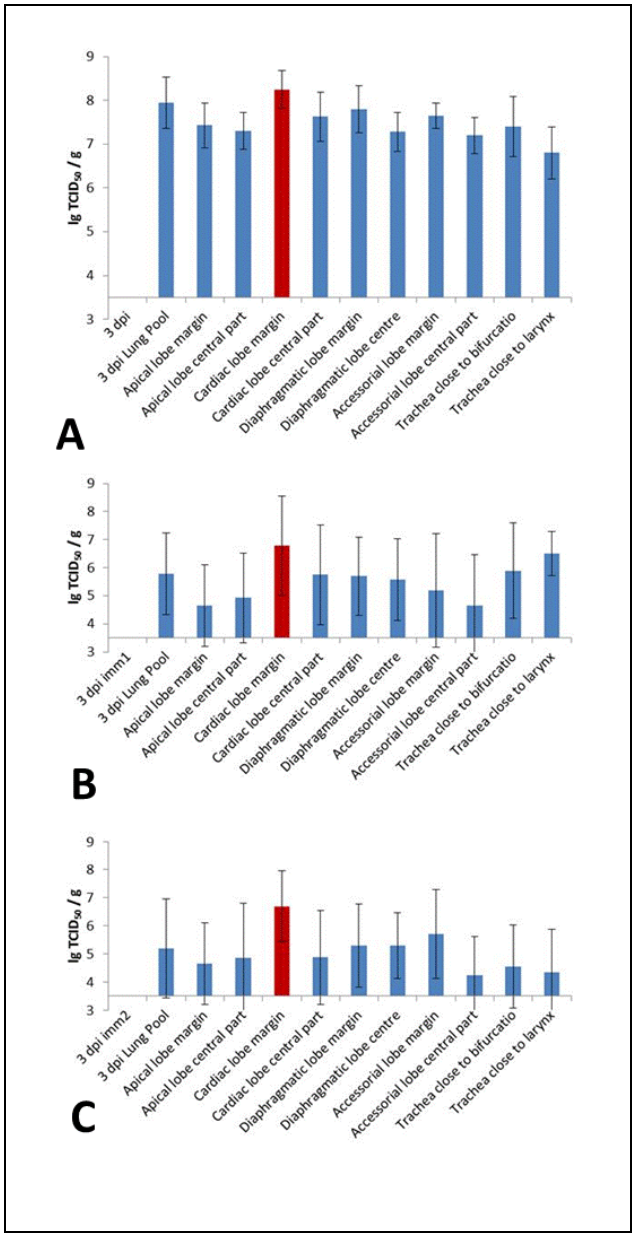


Figure 35: Fine analysis of virus distribution in lungs
reveals a higher viral lung load in the margins of the cardiac lobes 3 dpi after infection with FLUAV/sw/Schallern/19989/2014 ($H1_{pdm}N1$)
A, not immunised pigs; B, $H1_{pdm}N1$ 2009-immunised pigs (low potent batch); C, $H1_{pdm}N1$ 2009-immunised pigs (high potent batch); the high standard variations in immunised pigs indicate a strong individual variation characteristic for antigens at the border of protection; arithmetic mean values with standard deviation

To investigate the differences between regions with macroscopically visible lesions and those without, pieces of such regions were taken from pigs (n=5-10) at different times after infection (1, 3, 5 or 9 dpi) from trials in which $H1_{pdm}N1$ (A/Hamburg/NY1580/April 2009), $H1_{pdm}N2$ (A/swine/Papenburg/12653/2010) and $H1_{hu}N2$ (A/swine/Kitzen/6142/2007) virus was nebulised (Figure 37). The virus content was established by titration in cell culture. Immunhistological investigations were done and cytokine/chemokine (IL6, TNFα) profiles determined.

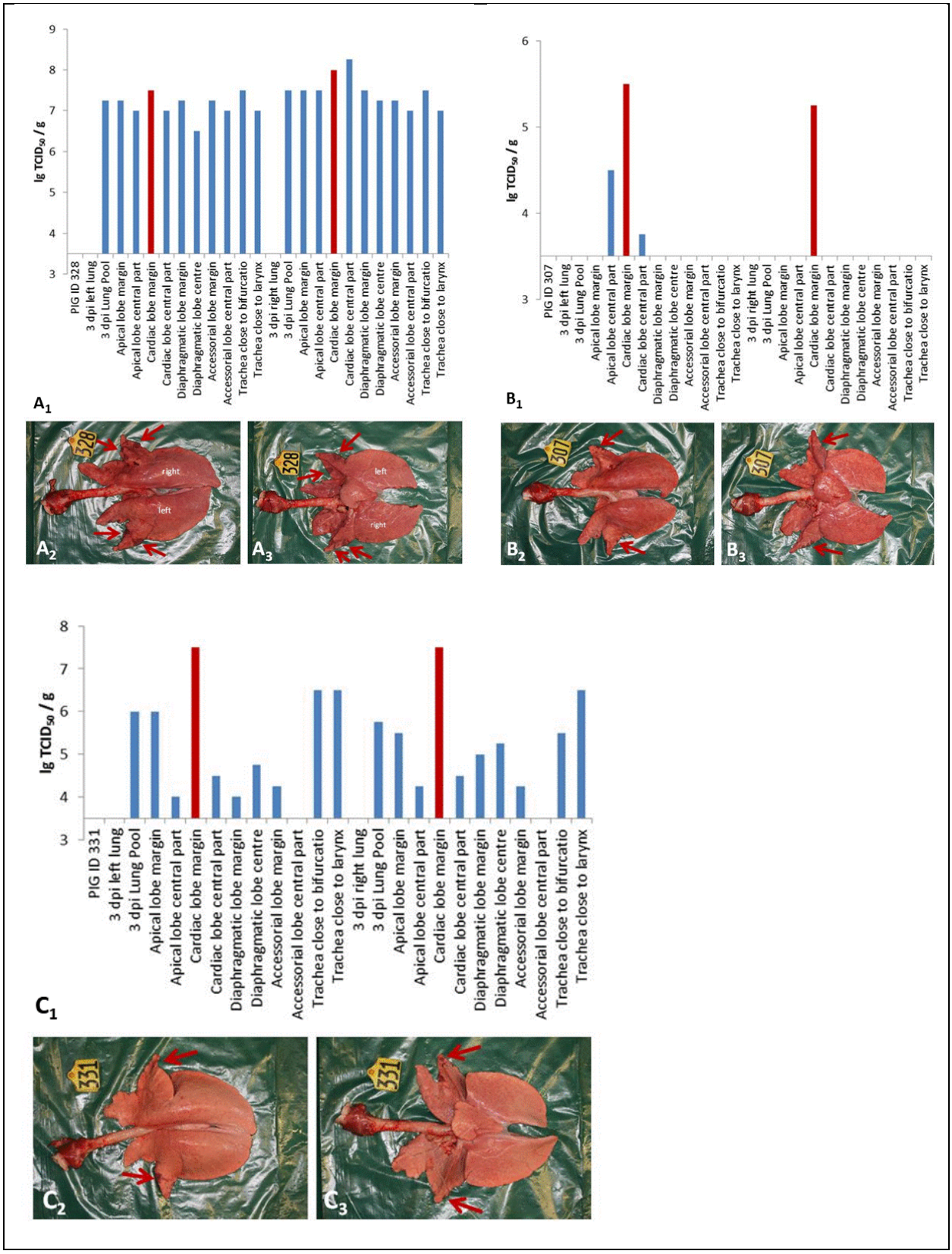


Figure 36: Fine analysis of virus distribution in the lungs ($H1_{pdm}N1$ 2014 virus) of immunised pigs 3 dpi after infection with FLUAV/sw/Schallern(19989/2014 ($H1_{pdm}N1$)

The margins of the cardic lobes which always reflect the first lung gross lesions display the highest amount of virus (red); A, pig not protected after immunisation; B, pig well-protected after immunisation; C; pig with intermediate protection by immunisation; the photographs show that all 3 pigs have lesions at the margins of the cardiac lobes ($_2$, dorsal view, $_3$, ventral view, red arrows point to the lesions); the extension of the lesions to the central part of the cardiac lobe may explain why the viral load of this part of the lung is so high at the right side of the lung of pig 328; arithmetic means are displayed

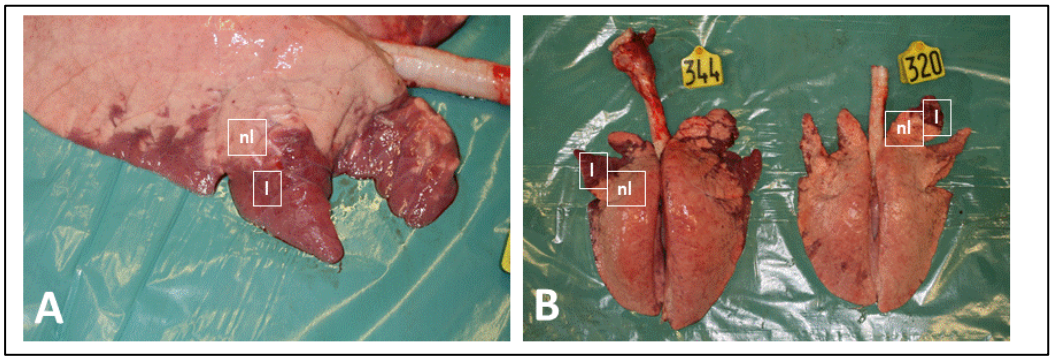


Figure 37: Sampling for comparative analysis of virus load (lesion vs. no lesions)
A, details of sampling; B, other examples of sampling; l, lesion; nl, no lesion

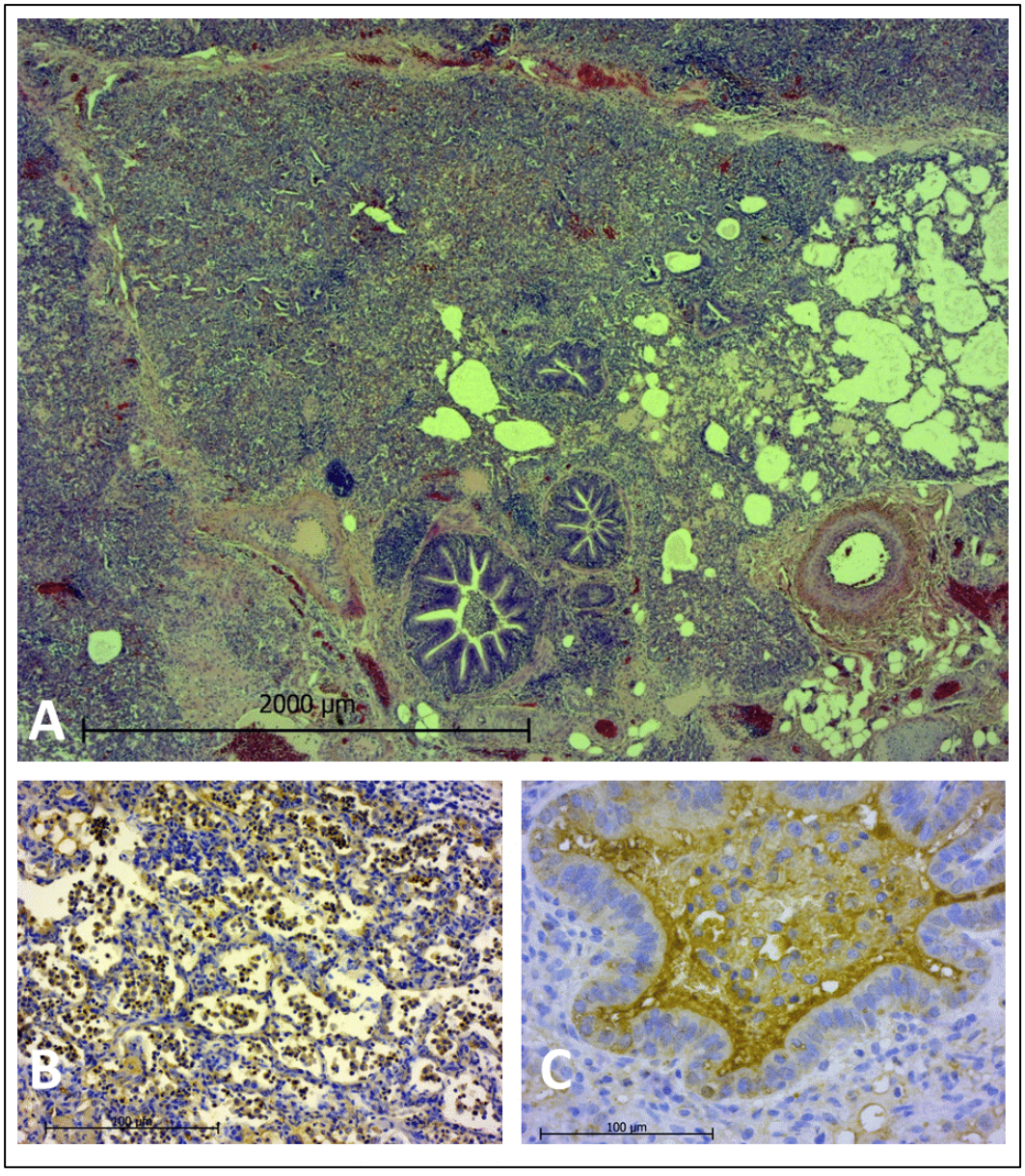


Figure 38: Immunhistological investigation of lung samples with lesions
A, the most remarkable histological sign within a lung with lesion in comparison to neighbored regions without lesion is the size of areas with an accumulation of interstitial macrophages (type A and B), here a picture is shown from an inflammation herd of 7000 µm of a pig infected with A/Hamburg/NY1580/2009 $H1_{pdm}N1$ April 2009 virus 5 dpi (HE staining); B, interstitial macrophages within an inflammation herd of a region 5 dpi (SABC staining), note the staining of macrophages which carry virus antigen although no infectious virus could be measured at that time after infection; C, alveolar macrophages within an bronchiolus within a lung area with macroscopic visible lesions 5 dpi (SABC staining), note the accumulation of viral antigen in the bronchioles (brownish colour)

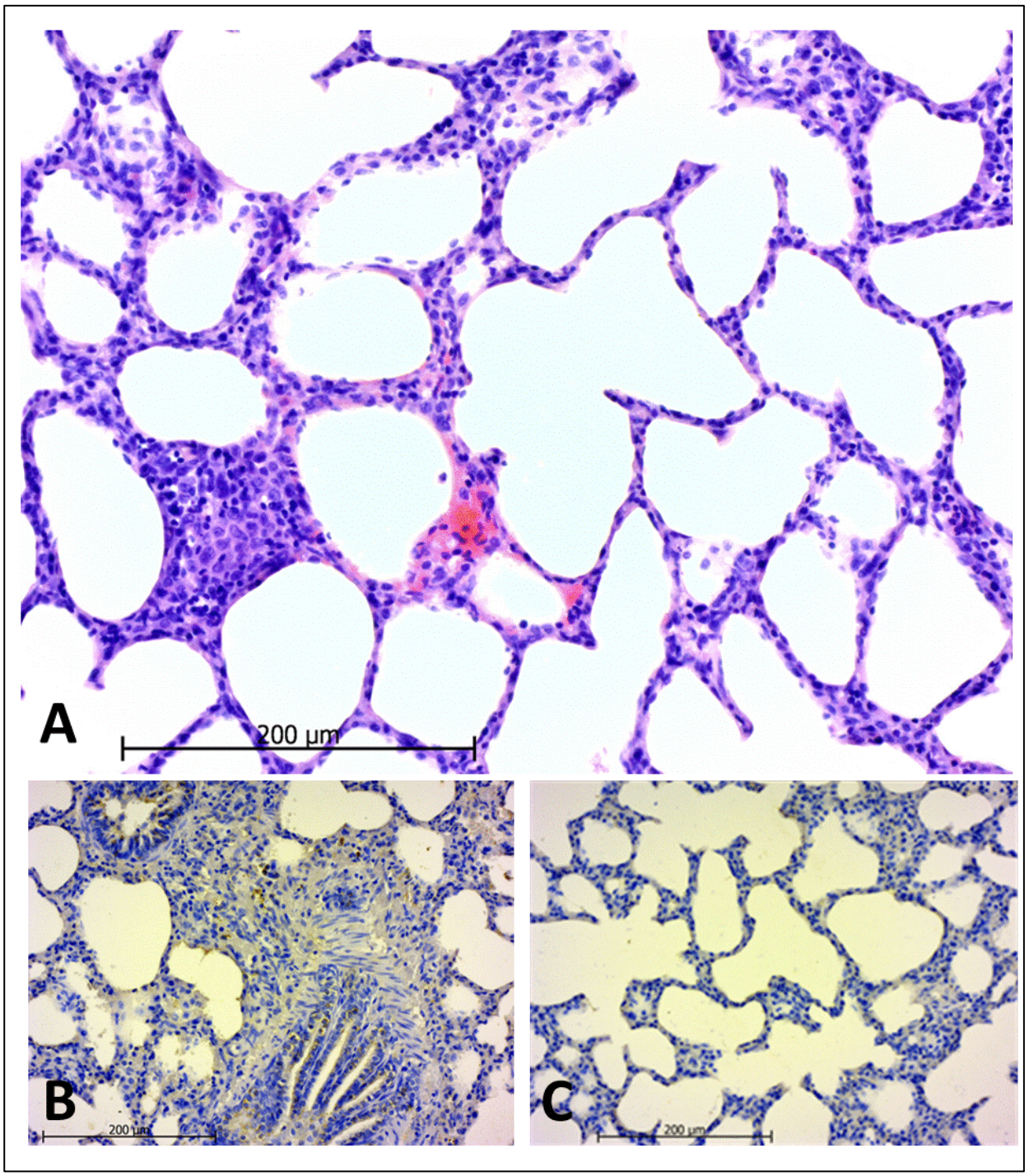


Figure 39: Microscopic analyses of lung regions without lesion
The major differences is the smaller size or the lack of herds with intense infiltration of macrophages, A, HE staining, some interstitial macrophages, mononuclear peribronchitis and slight alveolar edema; B, SABC staining, interstitial and bronchoepithelial virus-specific staining of cells; C, SABC staining, negative control

The main difference between lung regions with macroscopically visible lung lesions and those without is the higher expression of cytokines and chemokines in the first days after infection and the higher infiltration of macrophages in areas with lesions (Figure 38, Figure 39, Figure 40).

There was always a large individual variation in the size of the lung lesions, even though the pigs were from the same farrowing batch and had been infected at exactly the same

time with the same dose of virus. When lung lesions covered more than 50% of the lung, this was associated with greater lung weight. Cytokine expression (detected by IL6) was higher in pigs with larger lesions.

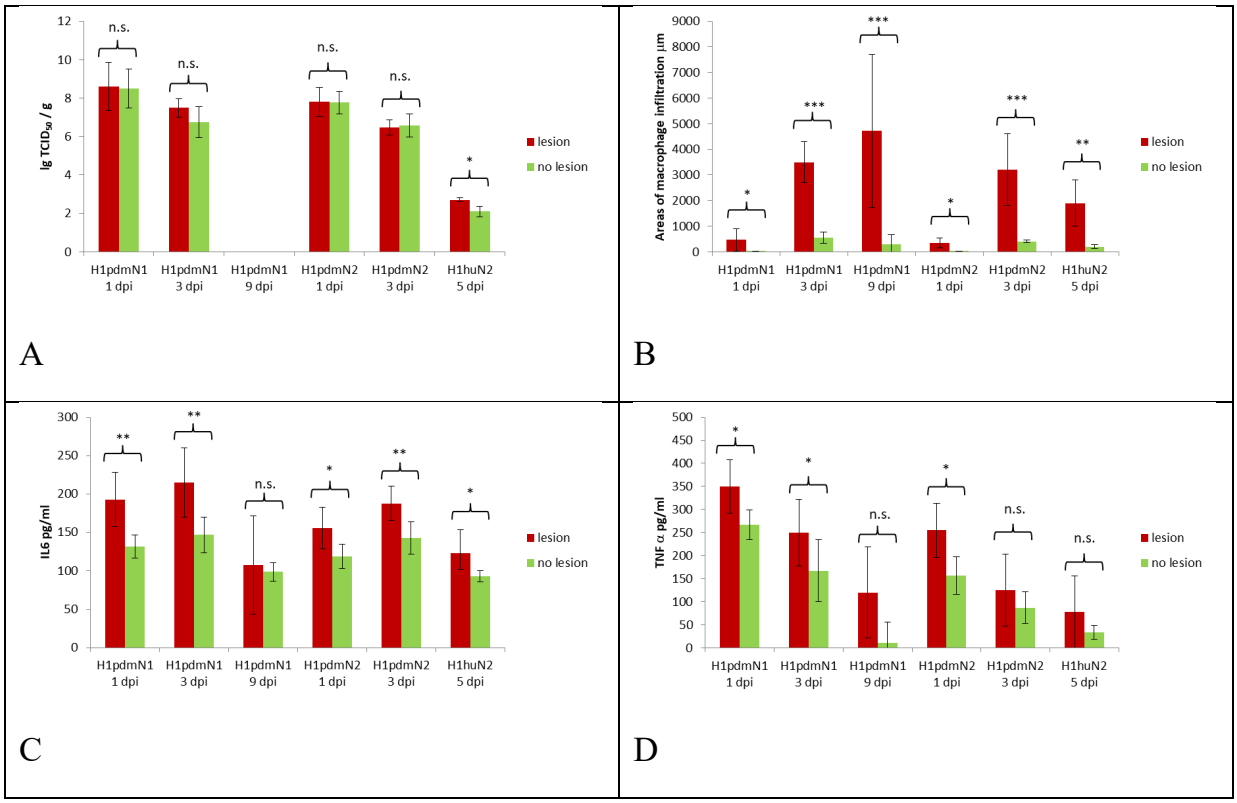


Figure 40: Comparison of adjacent pieces of lung with and without lesions (macroscopically visible lesions) taken from pigs at different times after experimental aerosol infection with $H1_{pdm}N1$ (1, 3, 9 dpi), $H1_{pdm}N2$ (1, 3 dpi) and $H1_{hu}N2$ (5 dpi) viruses; A, virus content of lung (lg $TCID_{50}$/g); B, inflammation as reflected by areas of interstitial macrophage infiltration within a section of a 10 mm piece of lung (herd size in µm); C, Il6 content in 1:10 diluted lung suspensions (pg/ml); D, TNFα in 1:10 diluted lung suspensions (pg/ml)

Short interpretation of results. The data show a clear pattern of distribution and extent of lung lesions after infection. Lung lesions vary according to the virulence of the infecting strain. The viral content of lung sections with lesions is not lower than that of lung sections without gross lesions (except in vaccinated pigs, where there were larger virus titres in the lesions in the apical areas). The higher affinity of lesions to the peripheral parts of the cardiac lobes, even in immunised and otherwise well-protected pigs, indicates a reduced local protective capacity in this region of the lung. Cytokine induction was higher in the lesions, suggesting a stronger interaction between type II pneumocytes and alveolar macrophages in these regions, as evidenced by the large size of inflammatory herds within the lesions. Despite the strong inflammation in these areas, the changes are tolerated by the infected pigs if the areas of lung lesions are not too large (not larger than 1/3 of the lung). There is a strong individual variation in lesion size between individuals.

5.1.6 APPLICATION OF THE AEROSOL MODEL FOR SCIENTIFIC INVESTIGATIONS

5.1.6.1 INVESTIGATION OF PROTECTIVE EFFECTS OF IMMUNOGENIC COMPONENTS OF FIELD ISOLATES OF INFLUENZA A VIRUSES

Background. To investigate the protective effects of immunogenic components of isolated field influenza A virus strains (haemagglutinin, neuraminidase, matrix protein), pigs were immunised with different viruses that differed in their relationship to the H3N1 influenza virus strain which was used for experimental challenge infection (Table 12, Table 13).

Study design. The pigs came from a pig herd that was free of influenza virus infection and had no antibodies to influenza A viruses (ELISA against M protein was negative). Prophylactic treatment with tulathromycin (Draxxin™) was given at 5 and 8 weeks of age. Different influenza viruses were cultured, inactivated with ethylenimine, deactivated with sodium thiosulphate, adjusted to 64 haemagglutinating units per ml, adjuvanted with carbomer and used for immunisation. Six groups of pigs were tested, each consisting of 15 pigs (Table 12). In addition to the immunised groups, two control groups were used (negative control without immunisation; contact control without immunisation and experimental infection: natural infection by contact with the others, achieved by placing the pigs with infected pigs 1 day after aerosol infection of the other pigs). The pigs were 68 days old at the time of the first immunisation and 89 days old at the time of the second immunisation (1 ml was administered at each immunisation). Challenge infection was performed 1 week after the second administration of the corresponding antigen (96 days of age). The H3N1 virus was nebulised at a high dose and induced severe disease ($10^{9.4}$ $TCID_{50}$ $MDBK/m^3 = 10^{10.65}$ $TCID_{50}$ $MDCK/m^3$). The infectious strain was isolated from pigs with respiratory disease in Germany. This was one of the two cases of H3N1 virus spreading from one pig herd to another. The lungs of 5 pigs from each group were examined on 1, 3 and 9 days after infection.

Table 12: Details of the experiment investigating immunogenic effects of proteins of influenza A viruses

Group	Virus used for immunisation	Main component of the vaccine related to the infection strain	Number of pigs included
1	FLUAV/sw/Bakum/1769/2003 (H3N2)	haemagglutinin	15
2	FLUAV/Jena/VI5258/2009 ($H1_{pdm}N1$)	neuraminidase	15
3	FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$)	matrix protein	15
4	FLUAV/sw/Coesfeld/19499/2014 (H3N1)	all proteins, homologous virus	15
5	None (control group, not infected)	-	15
6	None (indirect contact control)	-	15

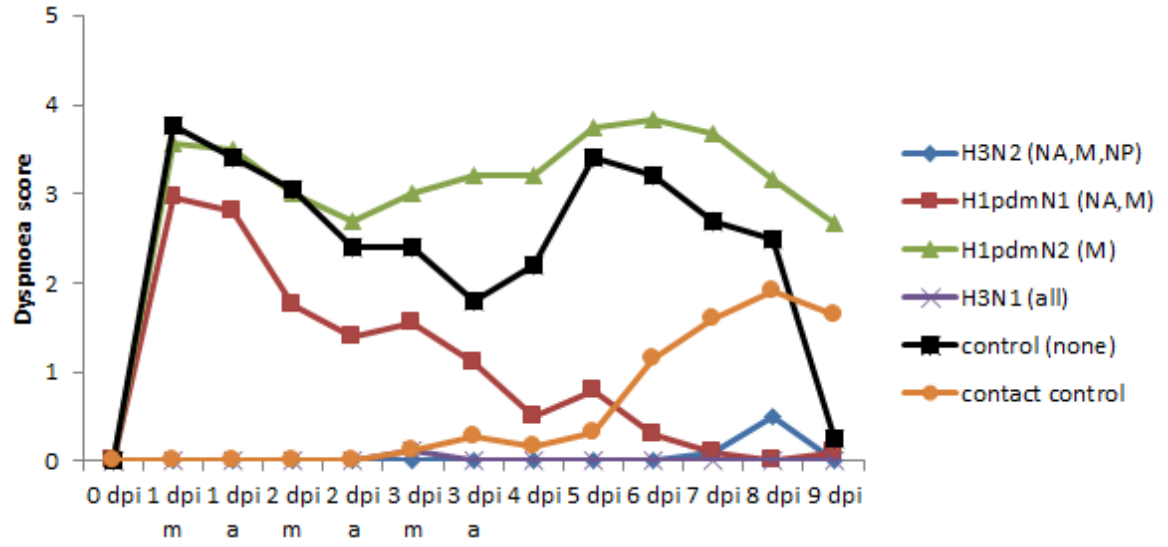
Table 13: Overview of the antigens used for immunisation
and their antigenetic relation to the H3N1 infection strain as deduced from sequence data
(green: antigens closely related to the infecting strain; red: major immunogenic components)

Strain	Subtype	PB2	PB1	PA	HA	NP	NA	M	NS
1769/03	H3N2	-	✓	✓	✓	✓	-	✓	✓
5258/09	$H1_{pdm}N1$	-	-	-	-	-	✓	✓	✓
12653/10	$H1_{pdm}N2$	-	-	-	-	-	-	✓	✓
19499/14	H3N1	✓	✓	✓	✓	✓	✓	✓	✓
Not immunised control		-	-	-	-	-	-	-	-

✓related to the gene of the infection strain at the antigenic level, i.e. vaccine 12653/2010 ($H1_{pdm}N2$) represents the protection provided by the matrix protein among the main immunogenic components, while vaccine 5258/2009 ($H1_{pdm}N1$) represents the protection provided by the neuraminidase (and matrix protein); vaccine 1769/2003 (H3N2) represents protection provided by haemagglutinin (and matrix protein and nucleoprotein) without neuraminidase and vaccine 19499/14 (H3N1) represents homologous protection (maximum possible protection based on this type of vaccine, all viruses were inactivated, all vaccines were carbopol-adjuvanted; the infection strain A/Coesfeld/19499/2014 consists of PB2 and NA of $H1_{av}N1$ 1C.2.2 viruses (PB2 A/swine/Frankfurt/14693/2012-like, NA A/swine/Ankum/14132/2011-like) and six segments of H3N2 viruses (A/swine/Nottuln/18090/2013-like), for GenBank accession numbers see tables in the supplement of volume 1 of this monograph[1]

Results. Infection caused severe disease in the control group, with 3 cases resulting in death (Figure 41 B). Pigs that were immunised with an H3 antigen were protected against high levels of virus in the lungs and against disease (Figure 41 A, D). Pigs immunised with an antigen covering only the neuraminidase of H3N1 ($H1_{pdm}N1$) or the matrix gene ($H1_{pdm}N2$) had no differences in viral lung load compared to unvaccinated control pigs, but viral lung load decreased sharply at 3 dpi, indicating a faster immune response than in unvaccinated pigs (Figure 41 D). Viruses in the contact control pigs replicated at a later stage in the lungs and at a lower titre (Figure 41 E, G). Lung lesions were pronounced in aerosol-infected controls, but also in immunised pigs protected only by the matrix antigen. This was due to the configuration of the antigens of the immunogen ($H1_{pdm}N2$ group; Figure 41 F). Contact controls also developed lung lesions, but to a lesser extent than aerosol-infected controls (Figure 41 G). Inflammation (interstitial pneumonia) was severe in all groups except those immunised with H3 antigens (Figure 41 H). Virus shedding was significantly lower in H3-immunised pigs and delayed in contact controls (Figure 41 C). Pigs immunised with $H1_{pdm}N1$ virus were protected from severe disease despite high lung burden and responded similarly to oseltamivir-treated animals in other studies (compare with data of Dürrwald et al., 2013[88]). Pigs immunised with an antigen covering only the matrix gene of H3N1 developed severe disease and 2 pigs died after infection (4 + 6 dpi; the pig that died at 6 dpi had bacterial co-infection; Figure 41 B; Supplement 82, page CLXX; Figure 42, Figure 43). This group showed effects of an enhancement of disease regarding the lung lesions (Figure 41 F). Pigs without bacterial co-infection and those with bacterial co-infection also showed severe lung pathology (Figure 42, Figure 43). The following bacteria were detected in the pig with bacterial co-infection: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus sp.* (it was negative for *Haemophilus parasuis*,

Actinobacillus pleuropneumoniae). Pigs vaccinated with H3N2 and H3N1 virus developed high titres of HI antibodies prior to challenge. $H1_{pdm}N1$ and $H1_{pdm}N2$ immunised pigs behaved in the same way as unimmunised controls and produced specific H3 antibodies between 4-7 dpi. The antibody response in the contact control group was delayed by two days, indicating that infection occurred within the first two days after contact. Individual data are shown in Supplement 72 to Supplement 82, pages CLX-CLXX.



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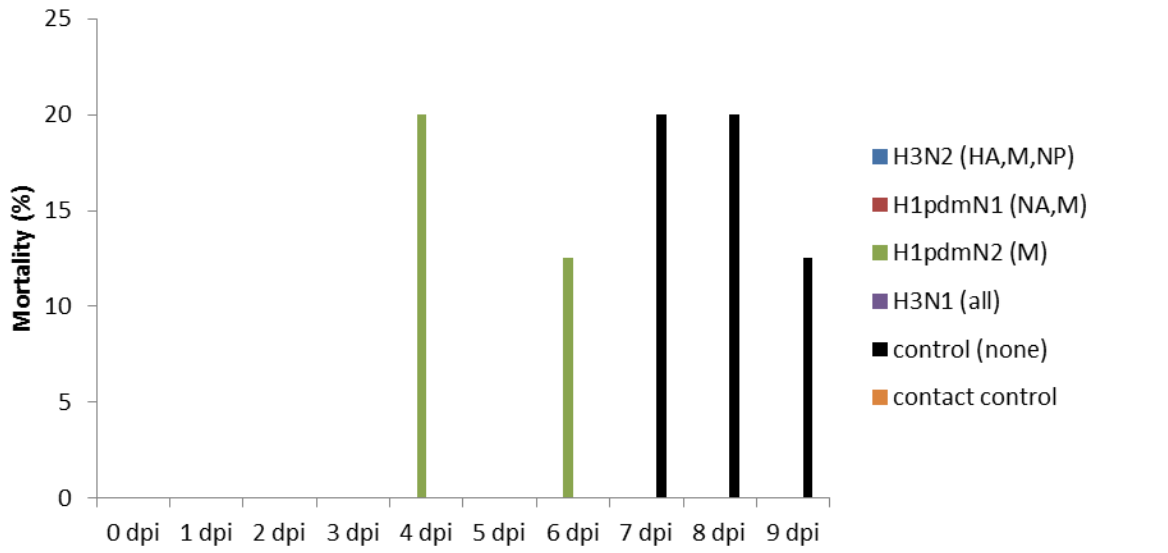

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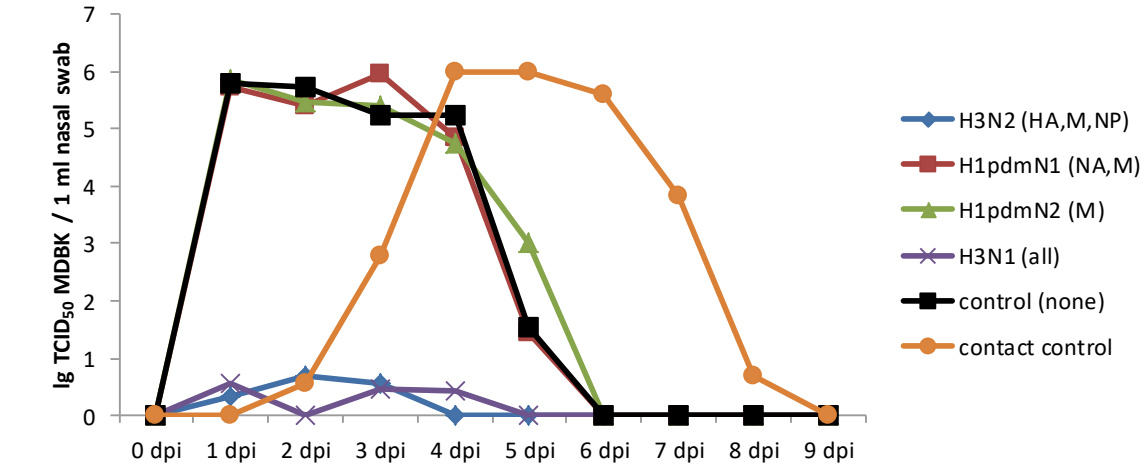

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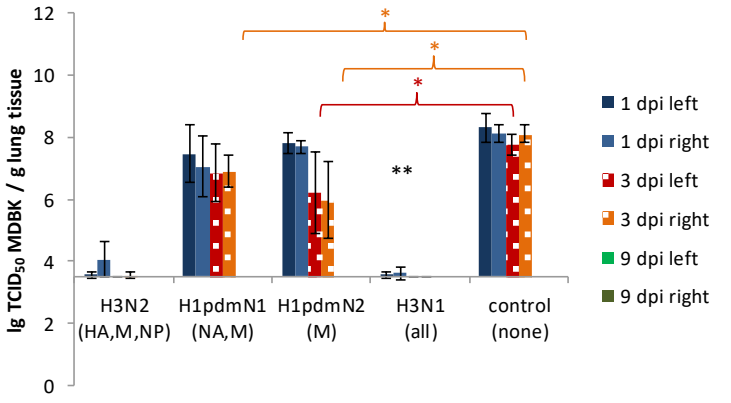

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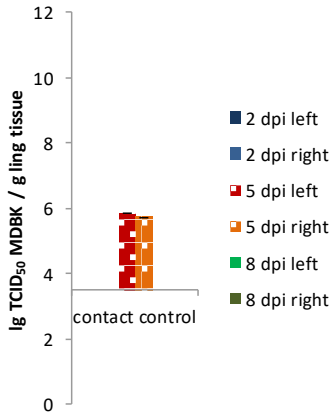

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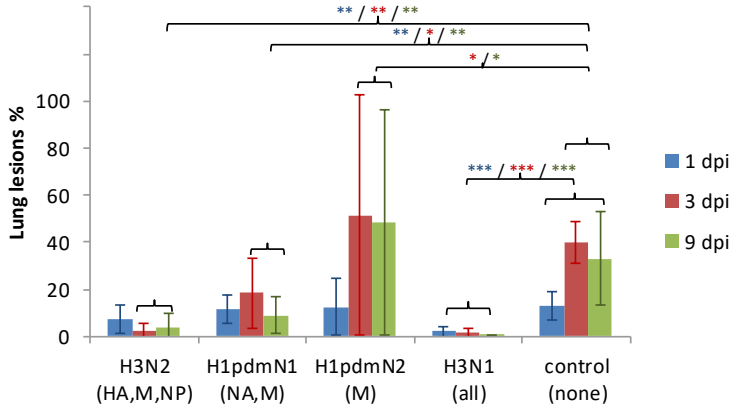

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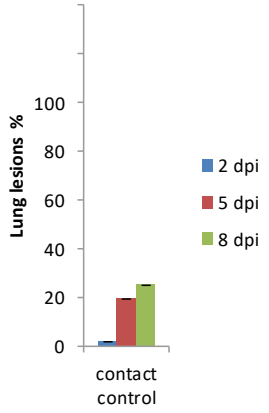

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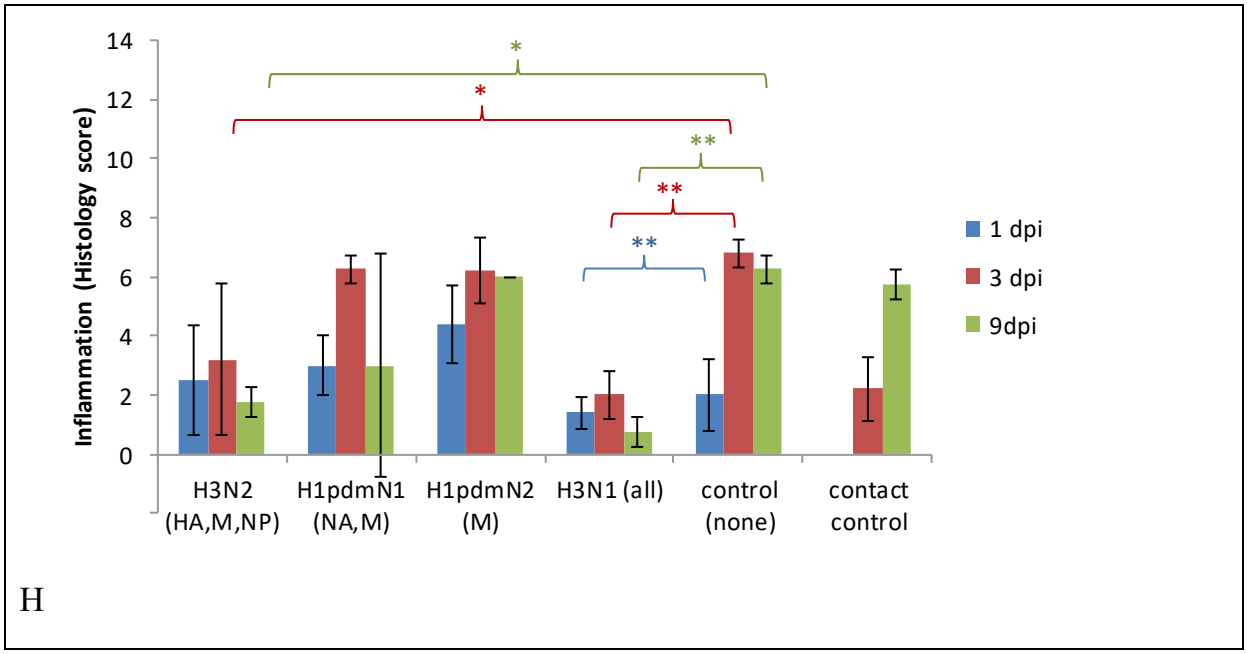


Figure 41: Investigation of the effects of immunogenic components of influenza viruses against high-dose infection with H3N1 virus

H3N1 virus is homologous to vaccine group H3N1 (all); A, Symptoms (dyspnoea score); B, Mortality (%); C, Virus excretion (lg $TCID_{50}$/0,1 ml); D, E, Viral lung load (lg $TCID_{50}$/g); F, G, lung lesions (%); H, Inflammation (histology score); HA, hemagglutinin; M, matrix protein; NP, nucleoprotein; NA, neuraminidase, all, entire virion; arithmetic means with (D-H) and without (A-C) standard deviation are displayed

Explanations to Figure 41

Symptoms (dyspnoea score) *of pigs immunised with different antigens after challenge with H3N1 virus; dpi, days after infection; for better visualisation only arithmetic mean values are shown (1 dpi 15 pigs each group, 2-3 dpi 10 pigs each group, 4 dpi onwards 5 pigs each group); there was highly significant less dyspnoea in H3N2- and H3N1-immunised pigs in comparison to controls as well as to $H1_{pdm}N1$- and $H1_{pdm}N2$-immunised pigs ($p<0.001$); not immunised controls and $H1_{pdm}N2$-immunised pigs reflected a similar pattern of severe respiratory disease which did not differ significantly; $H1_{pdm}N1$-immunised pigs developed significantly less dyspnoea in comparison to controls on 1 dpi ($p<0.05$) and recovered quickly thereafter; contact control displayed a delay in the occurance of dyspnoea which is due to natural contact infection; contact controls had a highly significantly lower degree of dyspnoea ($p<0.01$)in comparison to aerosol-infected controls (control none); H3N2, $H1_{pdm}N1$, $H1_{pdm}N2$, H3N1, antigens used for immunisation; H3N1 is homologous to challenge strain; (HA,NA,M,NP), antigens related to those of challenge strain (Hemagglutinin, Neuraminidase, Matrix protein, Nucleoprotein);* ***Mortality*** *was only seen in groups $H1_{pdm}N2$ (M) and not vaccinated control group;* ***Virus shedding (lg $TCID_{50}$ MDBK/1 ml nasal swab)*** *in pigs immunised with different antigens after challenge with H3N1 virus; dpi, days after infection; for better visualisation mean values are shown (1 dpi 15 pigs each group, 2-3 dpi 10 pigs each group, 4 dpi onwards 5 pigs each group); there was highly significant less virus shedding in H3N2- and H3N1-immunised pigs in comparison to controls as well as to $H1_{pdm}N1$- and $H1_{pdm}N2$-immunised pigs on 1-4 dpi ($p<0.001$), there were no differences in between controls and $H1_{pdm}N1$- and $H1_{pdm}N2$-immunised pigs (not significant); contact control displayed a delay in their shedding curve by 3 days which is due to natural contact infection but show the same height and pattern of virus excretion****; Viral lung load (lg $TCID_{50}$ MDBK/g lung tissue)*** *of pigs immunised with different antigens after challenge with H3N1 virus; dpi, days after infection; for better visualisation only significant differences in comparison to not immunised control pigs are shown by asterisks, *, $p<0.05$; **, $p<0.01$; H3N1- and H3N1-immunised pigs displayed an highly significantly lower viral lung load 1 and 3 dpi whereas $H1_{pdm}N2$- and $H1_{pdm}N2$-immunised pigs reflected significantly lower viral lung loads only on 3 days dpi; 3.5, detection limit (due to predilutions of lung samples);* ***Lung lesions (%)*** *in pigs immunised with different antigens after challenge with H3N1 virus; dpi, days after infection; for better visualisation only significant differences in comparison to not immunised control pigs are shown; all other parameters reflected not significant differences in comparison to not immunised pigs, $p\geq0.05$; H3N2, $H1_{pdm}N1$, $H1_{pdm}N2$, H3N1, antigens used for immunisation;* ***Inflammation (histology score)*** *in lungs of pigs immunised with different antigens after challenge with H3N1 virus; dpi, days after infection; for better visualisation only significant differences in comparison to not immunised control pigs are shown (*, $p<0.05$; **, $p<0.01$); H3N2, $H1_{pdm}N1$, $H1_{pdm}N2$, H3N1, antigens used for immunisation; H3N1 is homologous to challenge strain; (HA,NA,M,NP), antigens related to those of challenge strain (Hemagglutinin, Neuraminidase, Matrix protein, Nucleoprotein); for* ***Rectal temperatures*** *see Supplement 72, page CLX (Rectal temperatures were significantly higher in pigs of the $H1_{pdm}N1$ (NA, M) and $H1_{pdm}N2$ (M) groups on 1 dpi; for* ***Antibodies*** *see Supplement 80, page CLXVIII and Supplement 81, page CLXIX*

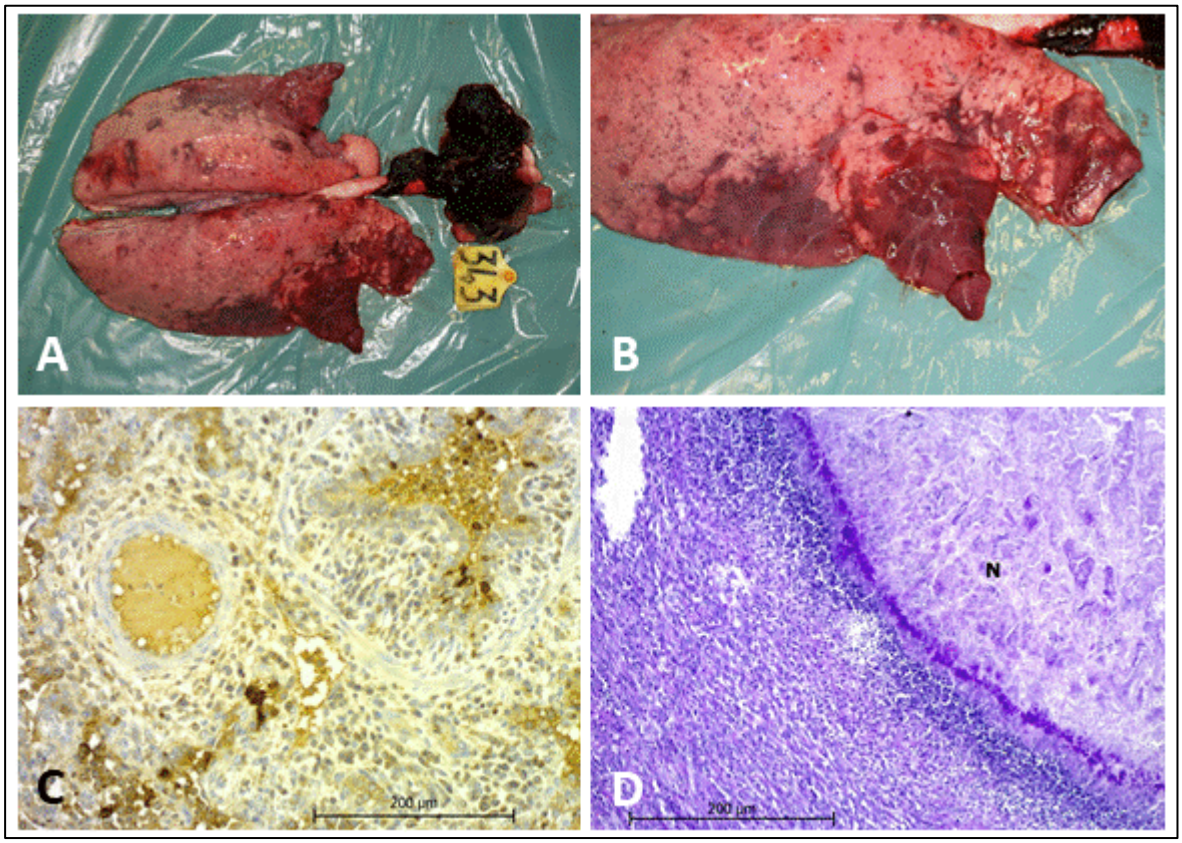


Figure 42: Lung from $H1_{pdm}N2$-immunised lethal case after H3N1 infection 4 dpi (4 days after infection)
A, B, lesions cover the entire apical and medial lung lobes and rostral parts of the diaphragmatic lung lobe; C, SABC-staining: strong inflammation and oedema, obstruction of bronchi, virus-specific staining of macrophages in interstitial tissue and broncho-luminal tissue; D, massive inflammation and necrosis (N), neutrophil and eosinophil granulocytes, hyperaemia, oedema; bacteria were not detected in this lung after Gram-staining

Short interpretation. Major findings of this trial:

i) The H3N1 field strain was highly virulent and caused severe disease with a high lethality (case fatality rate),

H3N1 viruses are rare in nature. During the surveillance of swine influenza in the years 2003 - 2015, only 9 events were observed in which an H3N1 virus was involved. In 7 of these events, H3N1 was confirmed together with H1N1 and H3N2 viruses in the same sample, indicating a recent reassortment after simultaneous infection of the pig herd with H1N1 and H3N2 viruses. In 2 cases, H3N1 virus was isolated but no other virus. These two H3N1 viruses spread within a pig herd and to neighbouring pig herds, but then disappeared. H3N1 viruses are very unlikely to form stable lineages. Therefore, the H3N1 virus isolated from the last event was most likely the result of a recent reassortment. This virus was used in this challenge study. It unexpectedly caused more severe disease than the H3N2 viruses which may indicate that freshly reassorted viruses containing N1 may be more virulent shortly after reassortment.

ii) Field strains provide varying degrees of protection against reinfection,

Protection by the corresponding haemagglutinin includes protection against high viral lung load, high virus shedding, high lung inflammation and severe lung lesions and disease. Protection by neuraminidase alone did not protect against high lung viral load, shedding, lung pathology and respiratory disease, but lung titers declined rapidly and pigs recovered quickly from disease. There was no protection conferred by the matrix protein.

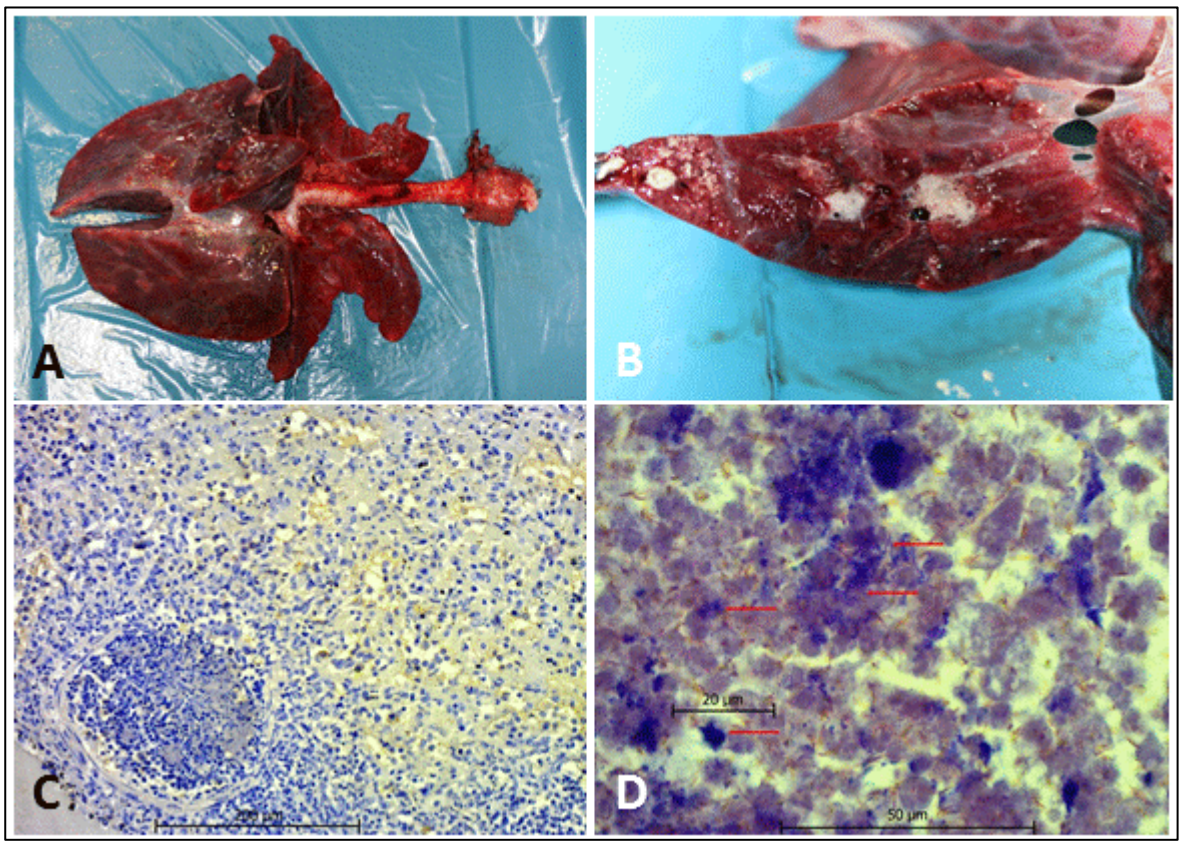


Figure 43: Lung from $H1_{pdm}N2$-immunised lethal case after H3N1 infection 6 dpi (6 days after infection with H3N1 virus)
A, B, lesions cover the entire apical and medial lung lobes and rostral parts of the diaphragmatic lung lobe; C, SABC-staining: strong inflammation, virus-specific staining of macrophages in interstitial and broncholuminal tissue; D, Gram-staining: gram-positive rods and cocci (red arrows); because no others pigs had bacterial infection this pig represents a single case of secondary activation of bacterial growth in the course of viral infection

Pigs vaccinated with $H1_{pdm}N2$ antigen, which only covered the matrix protein in terms of protection against the H3N1 challenge strain, developed symptoms as severe as in the unvaccinated control group. Lung viral load decreased significantly faster in $H1_{pdm}N2$-immunised pigs, but all other parameters did not reflect protection. Lung lesions were more pronounced in $H1_{pdm}N2$-immunised pigs than in control pigs. This may indicate a phenomenon known as "vaccine-induced disease enhancement" (Gauger et al., 2011[156]) where more severe lung lesions were observed in pigs immunised with vaccines whose immunogenic components, such as haemagglutinin and neuraminidase, did not match the infecting strain.

iii) Natural infection, such as contact infection, does not cause as severe disease as high-dose aerosol infection,

The contact control group showed a delay in the onset of symptoms compared with the aerosol-infected control group. This can be explained by the longer time required for virus replication to reach higher titres in the lungs. Despite this, the contact control pigs had a similar disease pattern but at a lower level.

iv) Bacterial infections act in concert with virus infection.

Bacteria were found in one of the pigs that died. Therefore, it is possible that bacterial co-infection contributed to the severe disease outcome in this pig.

5.1.6.2 Investigation of the efficacy of antivirals

Background. There is still a lack of infection models to demonstrate the efficacy of antivirals. Swine influenza A viruses are resistant to amantadine (Krumbholz et al., 2009)[345]. Oseltamivir continues to be effective (Bauer et al., 2012)[358]. The high-dose aerosol infection model was successfully used to investigate the efficacy of oseltamivir against European swine influenza A viruses (Dürrwald et al., 2013)[88], but information on the efficacy of oseltamivir against $H1_{pdm}N1$ has not yet been obtained in porcine infection models.

Study design. 54 crossbred pigs (Piétrain x LargeWhite, Germany) were obtained from a herd that had been free of H1N1 infection for the past 10 years. All pigs were from the same farrowing event and were 76 days old at the time of experimental aerosol infection or 78 days old at the time of contact infection.

Division into groups. Pigs were randomly assigned to 3 main groups (vaccinated, oseltamivir-treated, unvaccinated and untreated control). Pigs in each group were exposed to infectious aerosol (strain FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$); infectious dose 5.75 lg $TCID_{50}/m^3$). 5 pigs from the control group were used as seeder pigs to infect contact pigs, which were moved to the infection unit two days after aerosol infection (Table 14).

Immunisation of swine. 17 pigs were vaccinated at 48 and 69 days of age with inactivated $H1_{pdm}N1$ antigen (isolate from a human patient: FLUAV/Jena/VI5258/2009).

Treatment of swine. 15 pigs were treated orally with Tamiflu® capsules starting one hour before aerosol infection or before exposure to infected pigs with an initial dose of 150 mg oseltamivir (2 capsules of Tamiflu®), followed by 75 mg oseltamivir (1 capsule of Tamiflu®) twice daily for 4 days according to the Tamiflu® SPC. Commercially available Tamiflu® capsules (F. Hoffmann-La Roche AG, Basel, CH, batch B113313) were used for *in vivo* testing in pigs. Each capsule contains 75 mg oseltamivir (98.5 mg oseltamivir phosphate).

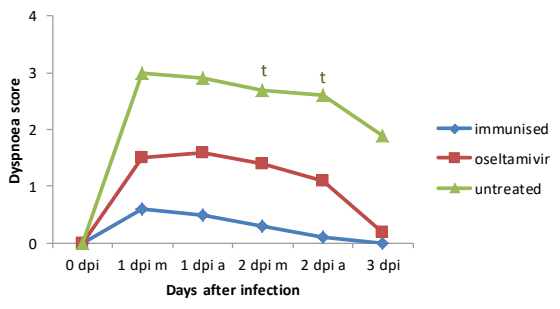
Untreated groups. For each infection model, at least 5 pigs remained unvaccinated and untreated as controls.

Course of the trials. Lung samples were collected from half of the pigs in each group of the high-dose aerosol infection study at 1 dpi and 3 dpi. Two days after infection, the direct contact pigs were moved to the infection unit and housed with the seeder pigs. After 14 days, lung samples were collected.

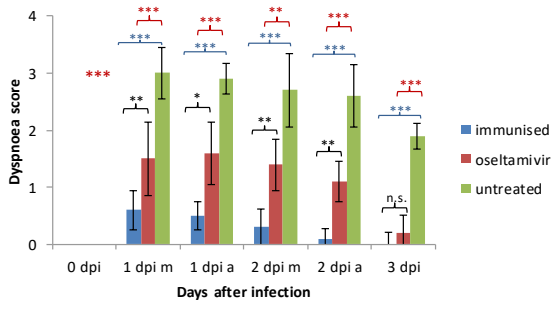
Table 14: Overview of the experimental design of the oseltamivir trial investigating the efficacy of Tamiflu®

Group	Infection model	Treatment	Number of pigs included
1	High-dose aerosol	Immunisation $H1_{pdm}N1$	12
2		Oseltamivir	10
3		None	12
4		None (seeder)	5
5	Direct contact	Immunisation $H1_{pdm}N1$	5
6		Oseltamivir	5
7		None	5

Results of high-dose aerosol infection. Untreated and unimmunised control pigs developed severe respiratory distress after infection (Figure 44 A, B). On 2 dpi, two pigs died (Figure 44 A). Oseltamivir-treated pigs showed significantly fewer symptoms, despite high virus titres measured in nasal swabs and lungs (Figure 44 C, D, E). Oseltamivir-treated pigs had significantly fewer lung lesions than untreated pigs (Figure 44 F). Oseltamivir treatment did not prevent fever (Figure 44 H). Immunised pigs were better protected from becoming infected than those treated with oseltamivir.



A



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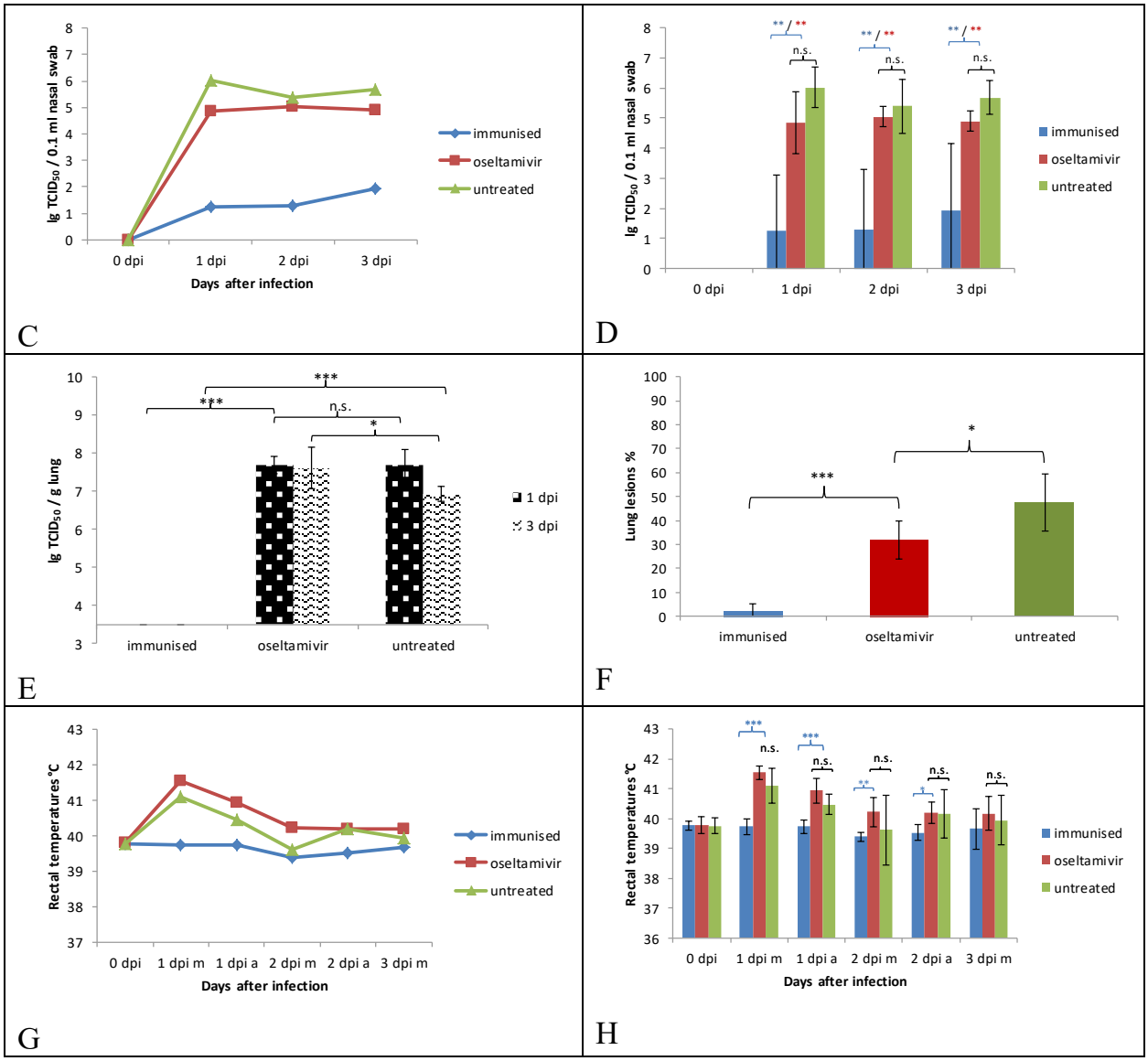


Figure 44: Comparison of immunisation and treatment with oseltamivir after infection with very highly virulent H1$_{pdm}$N1 2009 virus

A, Symptoms (dyspnoea score, trends of arithmetic means); B, Symptoms (dyspnoea score; arithmetic means with standard deviations and results of statistical analysis); C, Virus excretion, lg TCID$_{50}$/0.1 ml (trends of arithmetic means); D, Virus excretion (lg TCID$_{50}$/0.1 ml; arithmetic means with standard deviations and results of statistical analysis); E, Viral lung load (lg TCID$_{50}$/g); F, Lung lesions 1 dpi (%); G, Rectal temperatures (°C, trends of arithmetic means); H, Rectal temperatures (°C, arithmetic means with standard deviations and statistics)

Symptoms (dyspnoea score) in pigs after high-dose aerosol infection with FLUAV/Hamburg/NY1580/2009 (H1$_{pdm}$N1); arithmetic mean values; immunised and oseltamivir-treated pigs displayed significantly lower symptoms in comparison to unimmunised+untreated controls (*, p<0.05); t, pig died (two pigs of the untreated group died 2 dpi; **Virus shedding (lg TCID$_{50}$/0.1 ml nasal swab)**; arithmetic mean values; immunised pigs shed significantly lower virus in comparison to oseltamivir-treated and unimmunised+untreated controls on 1-3 dpi (**, p<0.01); **Viral lung load (lg TCID$_{50}$/g)** in pigs after high-dose aerosol infection with FLUAV/Hamburg/NY1580/2009 (H1$_{pdm}$N1); arithmetic mean values with standard deviation; immunised pigs displayed significantly lower virus titres in the lungs in comparison to oseltamivir-treated and unimmunised+untreated controls on 1+3 dpi (***, p<0.001); oseltamivir-treated pigs started to clear virus earlier from the lung than untreated pigs (*, p<0.05), **Macroscopic visible lung alterations (lesions, %)** in pigs after high-dose aerosol infection with FLUAV/Hamburg/NY1580/2009 (H1$_{pdm}$N1); arithmetic mean values with standard deviation; immunised pigs (***, p<0.01) and oseltamivir-treated pigs (*, p<0.05) displayed significantly lower lesions than unimmunised+untreated controls on 1 dpi; **Rectal temperatures (°C)** in pigs after high-dose aerosol infection with FLUAV/Hamburg/NY1580/2009 (H1$_{pdm}$N1); arithmetic mean values with standard deviation; immunised pigs had no fever and reflected significantly lower temperatures in comparison to oseltamivir-treated and unimmunised+untreated controls on 1 dpi (***, p<0.001); there were also significant differences between immunised and oseltamivir-treated pigs on 2 dpi but none between immunised and untreated due to higher variance in the latter

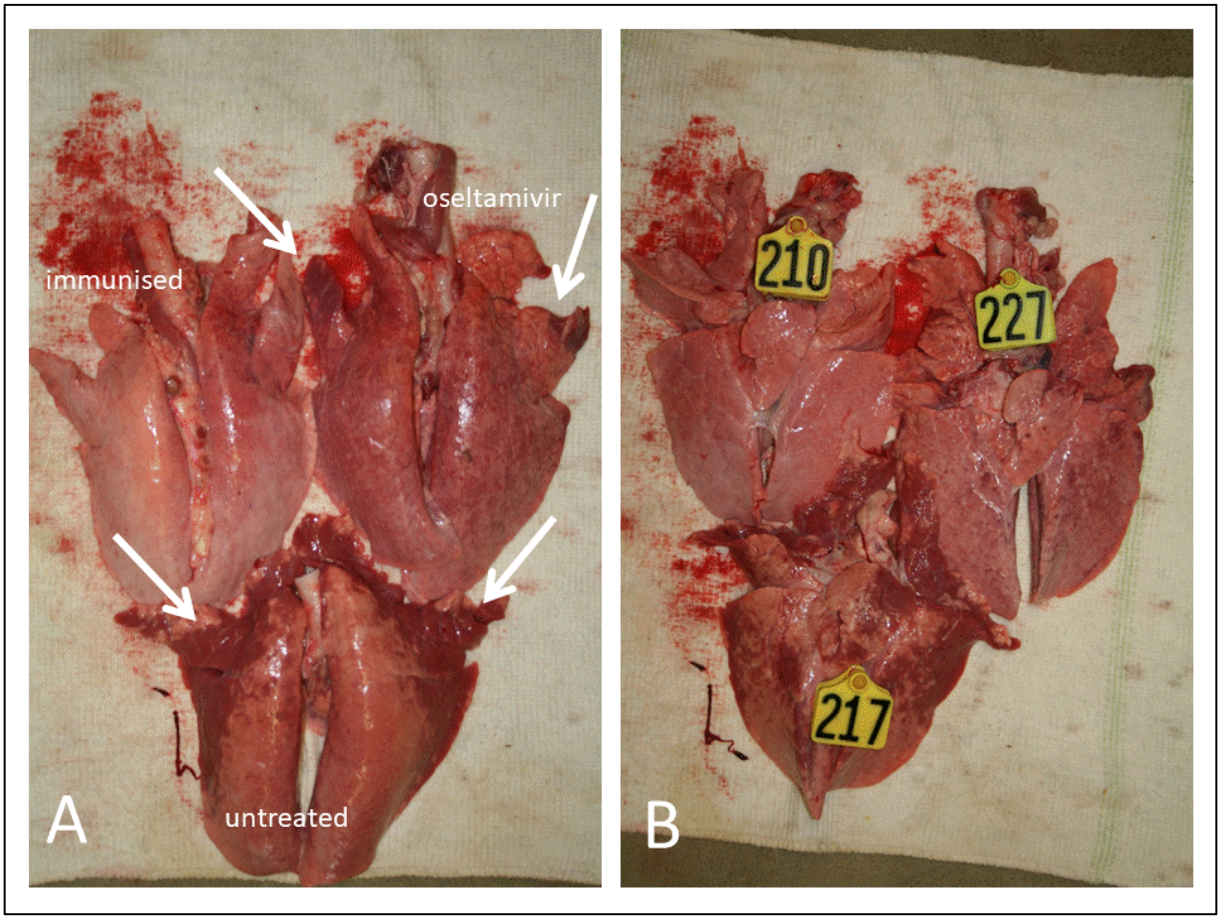


Figure 45: Lung lesions after infection with $H1_{pdm}N1$ April 2009 virus FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$)
A, dorsal view; b, ventral view; arrows indicate position of lesions

Results of contact infection. Pigs infected by contact did not develop severe disease. Comparison with high-dose aerosol-infected seeder pigs reflects the difference in disease induction between the two infection models (Figure 46). As a result of the lower level of disease induced by contact exposure, no significant differences were observed between the groups. Pigs in the untreated group had higher mean rectal temperatures at 5 and 7 dpi, but no significant differences were observed (Figure 47).

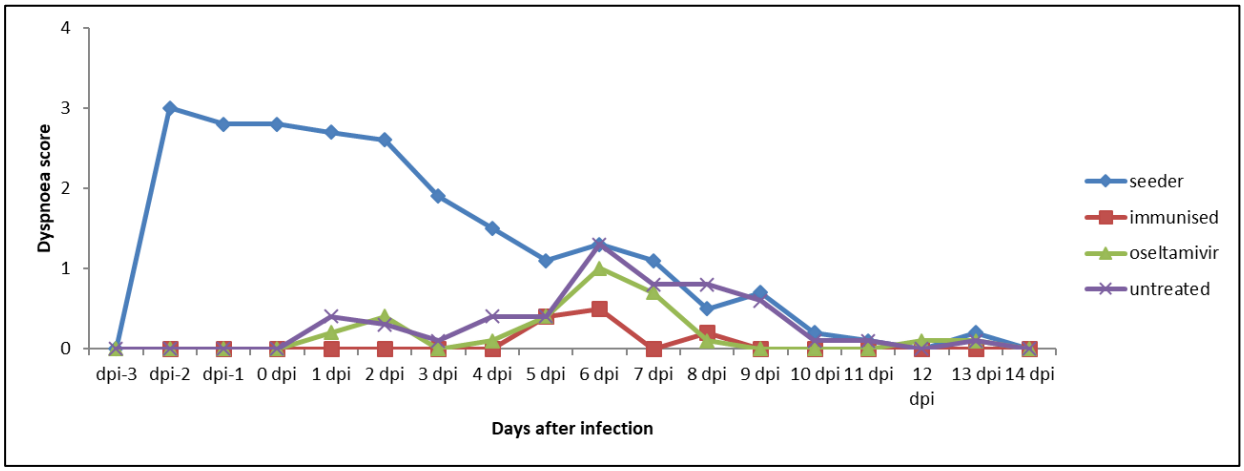


Figure 46: Effect of vaccination or oseltamivir treatment on symptoms
Symptoms (dyspnoea score) in pigs after contact infection by high-dose aerosol infected pigs
strain FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$); arithmetic mean without standard deviation; no significant differences between immunised, oseltamivir-treated and not immunised+untreated pigs were seen

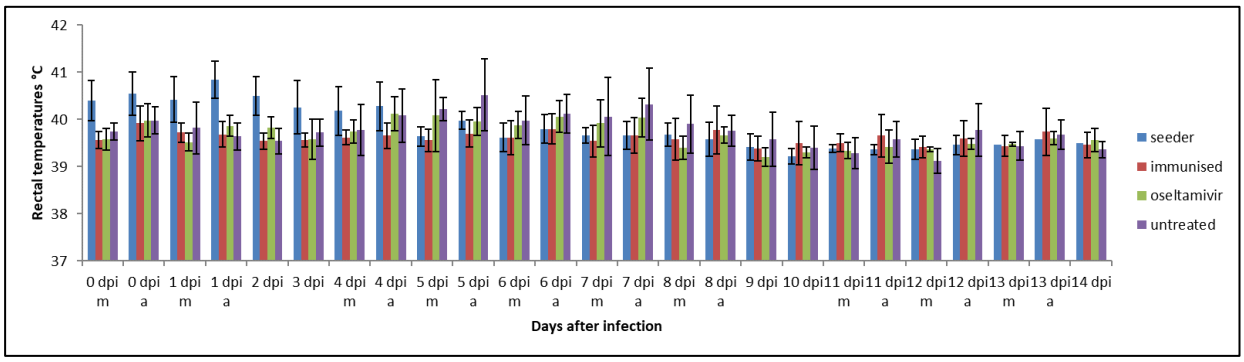


Figure 47: Effects of vaccination or oseltamivir on body temperatures
Rectal temperatures (°C) in pigs after contact infection by high-dose aerosol infected pigs
strain FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$); arithmetic mean with standard deviation; despite higher temperatures in untreated pigs no significant differences between immunised, oseltamivir-treated and not immunised+untreated pigs were observed

Seeder pigs shed high levels of virus 2 days after high-dose aerosol infection. Contact pigs were immediately infected, as shown by virus detection in nasal swabs on the same day. While the seeder pigs cleared the infection, the contact pigs followed the normal course of influenza A virus shedding. Vaccinated and oseltamivir-treated pigs had significantly lower virus shedding, but virus shedding increased again at 5 dpi when oseltamivir treatment was stopped (Figure 48, Figure 49).

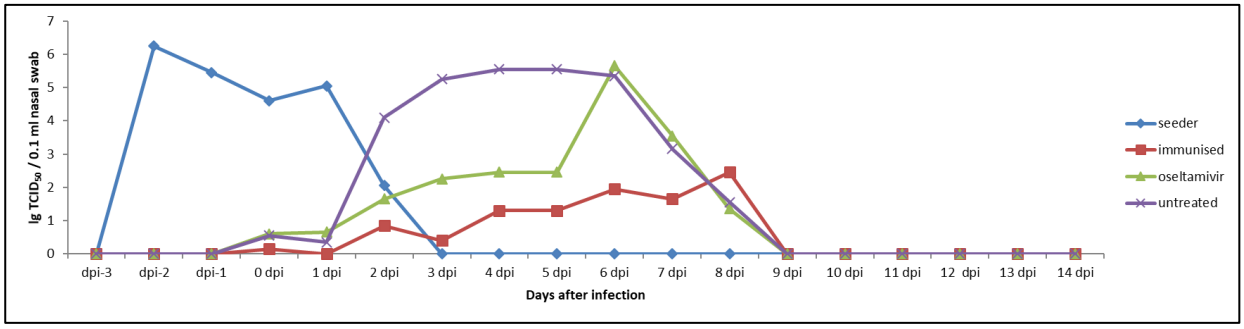


Figure 48: Effects of vaccination or oseltamivir on virus shedding
Virus excretion (lg $TCID_{50}$/0,1 ml nasal swab) after infection of pigs with FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$)
*arithmetic mean without standard deviation; shedding in immunised pigs was significantly lower over the entire investigation period (**, p<0.01); oseltamivir-treated pigs shed significantly lower virus (*, p<0.05) in comparison to untreated pigs until 4 dpi when oseltamivir-treatment stopped*

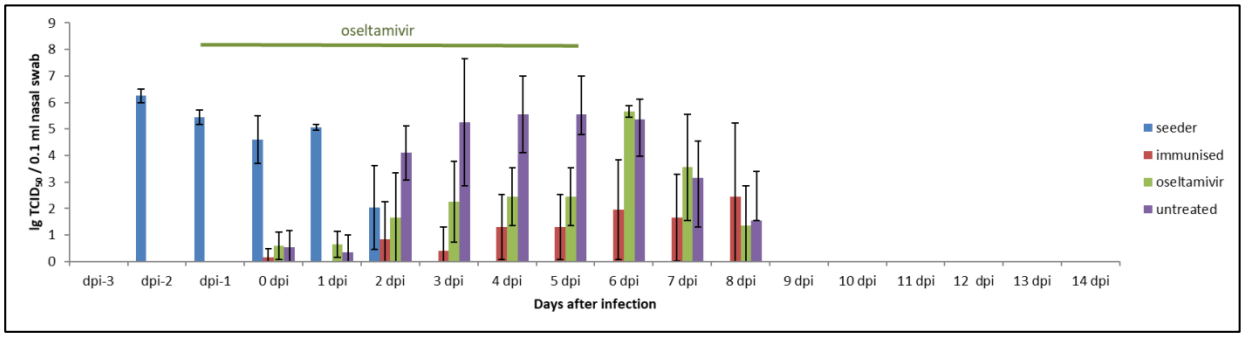


Figure 49: Effects of vaccination or oseltamivir on virus shedding (with statistics)
Virus excretion (lg $TCID_{50}$/0,1 ml nasal swab) after contact infection of pigs with FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$)
The figure shows the same data as in the previous figure, but as a bar chart with standard deviation.
*arithmetic mean with standard deviation; shedding in immunised pigs was significantly lower over the entire investigation period (**, p<0.01); oseltamivir-treated pigs shed significantly lower virus (*, p<0.05) in comparison to untreated pigs until 4 dpi when oseltamivir-treatment stopped; green line, periof of treatment with oseltamivir*

All pigs developed antibodies against the infecting strain between 6 and 8 dpi. This coincided with the cessation of virus shedding. The seeder pigs responded earlier because the aerosol infection was 2 days earlier. Immunised pigs were boosted by the infection. Oseltamivir-treated pigs responded to infection with antibodies, as did untreated pigs (Figure 50).

Short interpretation. The high-dose aerosol infection model was superior in disease induction. The contact infection model provided more insight into shedding kinetics. It was clearly demonstrated that pigs treated with oseltamivir shed less virus until treatment was stopped. Treatment with oseltamivir should be continued until 7 dpi, when antibodies rise. Antibody induction is not inhibited by oseltamivir treatment. This means that oseltamivir treatment is a good application when infected patients cannot be isolated in hospitals. Oseltamivir prevents illness but does not interfere with the development of immunity to influenza.

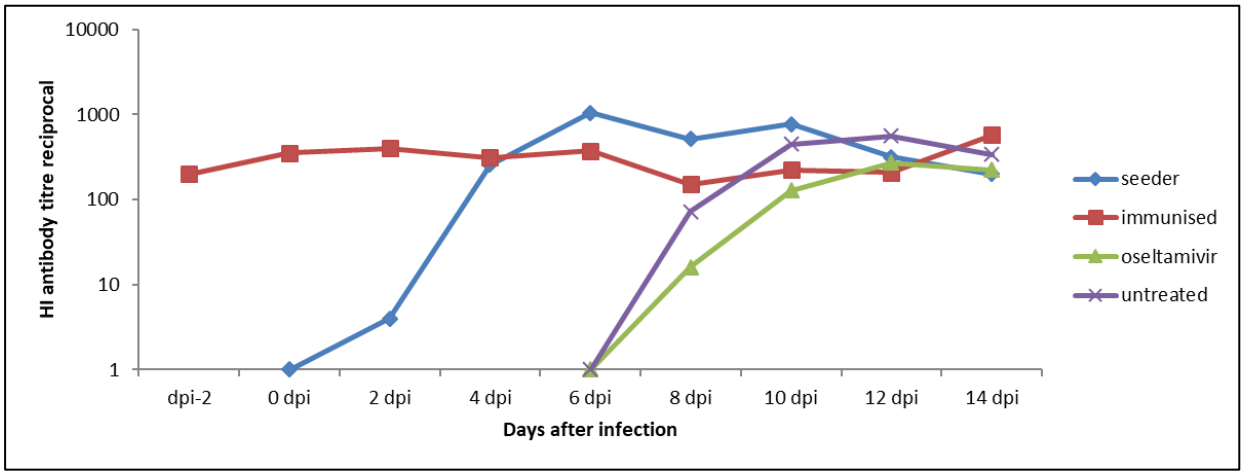


Figure 50: Antibodies (HI against infection strain) after $H1_{pdm}N1$ contact infection FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$)
geometric mean without standard deviation

5.1.6.3 Investigation of the effects of immunosuppression on the course of influenza

Background. Patients undergoing organ transplantation are often treated with immunosuppressive drugs. Influenza virus infections may happen during this treatment. Therefore, the effect of this infection on immunocompromised pigs was studied to gain insight into what might happen in human patients.

Study design. The pigs were treated according to a regimen used for low-risk kidney transplant patients (Ott et al., 2007[359]). Treatment was started 3 days prior to experimental infection. Pigs received cyclosporine A (Sandimmune™, Novartis Pharma GmbH) 2.5 mg/kg body weight orally twice daily from 3 days before infection until the end of the study, mycophenolatmofetil (Myfenax™, Teva GmbH) 1 g orally daily from 3 days before infection until the end of the study, and prednisolone (Prednisolon AL™, ALUID Pharma GmbH) orally (500 mg 3 days before infection, 50 mg 2 days before infection, and 5 mg daily from 1 day before infection until the end of the study).

A virus with low virulence was selected for the trial. The classical swine influenza A H1N1 virus, FLUAV/sw/England/117316/1986 ($H1_{cl}N1$), was cultivated on MDBK cells. The virus replicated to titres of 5.5 lg $TCID_{50}$/ml in the MDBK cell monolayer of roller bottles.

Pigs from the same farrowing event were randomised to different groups and infected on the day 96th day of life (Table 15). Two groups were exposed to high-dose aerosol nebulisation. Two other groups served as indirect contact controls and were housed with the other pigs in the same infection room after aerosol nebulisation to mimic natural infection. A strict control group of 4 pigs was kept on the farm outside the infection unit. At dpi 1 and 3, two pigs from each group were removed for lung sampling. On dpi 9, the remaining pigs were examined. For all other parameters, the experimental procedure followed the general

outline of the other trials (observation and recording of clinical symptoms, daily nasal swabs and blood sampling, weighing).

Results. The virus induced a moderate level of symptoms in pigs. At 3 dpi, significantly more symptoms were observed in pigs receiving immunosuppressive treatment following high-dose aerosol nebulised infection (Figure 51 A), whereas this was not observed following indirect contact infection (Figure 51 B). Pigs had a higher viral lung load at 1 dpi after high dose aerosol infection and at 3 dpi after indirect contact infection, but this was not significant due to the low number of pigs tested = 2 pigs at each time point (Figure 51 C, D).

There were remarkably large differences in virus shedding between immunocompromised and untreated pigs. In both infection models, immunocompromised pigs shed virus until the end of the study (Figure 51 E, F).

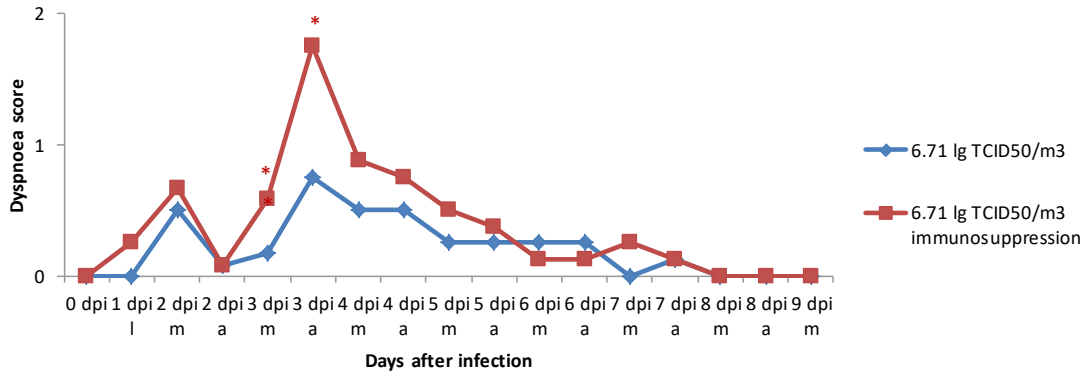
Table 15: Overview of the experiment investigating the effects of immunosuppression

Group	Infection dose lg $TCID_{50}$ / m^3	Immunosuppressive treatment*	Number of pigs included
1	6.71	none	8
2	6.71	yes	8
3	Indirect contact	none	8
4	Indirect contact	yes	8
5	Strict control (no infection)	none	4

* pigs were administered Cyclosporine A 2.5 mg/kg body weight 2x orally daily 3 days before infection until the end of the trial, Mycophenolatmofetil 1 g orally daily 3 days before infection until the end of the trial, and Prednisolon orally (500 mg 3 days before infection, 50 mg 2 days before infection, and 5 mg daily from 1 day before infection until the end of the trial)

Throughout the study (data not shown), the strict control pigs showed no symptoms, no lung changes and no antibodies to influenza.

Macroscopically visible lung changes (lesions) were more pronounced in the immunocompromised groups in both studies, but this could not be statistically proven due to the small number of pigs studied = 2 pigs at each time point (Figure 51 G, H). For the same reason, pigs did not differ significantly in the degree of interstitial pneumonia, but there was a higher standard deviation in immunocompromised pigs from both trials at 3 dpi, reflecting stronger responses in individual pigs (Figure 51 I, J).



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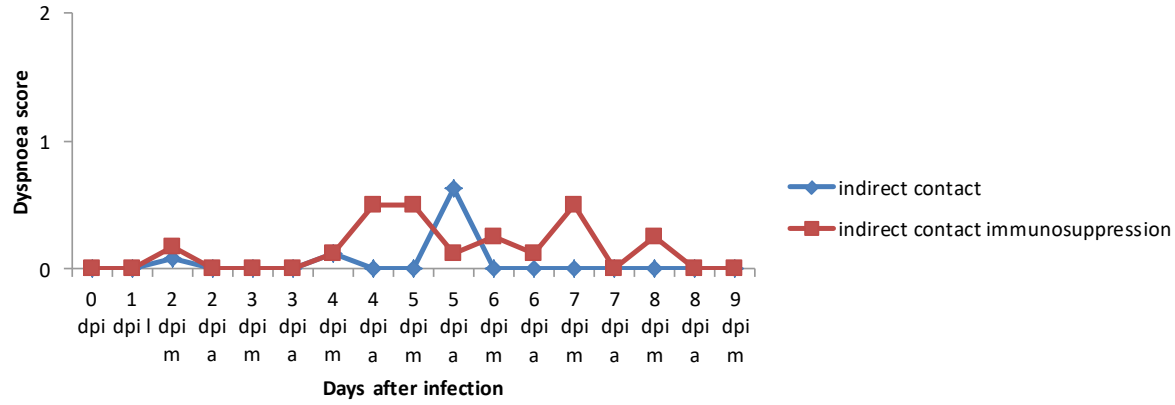

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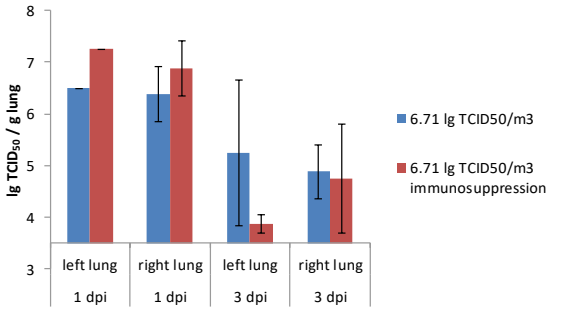

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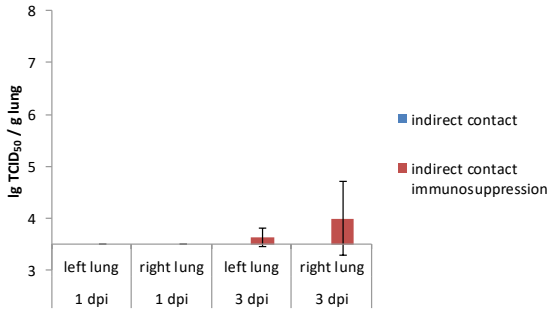

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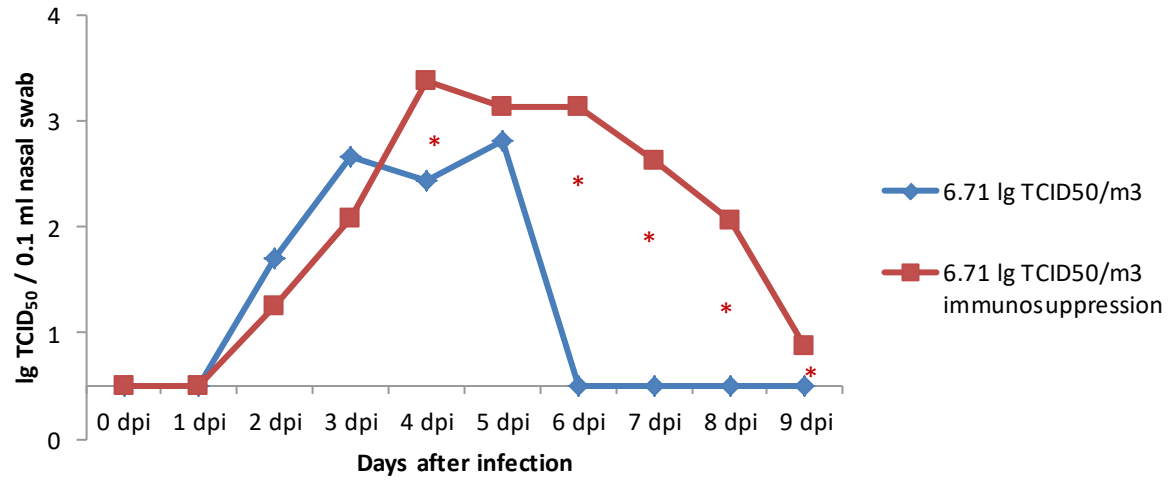

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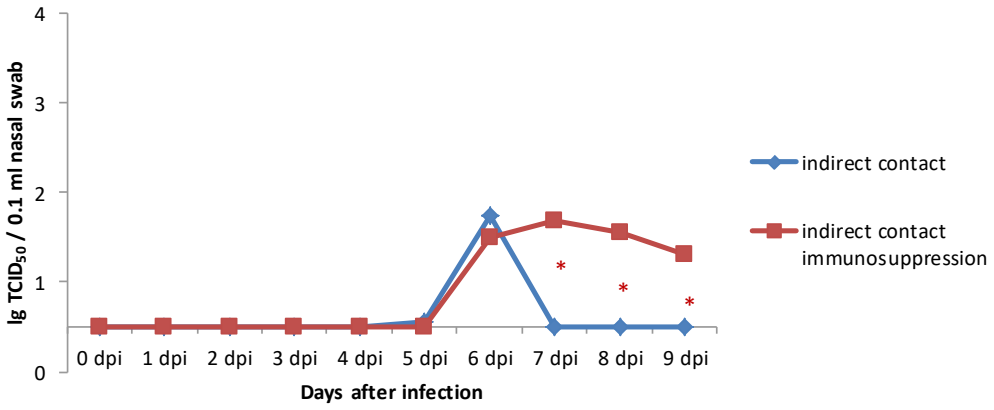

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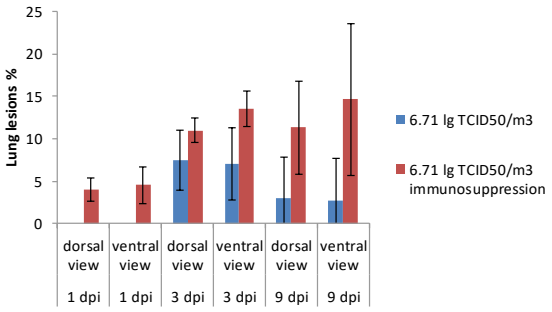

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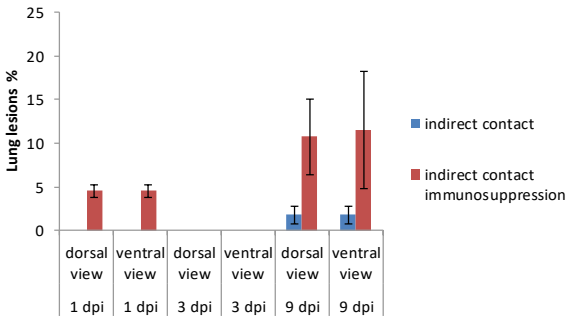

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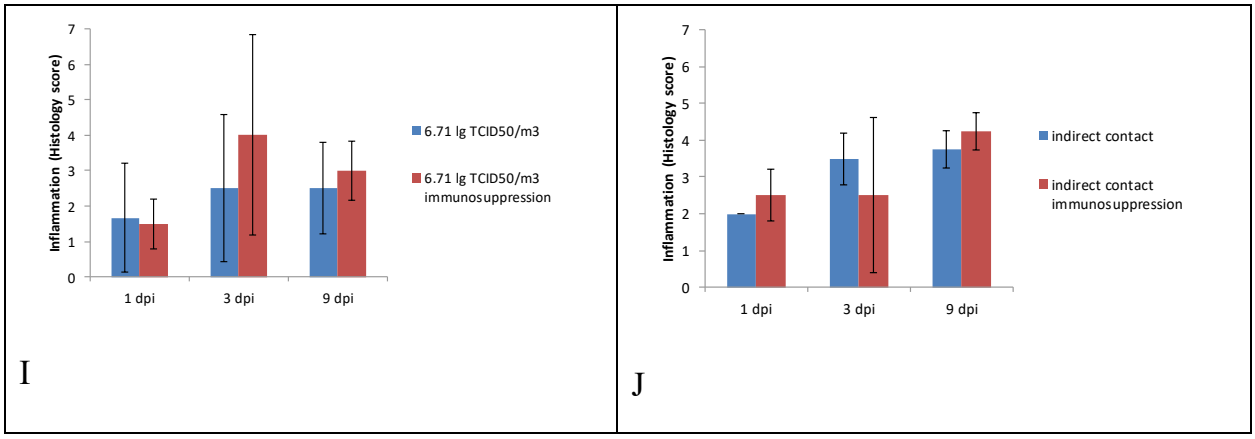
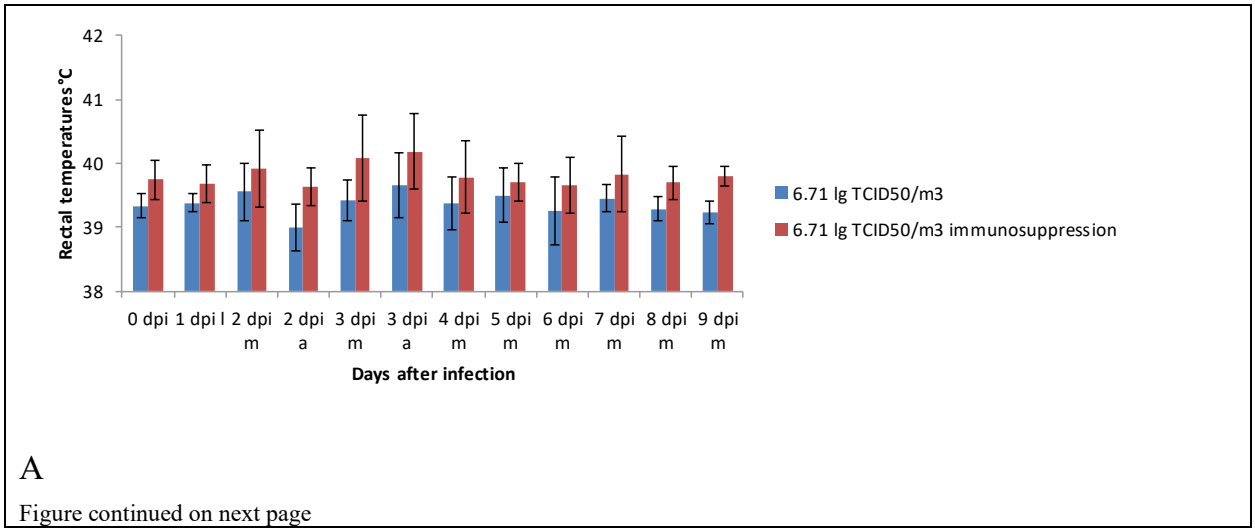


Figure 51: Comparative analysis of immunosuppressive treatment in H1$_{cl}$N1 influenza A virus-infected pigs in two infection models: high-dose aerosol and indirect contact infection

*A, Symptoms after high-dose aerosol infection (dyspnoea score, arithmetic mean); B, Symptoms after indirect contact infection (dyspnoea score, arithmetic mean); C, Viral lung load after high-dose aerosol infection (lg TCID$_{50}$/g, arithmetic mean); D, Viral lung load after indirect contact infection (lg TCID$_{50}$/g, arithmetic mean); E, Virus shedding after high-dose aerosol infection (lg TCID$_{50}$/0.1 ml, arithmetic mean); F, Virus shedding after indirect contact infection (lg TCID$_{50}$/0.1 ml, arithmetic mean); G, Lung lesions after high-dose aerosol infection (%, arithmetic mean); H, Lung lesions after indirect contact infection (%, arithmetic mean); I, Inflammation after high-dose aerosol infection (histology score, arithmetic mean; J, Inflammation after high-dose aerosol infection (histology score, arithmetic mean; *, p<0.05, **, p<0.01, ***, p<0.001*

The virus did not induce fever, but some of the immunocompromised pigs developed elevated rectal temperatures on several days after infection, as indicated by the higher standard deviations (Figure 52 A, B). Added to this, immunocompromised pigs did not respond to infection by producing antibodies until the end of the study (Figure 52 C, D). The mean starting point of body weight was lower in the immunocompromised groups before infection and the number of animals was too small to apply statistics, but calculation of body weight gains showed that these were impaired in immunocompromised pigs with influenza A virus infection (Figure 52 E, F, G, H).



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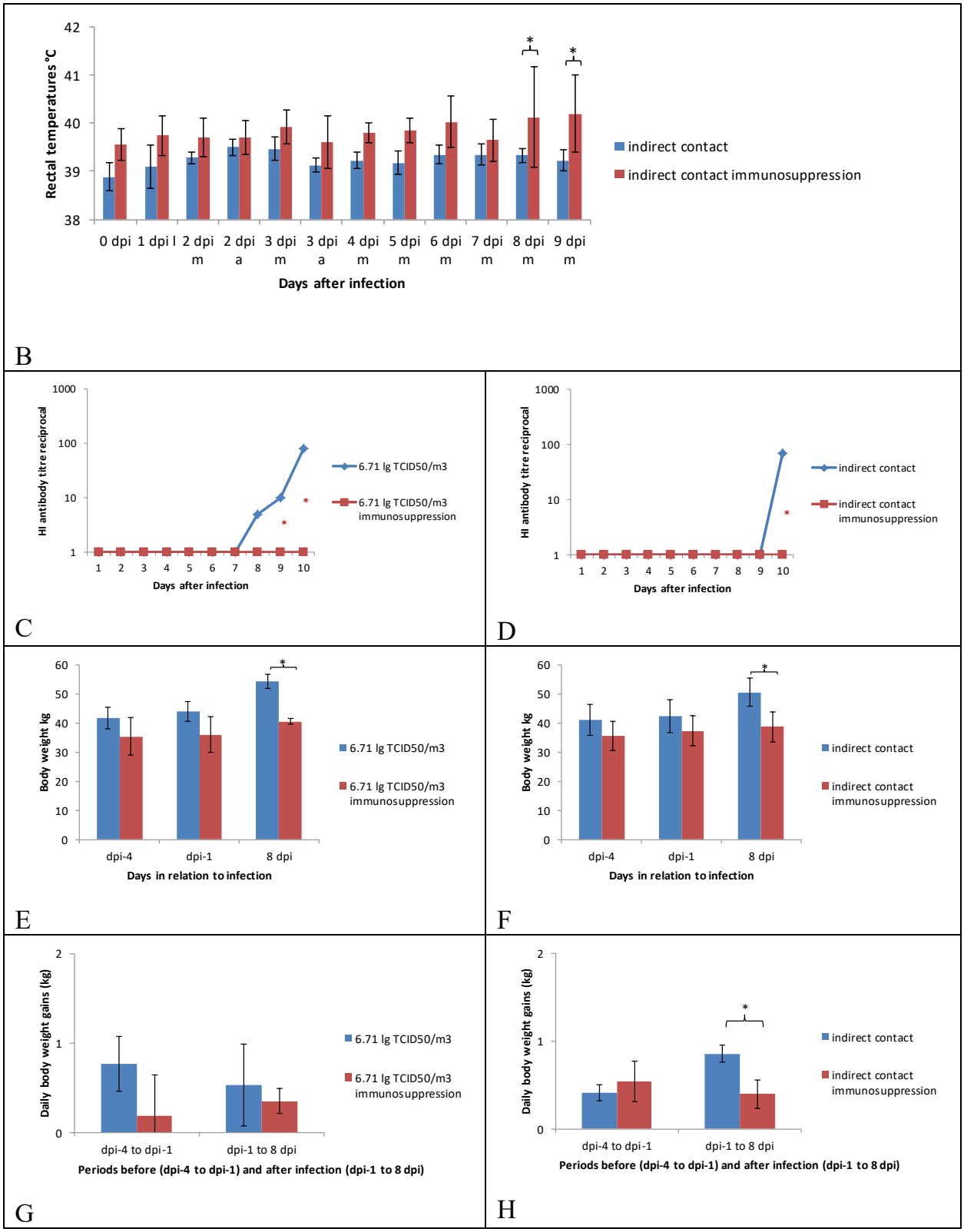


Figure 52: Comparative analysis of immunosuppressive treatment continued in H1$_{cl}$N1 influenza A virus-infected pigs in two infection models: high-dose aerosol and indirect contact infection

A, Rectal temperatures after high-dose aerosol infection (°C, arithmetic mean); B, Rectal temperatures after indirect contact infection (°C, arithmetic mean); C, Antibody kinetics after high-dose aerosol infection (HI titre, geometric mean); D, Antibody kinetics after indirect contact infection (HI titre, geometric mean); E, Body weights before and after high dose aerosol infection (kg, arithmetic mean), F, Body weights before and after indirect contact infection (kg, arithmetic mean); G, Daily body weights before and after high dose aerosol infection (kg, arithmetic mean), H, Daily body weights before and after indirect contact infection (kg, arithmetic mean)

Short interpretation of results. In general, it can be concluded from these infection studies that immunocompromised patients can cope with influenza A virus infections of moderate virulence. Such patients must be considered as virus shedders. The results of the high-dose aerosol infection suggest that influenza A virus infections are tolerated in immunocompromised patients, but prolonged virus shedding occurs.

5.2 APPLICATION OF HIGH-DOSE AEROSOL NEBULIZATION TO INVESTIGATE EFFECTS OF MATERNALLY-DERIVED IMMUNITY

A summary of the results is provided in Table 16.

Table 16: Overview of the results regarding maternal immunity

Topic	Subtopic	Major results
Transfer of antibodies	Antibody concentration	Sows concentrate antibodies in their colostrum; vaccinations should be carried not later than 2 weeks before farrowing (pages 97 - 98)
	Primary antigen contact	Even in cases of no measurable antibody titers sows will provide antibodies if they had contact to influenza viruses prior in their lives (Table 17, page 98)
Protection of piglets	Disease prevention	Maternal antibodies prevent dyspnoea and fever (pages 109-116)
	Duration of protection	Kinetics of maternally-derived antibodies differ depending on virus strains and number and time of exposure of the mother sows (pages 100 + 102)
Interference with antibody response to vaccination	Maternally-derived immunity suppresses antibody formation after vaccination	Maternally-derived immunity suppresses serological response to immunisation even long-term after maternal HI antibodies disappeared (pages 100 - 104); despite this interaction, immunisation primes the immune system and protective effects are seen in pigs without antibodies (page 104)
	Vaccination and maternal immunity have a synergistic effect	In pigs immunised in the presence of maternal antibodies synergistic effects of immunisation and maternal immunity on disease suppression occur despite lacking prevention of virus replication in abscense of antibodies (pages 113 - 116)

5.2.1 MATERNAL IMMUNITY AND ANTIBODY RESPONSE TO IMMUNISATION

5.2.1.1 SOWS CONCENTRATE ANTIBODIES IN THEIR COLOSTRUM PRIOR TO BIRTH

Background. Sows transmit antibodies to their piglets via colostrum. In order to investigate the right time for vaccination sows were immunised at two different points in time before farrowing.

Study design. Five sows were immunised 5 and 2 weeks before farrowing, 5 other sows of the same age were immunised 4 and 1 week before farrowing (inactivated antigens of

$H1_{av}N1$ 2003+$H1_{hu}N2$ 2000+H3N2 2003 viruses, Carbopol-adjuvanted). Antibodies in serum and colostrum were measured by HI 1 day after birth.

Results. The antibodies in all sows were higher in colostrum compared to serum. Sows immunised 1 week before farrowing had no or low HI antibody titres in their sera but concentrated antibodies in serum (Table 17).

Table 17: HI antibodies in individual sows after antigenic contact with influenza A viruses $H1_{av}N1$, $H1_{hu}N1$, H3N2

$H1_{av}N1$		**$H1_{hu}N2$**		**H3N2**	
Serum	**Colostrum**	**Serum**	**Colostrum**	**Serum**	**Colostrum**
Immunisation 5 + 2 weeks before farrowing					
80	5120	320	10240	160	2560
80	1280	160	5120	80	2560
80	640	320	2560	80	2560
80	1280	80	1280	40	320
80	2560	640	20480	80	1280
160	1280	160	5120	0	320
Immunisation 4 + 1 week before farrowing					
<10	320	80	640	<10	640
<10	640	640	20480	<10	320
<10	320	40	160	40	640
<10	80	<10	160	<10	320
80	5120	320	10240	160	2560

Short interpretation of results. The data indicate that sows concentrate antibodies in colostrum. Despite low or not measurable HI antibodies in serum sows are able to concentrate antibodies in colostrum. This is most probably an active process involving activation of immune cells. Antigenic contact should be earlier than 1 week before farrowing in order to guaranty high colostral antibodies. The active character of serum concentration arises the possibility that even sows in which no antibodies are detectable in serum can transmit antibodies to their piglets via colostrum if they had contact to influenza A virus antigens in their life history.

5.2.1.2 MATERNALLY-DERIVED IMMUNITY INHIBITS SEROCONVERSION

Background. Sows transmit immunity by antibodies via colostrum to their piglets. These antibodies can interfere with antibody induction to immunisation. The study aims to investigate antibody kinetics and effects of repeated immunisations on pigs with maternally-derived antibodies in a long-term study.

Study design. Piglets from sows of a pig herd with prior $H1_{av}N1$ virus infection and of a pig herd that had no FLUAV infection before were investigated (Table 18).

Table 18: Interference of maternally-derived immunity with immunization
Overview of the trial

Group	Immunity	Abbreviation	Number of pigs included*
1	Pig herd with prior $H1_{av}N1$ 1999 infection	ma ab $H1_{av}N1$	10
2	Pig herd without contact to FLUAV	control	10

* age at begin of the study 1 week, age at first immunisation 8 weeks

The couse of maternally-derived antibodies was followed up by HI. The pigs were immunised with $H1_{av}N1$+H3N2 antigens adjuvanted with mineral oil and aluminum hydroxide (immunisation scheme see Table 19). The antibody response was monitored.

Results. Piglets of sows from a farm with prior $H1_{av}N1$ infection had antibodies against $H1_{av}N1$ virus but not against H3N2 (Figure 53 A). The maternal antibodies declined steadily and disappeared around 8 weeks after birth. Piglets of sows from a farm without prior FLUAV infection had no maternally-derived antibodies (Figure 53 B).

At the time of the first immunisation only 2 out of 10 piglets still had maternal antibodies at low titres (1:10; 1:20, data not shown). Pigs responded with high HI antibodies to immunisation but not the group that had had maternal antibodies (Figure 53 C, D). Despite the lack of measurable maternal antibodies, the piglets did not respond with antibodies to immunisation. After third immunisation these pigs responded in the same way and pattern as those which had no maternally-derived immunity prior to immunisation (Figure 53 C).

Table 19: Immunisation scheme

Group	Immunity	Immunisation*	Age of pigs at immunisation Weeks of life
1	ma ab $H1_{av}N1$	1^{st}	8
		2^{nd}	11
		3^{rd}	35
		4^{th}	61
2	control	1^{st}	8
		2^{nd}	11
		3^{rd}	35
		4^{th}	61

* inactivated FLUAV antigens $H1_{av}N1$ 1992+H3N2 1992 viruses adjuvanted with mineral oil + alumiumhydroxide; ma ab $H1_{av}N1$, maternally-derived antibodies against $H1_{av}N1$

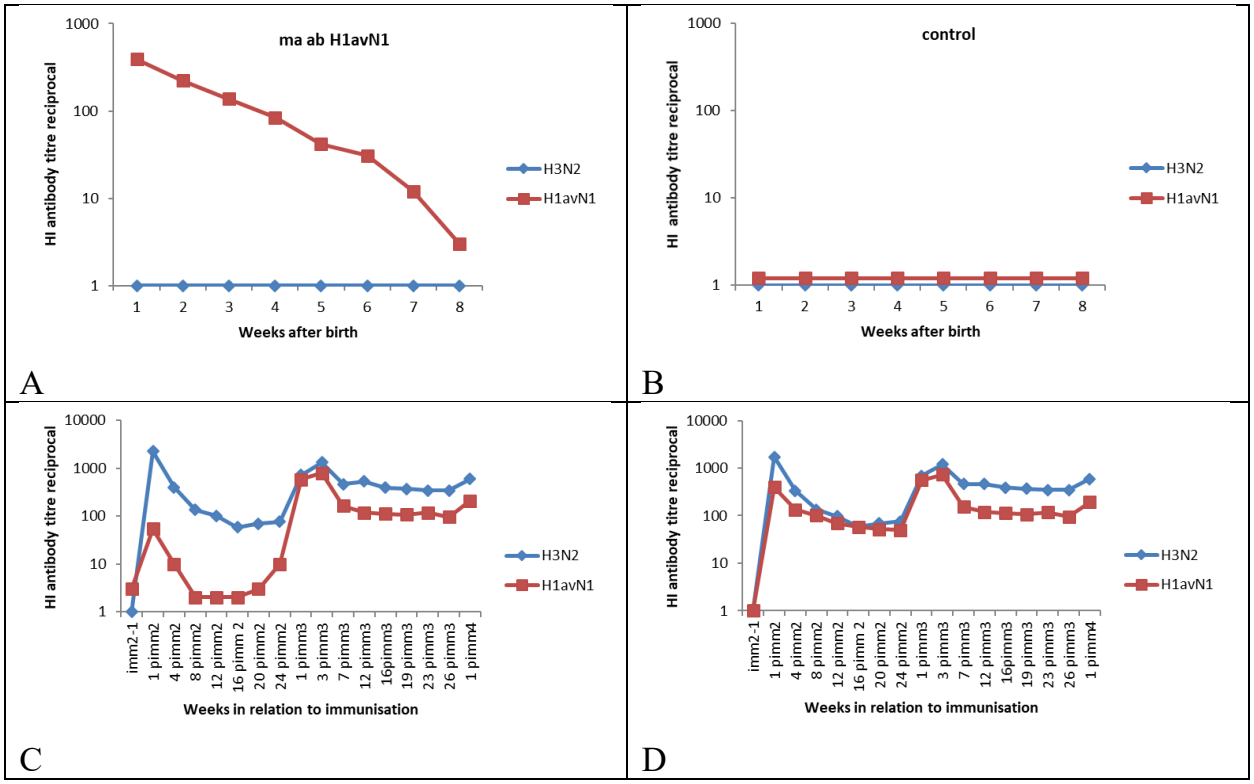


Figure 53: HI antibody kinetics in pigs with and without maternal immunity
(HI titre reciprocal, geometric mean of 10 pigs); A, piglets of sows of a pig herd with prior natural $H1_{av}N1$ 1999 virus infection (ma ab $H1_{av}N1$); B, piglets of sows from a pig herd without FLUAV infection (control); C, antibody response of the A-corresponding piglets after immunisation (inactivated $H1_{av}N1$+H3N2, aluminiumhydroxide+mineral oil adjuvant); D, antibody response of the B-corresponding piglets after immunisation (inactivated $H1_{av}N1$+H3N2, mineral oil adjuvant); imm2-1, 1 week before second immunisation, pimm2, time after second immunisation; pimm3, time after third immunisation; pimm4, time after fourth immunization)

Short interpretation of results. The data allow for following conclusions: i) maternally-derived immunity interferes with antibody induction by vaccination despite the lack of measurable HI antibodies prior to immunisation, ii) despite lacking serological responses to basic immunisation in piglets from sows which had been exposed to influenza virus infection prior to giving birth these piglets respond with antibodies to repeated immunisations in the same way as piglets that responded to the prior immunisation by antibodies; this indicates that immunisation primes the immune system in the presence of maternally-derived immunity (stimulation of memory B cells) despite lacking overt antibody induction.

5.2.1.3 OTHER FACTORS OF MATERNALLY-DERIVED IMMUNITY THAN ANTIBODIES DETERMINE MATERNALLY-DERIVED IMMUNITY IN LONG-TERMS

Background. The prior study showed that maternally-derived immunity interferes with antibody response to immunisation despite a lack of measurable HI antibodies at the time of first immunisation. This study is provided in order to investigate how long this interference lasts and which effects it has on protection.

Study design. Pigs were derived from a farm with prior H3N2 FLUAV 2001 infection one year ago. Ten sows were immunised 5 and 2 weeks before farrowing ($H1_{av}N1$ 2002 +

$H1_{hu}N2$ 2000 + H3N2 2003 inactivated FLUAV antigens + Carbopol adjuvant). Eighteen of their piglets were followed up for kinetics of maternally-derived antibodies. Thereafter the 18 piglets were divided into 3 groups of each 6 piglets and vaccinated at different times (Table 20). At the end of the study (1 week after the last immunisation of group 3) pigs were infected by high-dose aerosol infection in a simultaneous infection experiment in their 33rd week of life. The infection doses were as follows: FLUAV/sw/Freren-Ostwie/IDT8297/2008 ($H1_{av}N1$) 7.28 lg $TCID_{50}/m^3$ + FLUAV/sw/Bottrop-Kirchhellen/IDT8644/2009 ($H1_{hu}N2$) 6.53 lg $TCID_{50}/m^3$ + FLUAV/sw/Ostenfeld/IDT8082/2008 (H3N2) 7.78 lg $TCID_{50}/m^3$. The number of pigs in the groups at challenge were: group 1: 5 pigs, group 2: 6 pigs, group 3: 5 pigs, control group (unimmunised, same age): 7 pigs.

Table 20: Overview of immunisations carried out in order to investigate long-term interferences of maternally-derived immunity with antibody response to immunisation

Group	Immunisation*	Age of pigs at immunisation Weeks of life
1	1st	14
	2nd	17
	3rd	26
2	1st	17
	2nd	20
	3rd	29
3	1st	20
	2nd	23
	3rd	32

* inactivated FLUAV antigens $H1_{av}N1$ 2003+$H1_{hu}N2$ 2000+H3N2 2003 adjuvanted with Carbopol

Results. The maternally-derived HI antibodies in the piglets were highest in the first four weeks and fell steadily. They disappeared in the 3rd month of life. $H1_{av}$H1-antibodies disappeared first, followed by antibodies directed against $H1_{hu}N2$. Antibodies against H3N2 were still detectable in a few pigs at low titres in the 13th week of life (Figure 54).

Interactions of maternally-derived antibodies were detected for more than half a year after birth. Half a year after birth 50% of the pigs responded with antibodies to immunisation (Figure 55). Only in the 32th week of life all pigs developed antibodies after immunisation (Figure 55).

Due to the interference with maternally-derived antibodies antibody titres were only low and antibodies disappeared quickly after immunisation. At the time of challenge infection following percentage of pigs had antibodies against swFLUAV: group 1: $\alpha H1_{av}N1$: 0%, $\alpha H1_{hu}N2$ and αH3N2 20%; group 2: $\alpha H1_{av}N1$: 33%, $\alpha H1_{hu}N2$: 67%, αH3N2: 60%; group

3: all 100%. Despite lacking measurable HI antibodies in approximately one third of the pigs all pigs were protected against simultaneous infection (Figure 56). Due to the age of the pigs at the time of challenge infection symptoms were only low in the control group. The immunised pigs performed better regarding all parameters of protection (Figure 56).

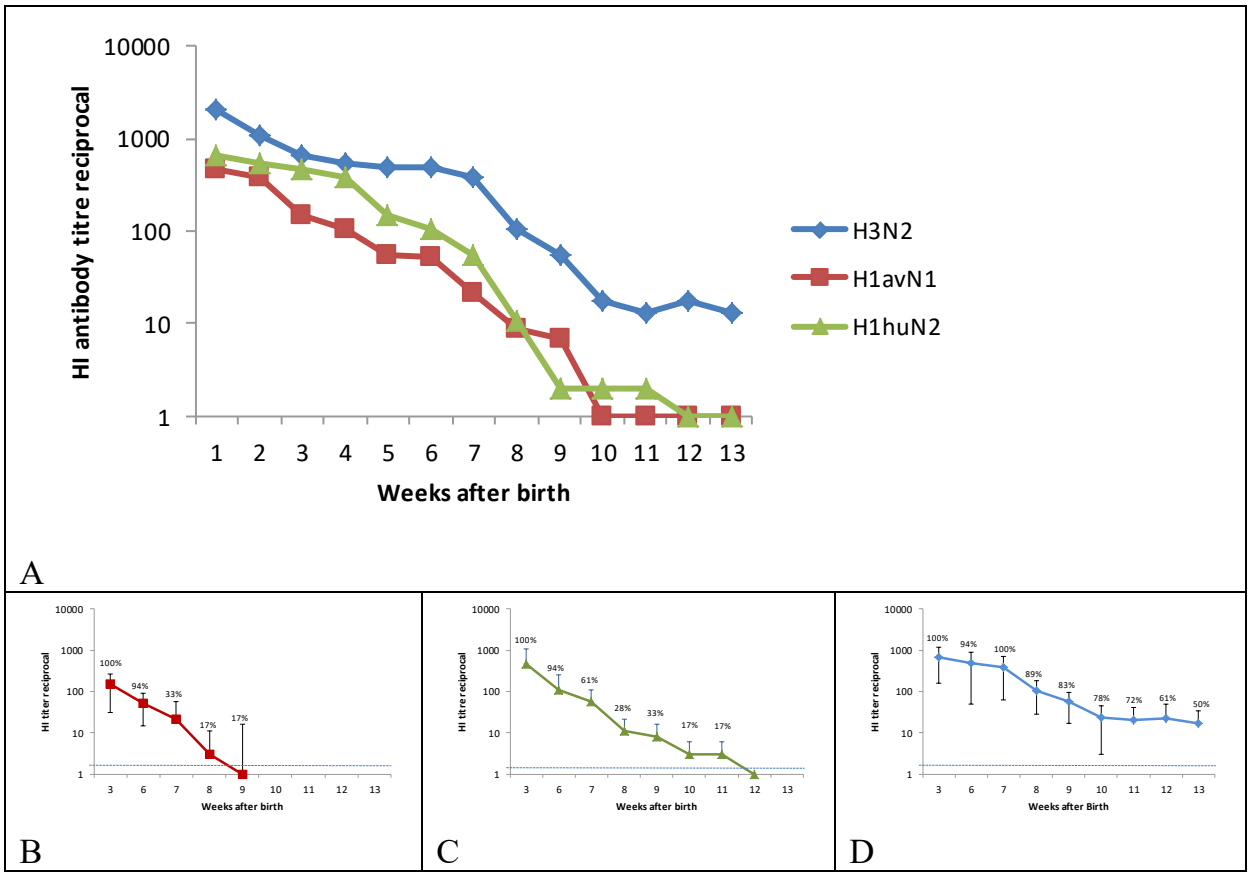


Figure 54: Kinetics of maternally-derived antibodies in piglets
from sows with two- to threefold antigen contacts to FLUAV prior to farrowing (2x $H1_{av}N1$, 2x $H1_{hu}N2$, 3x H3N2, H3N2: field infection + twofold immunisation, $H1_{av}N1$+$H1_{hu}N2$ twofold immunisation); A, trends (geometric mean values without standard deviation; B, $H1_{av}N1$ (geometric mean values with standard deviation + percentages of positives); C, $H1_{hu}N2$ (geometric mean values with standard deviation + percentages of positives); D; H3N2 (geometric mean with standard deviation + % of positives)

Short interpretation of results. The longer duration of the maternally-derived antibodies against H3N2 may be due to the three antigenic contacts of the sows to this antigen but also due to the higher antigenicity of H3N2 in general.

The interference with maternally-derived antibodies lasts long. Over more than half a year after birth piglets respond not or only poorly with antibodies to immunisation. Despite this, pigs are protected against infection. This indicates the existing of still unknown factors involved in maternally-derived immunity. The HI assay is not as sensitive as NT but even when using NT such a long circulation of antibodies is improbable. The reason for the lacking induction of antibodies may be a block of antibody production of B cells in order to safe resources. The pigs can afford this because the blocking factor does not inhibit priming of the immune system. It also may provide protection.

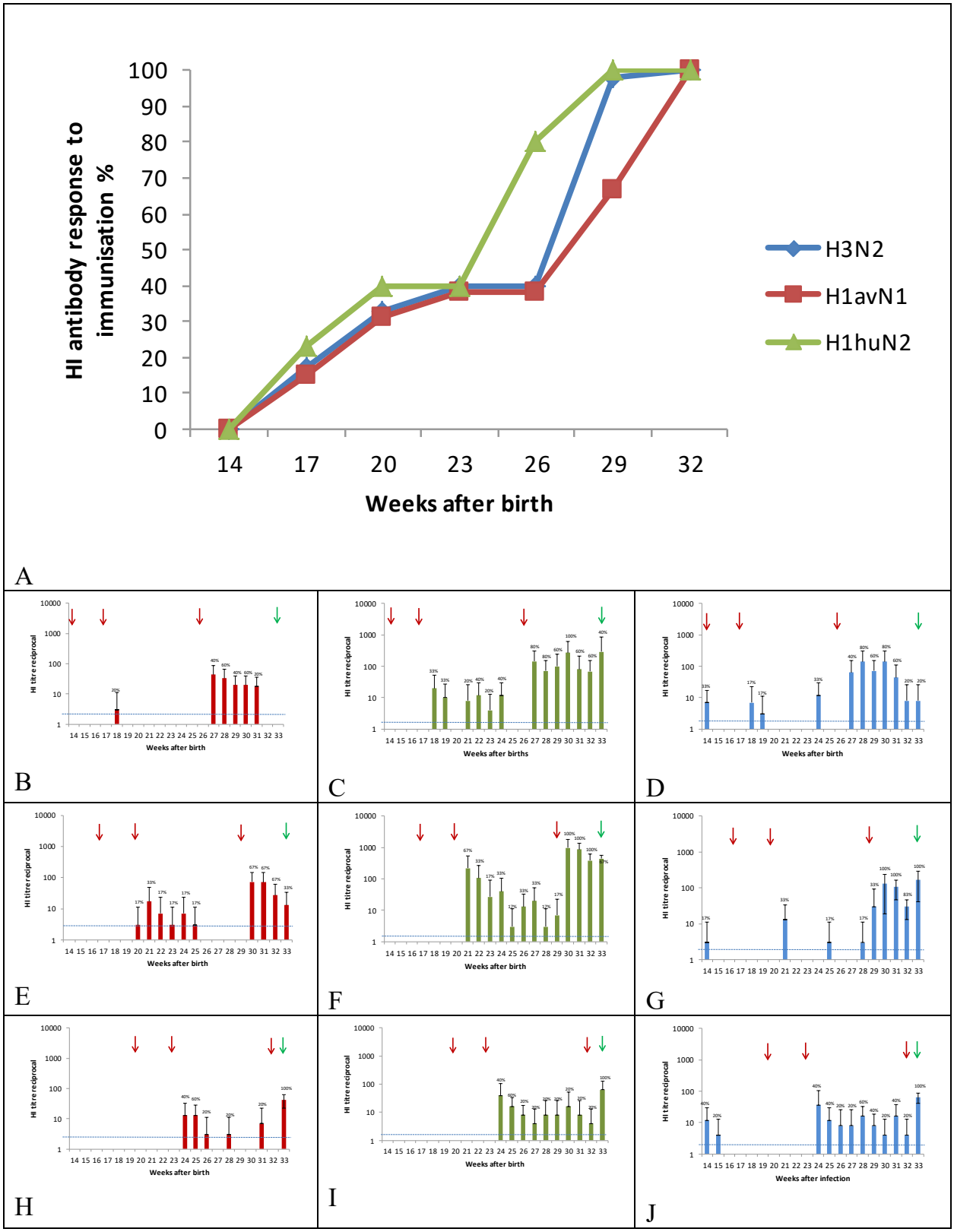


Figure 55: Interference of maternally-derived immunity with antibody response to immunization

A, half a year after birth 50% of the pigs respond with antibodies to immunisation; it takes 32 weeks until all pigs respond to immunisation with antibodies; B, E, H, geometric mean values with standard deviation and percentage of seroconversion for $H1_{av}N1$; C, F, I, geometric mean values with standard deviation and percentage of seroconversion for $H1_{hu}N2$; D, G, J, geometric mean values with standard deviation and percentage of seroconversion for H3N2; red arrows; time of immunisation; green arrow, experimental infection; dotted line, detection limit

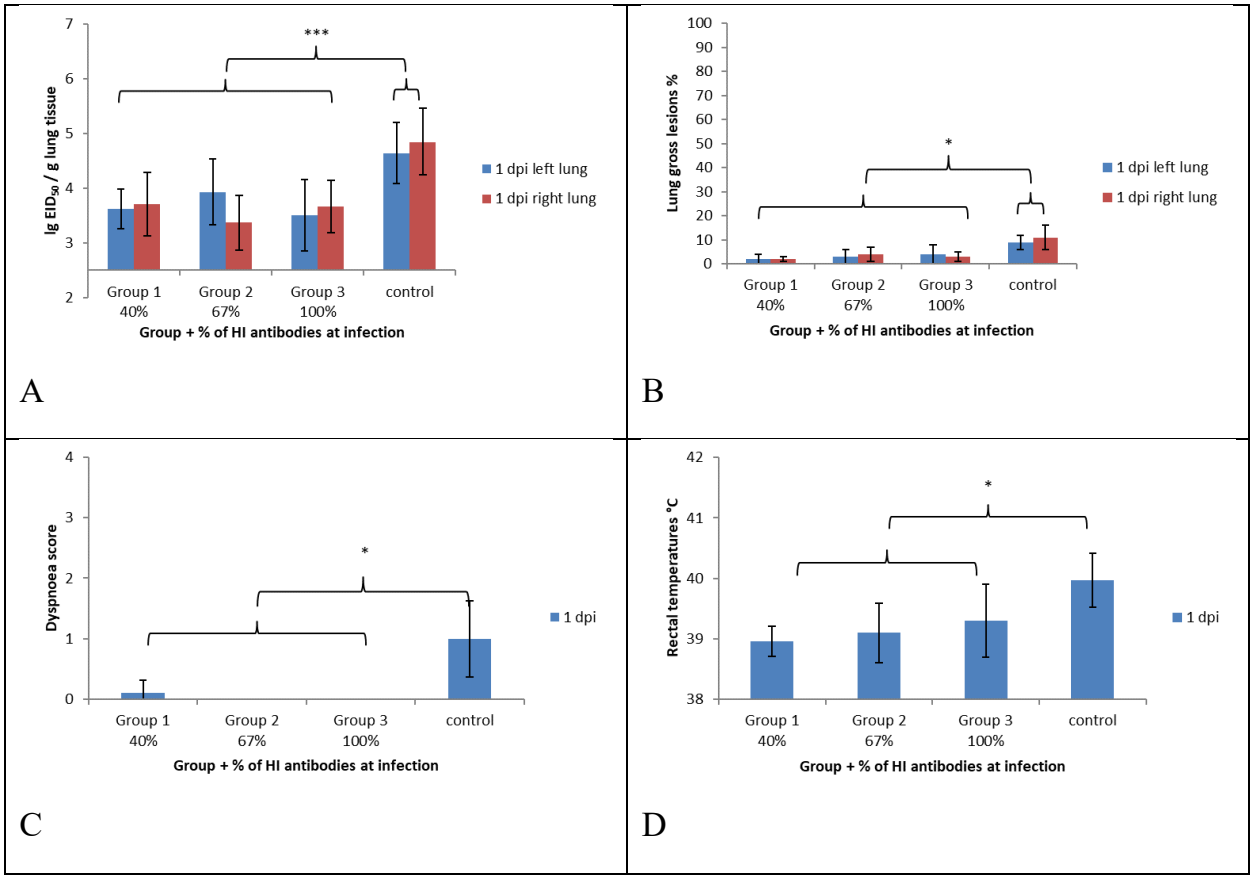


Figure 56: Protection despite low antibody response
1 dpi of pigs with poor antibody response to immunisation due to interference with maternally-derived immunity in comparison to not immunised control pigs
A, viral lung load (lg EID_{50}/g); B, lung lesions (%); C, symptoms (dysponea score); D, rectal temperatures (°C)

5.2.1.4 MATERNALLY-DERIVED IMMUNITY INDUCED BY $H1_{PDM}N1$ 2009 VIRUS

Background. Data for maternally-derived immunity induced by $H1_{pdm}N1$ 2009 virus are still lacking. Therefore, sows were immunised and antibodies in piglets were followed up.

Study design. 10 sows were immunised (inactivated antigen of 5555 $H1_{pdm}N1$ 2009 virus, Carbopol-adjuvanted) 5 and 2 weeks prior to birth. Piglets of these sows were investigated for HI maternally-derived antibodies from 6 to 15 weeks after birth (Table 21). Pigs were immunsed in their 11^{th} and 13^{th} week of life. Experimental high dose-aerosol infection was performed in their 15^{th} week of life using strain 1580 $H1_{pdm}N1$ 2009 virus (6.38 lg $TCID_{50}/m^3$).

Table 21: Overview of the groups included in the trial to study effects of maternal immunity against $H1_{pdm}N1$ virus

Group	Immunity	Abbreviation	Number of pigs included piglets
1	Piglets from immunised $H1_{pdm}N1$-sows with maternal immunity	Mat imm	8
2	Piglets from immunised $H1_{pdm}N1$-sows in which maternal immunity run out	Run out mat imm	8
3	Control (piglets of not immunised sows)	control	16

Results. Maternally-derived antibodies disappeared 11 weeks after birth in all piglets (Figure 57 A). The seroconversion after immunisation at an age of 13 weeks of life was 50% (Figure 57 B). All piglets of all 3 groups had maternally-derived antibodies in their 7th week of life (Figure 58 C). Piglets in which maternally-derived immunity had been run out before immunisation responded with HI antibodies to repeated immunisation, the others did not. Piglets with maternally derived-immunity which did not respond to immunisation by antibodies responded 1 day earlier with antibodies to infection than unimmunised piglets (Figure 58 C). Immunised piglets with antibodies were protected best and developed no dyspnoea and no fever (Figure 58 A, B). The displayed a significantly lower viral lung load than not immunised control pigs (Figure 58 D) and their shedding curve was reduced by 1-2 lg $TCID_{50}$/0.1 ml nasal swab (Figure 58 E). The immunised piglets which did not respond with antibodies to infection showed moderate symptoms but symptoms as well as rectal temperatures were lower than in not immunised controls (Figure 58 A, B). The viral lung load on 1 dpi was significantly lower in these piglets in comparison to unimmunised controls but did not dfffer significantly on 3 dpi (Figure 58 D). Virus shedding did not differ much between both groups with the exception of 1 dpi on which immunised piglets shed significantly less virus (Figure 58 E).

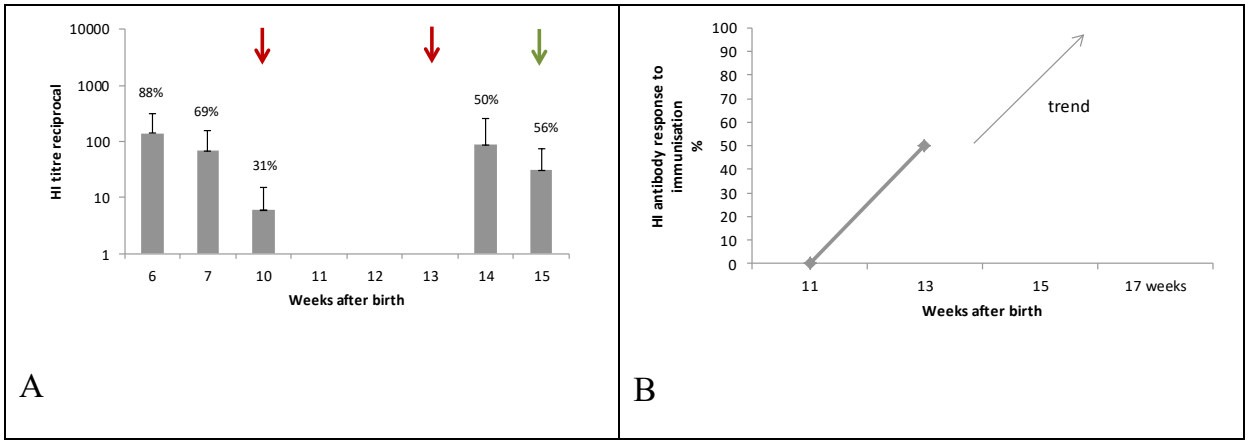


Figure 57: Kinetics of maternally-derived antibodies against $H1_{pdm}N1$ virus in piglets after twofold immunisation of their mother sows with $H1_{pdm}N1$ virus

A, Antibody kinetics, geometric mean with standard deviation and percentage of positive piglets, red arrows indicate immunisation with $H1_{pdm}N1$ antigen; green arrow indicates time of experimental infection; B, interference with maternally-derived immunity does not last as long as with the other porcine FLUAVs: already 13 weeks after birth 50% of the piglets respond to immunisation, the deduced trend indicates that around 16 weeks after birth all piglets could respond to immunisation with antibodies

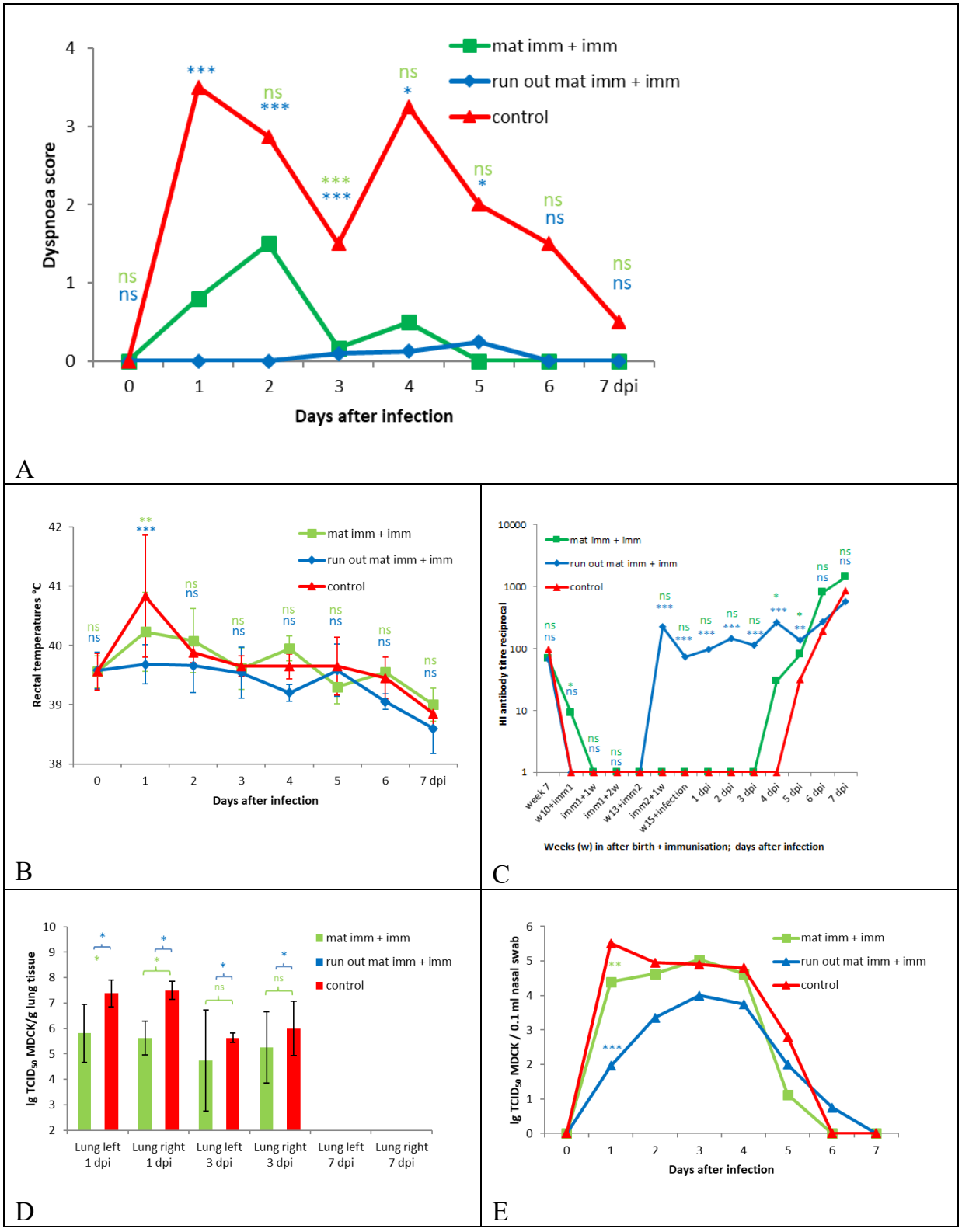


Figure 58: Effects of immunization during the phase of maternal immunity after infection with A/Hamburg/NY1580/April 2009 ($H1_{pdm}N1$) virus

Parameters of protection in piglets of the same farrowing event which did not respond to immunisation due to interactions with maternally-derived immunity and those in which maternally-derived immunity had been run out before immunisation; A, Symptoms (dyspnoea score, arithmetic mean), B, Rectal temperatures (°C, arithmetic mean with standard deviation); C, HI antibody response (titre reciprocal, geometric mean), D, viral lung load (lg $TCID_{50}$/g lung tissue, arithmetic mean with standard deviation); E, virus shedding (lg $TCID_{50}$/0.1 ml nasal swab, arithmetic mean)

Short interpretation of results. Interference of the antibody induction induced by the $H1_{pdm}N1$ vaccine with the maternally derived immunity did not last as long as with the

European swine FLUAVs $H1_{av}N1$, $H1_{hu}N2$, H3N2 viruses. At 13 weeks of life already 50% of the piglets developed antibodies to immunisation. Immunised piglets without HI antibodies responded 1 day earlier with antibodies to infection than not immunised controls which indicates that their memory cells had been primed at immunisation. Although piglets which did not respond with antibodies to immunisation due to interference with maternally-derived immunity performed significantly worser than those with antibodies the piglets were sufficiently protected from disease and had significantly less symptoms than not immunised controls. This hints to protection despite lacking antibody induction.

5.2.1.5 SEVERE DISEASE AND RECOVERY

Background. Immunisation into maternally-derived immunity does not always prevent or reduce disease. One single case of severe disease after immunisation into maternally-derived immunity is reported here.

Study design. Five pigs were vaccinated (laboratory batch containing inactivated antigen of strain 5555 $H1_{pdm}N1$ 2009 virus and Carbopol adjuvant) and investigated together with 6 unvaccinated pigs in high-dose aerosol-infection with strain 1580 $H1_{pdm}N1$ April 2009 virus (6.3 lg $TCID_{50}/m^3$). An overview of pig groups included is provided in Table 22. Clinical symptoms were observed as usual. Blood samples were taken after vaccination and every second day after infection.

Table 22: Overview of groups involved in the $H1_{pdm}N1$ efficacy trial

Group	Immunity	Abbreviation	Number of pigles included
1	Pigs immunised	Mat imm	5
2	Control (not immunised pigs)	Without mat imm	6

Results. It went out that two of the 5 vaccinated pigs did not respond with antibodies to vaccination. Further research revealed that both had been derived from a sow that went through a $H1_{pdm}N1$ field infection 1.5 year earlier. Whereas 1 of the 2 pigs without antibody response only developed moderate signs of disease and the pigs with maternally-derived antibodies were clearly protected the other vaccinated pig without antibodies developed severe disease but recovered suddenly with the emergence of antibodies. In order to investigate the priming effects in detail all sera were analysed by HI, NI, NT and antibody kinetics were compared with dyspnoea. It became obvious that pigs are strongly primed by immunisation despite lacking antibody induction and that antibody induction correlates with protection (Figure 59).

Short interpretation of results. The results show that there are strong priming effects caused by vaccination into maternally-derived immunity. Even in cases of severe disease piglets can respond quickly to infection and are able to recover from deadly sickness.

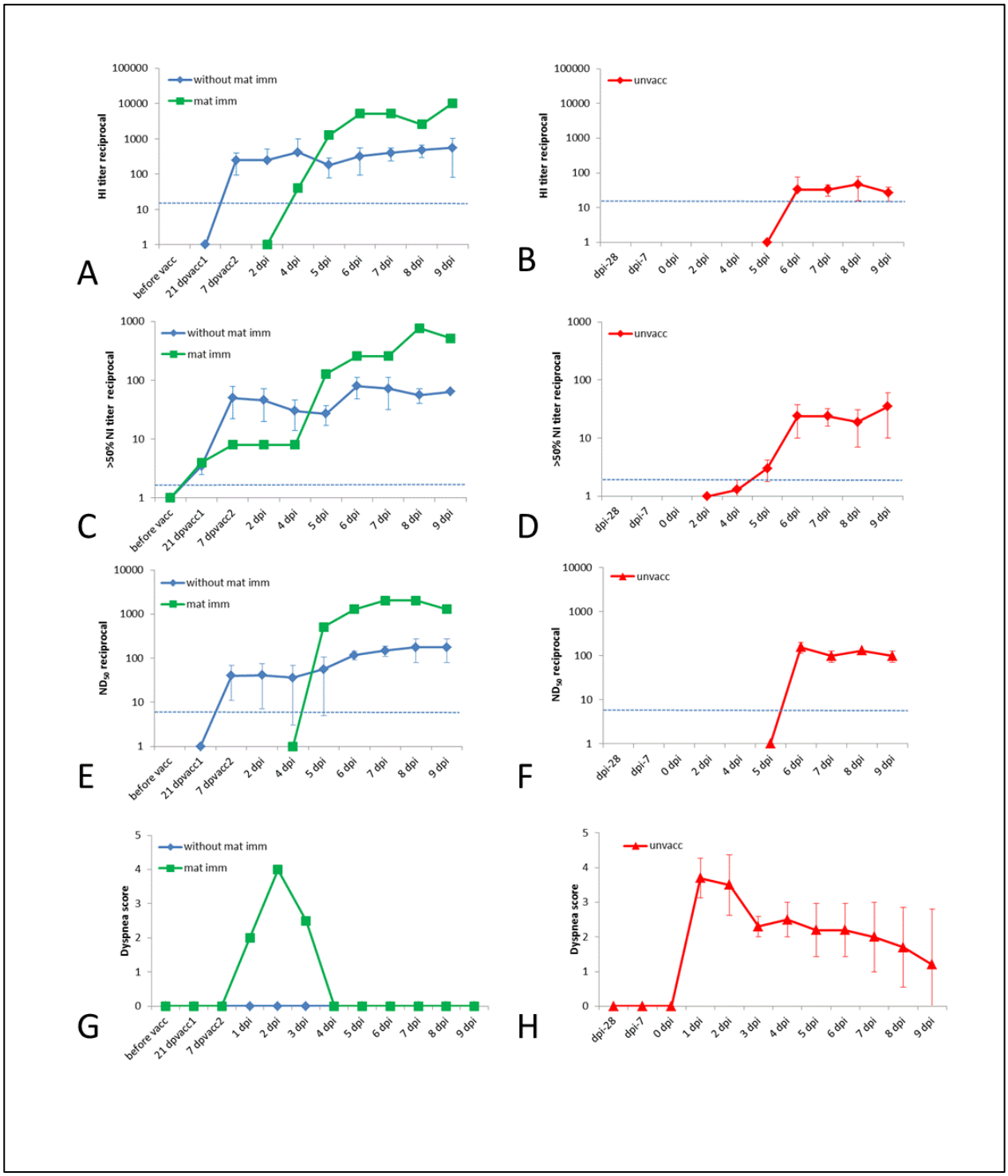


Figure 59: Antibody kinetics and effects of protection
Comparison of HI, NI, and NT antibody kinetics and dyspneoa after infection in vaccinated piglets with and without maternal immunity
mat imm, n=1 (no seroresponse to vaccination); without mat imm, pigs without maternally-derived immunity, n=3 (seroresponse to vaccination) in comparison to control pigs (unvacc, n=6), dpvacc, days after vaccination; arithmetic mean with standard deviation, for antibodies geometric mean

5.2.1.6 INVESTIGATION ON PIGLETS OF AN AGE OF 2 WEEKS AT INFECTION

Background. This trial served for the investigation of effects of infection with $H1_{av}N1$ 1998 virus on sows and their offspring.

Study design. Five sows were immunised 5 and 2 weeks before farrowing (inactivated strain FLUAV/Re220/1992 H3N2 + FLUAV/Re230/1992 $H1_{av}N1$), the other sows remained not immunised (Table 23). Fourteen dpi after farrowing 1 immunised and 1 not immunised sow with each 12 piglets were transported to infection units and FLUAV/Bakum/3543/1998 ($H1_{av}N1$) was nebulised at a dose of 8.45 lg $TCID_{50}/m^3$. Sows and piglets were observed clinically until 7 dpi. Body weights of the piglets were recorded. One dpi lungs of 5 piglets of each group were investigated. Nasal swabs were taken daily. As the unvaccinated sow showed signs of mastitis, milk samples were also taken from 3 teats of each sow from day 3 after infection. The virus content in nasal swab samples, lungs and milk was determined.

Table 23: Overview of the design of the trial for investigation of infection on piglets with and without maternally-derived antibodies with FLUAV $H1_{av}N1$

Group	Immunity	Abbreviation	Number of pigs included Sow + piglets
1	Immunised sow with piglets	imm+ma ab	1+12
2	Control (not immunised sow with piglets)	control	1+12

Results. Immunised sows provided piglets antibodies via colostrum (Table 24). There were no signs of respiratory disease detectable in the sows. Neither increases in rectal temperatures nor dyspnoea were seen in the sows. Despite this, the not immunised sow refused to eat, gave almost no milk and had no defecation (Table 25).

Table 24: HI antibodies against the $H1_{av}N1$ infection strain in sows and their piglets (HI titre reciprocal)

Sow ID	Serum	Colostrum
unimmunised		
118	<20	<20
128	<20	<20
immunised		
115	160	1280
125	5120	10240
197	320	1280
198	640	5120
199	5120	10240

Piglets at infection (individual data are shown, 14 days after birth)											
from unimmunised sow											
<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
from immunised sow											
640	40	640	160	1280	160	160	320	320	80	640	320

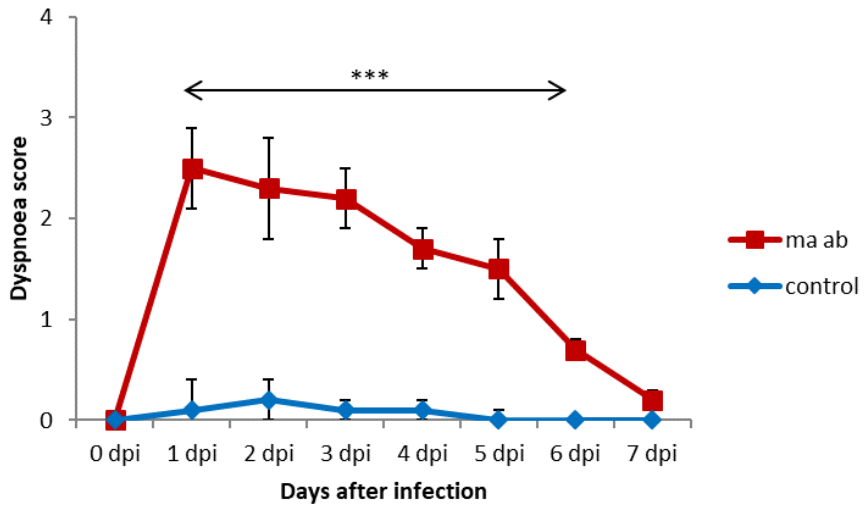
The piglets of the immunised sow remained healthy and showed no dyspnoea and no fever (Figure 60 A+B). The body weights gains remained high (Figure 60 C). The piglets of the not immunised sow developed dyspnoea, fever on 1 and 3 dpi and did not gain as much body weight as the piglets with maternally derived antibodies. The viral lung loads did not differ significantly between the groups (Figure 60 D) but lung gross lesions were significantly lower in piglets with maternally-derived immunity (Figure 60 E). The piglets with maternal antibodies shed virus for longer than those without. Milk samples from the non-immunised sow were infectious (Table 26).

Table 25: Symptoms in sows after infection with $H1_{av}N1$ FLUAV/sw/Bakum/3543/1998 ($H1_{av}N1$)

	0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi
imm sow								
Temperature °C	38.8	38.9	38.7	39	38.9	38.8	38.7	38.9
Dyspnoea	no	no	no	no	no	no	no	no
Food intake	yes	yes	yes	yes	yes	yes	yes	yes
Defecation	yes	yes	yes	yes	yes	yes	yes	yes
Lactation	yes	yes	yes	yes	yes	yes	yes	yes
control sow								
Temperature °C	39.1	38.8	38.9	39.1	39.0	38.9	38.8	38.9
Dyspnoea	no	no	no	no	no	no	no	no
Food intake	yes	yes	no	no	no	no	no	no
Defecation	yes	yes	no	no	no	no	no	no
Lactation	yes	yes	no	no	no	no	no	no

Table 26: Virus content in nasal swabs and milk after infection with $H1_{av}N1$ FLUAV/sw/Bakum/3543/1998 ($H1_{av}N1$); lg EID_{50} and standard deviation)

Days after infection	Nasal swabs				Milk	
	Sows		Piglets		Sows	
	imm	control	ma ab	control	imm	control
0 dpi	<0.5	<0,5	<0.5	<0.5	n.d.	n.d.
1 dpi	2.5	4.5	5.33±0.78	5.6±0.36	n.d.	n.d.
2 dpi	2.75	4.75	6±0.76	6.34±0.24	n.d.	n.d.
3 dpi	2.25	5.25	6.43±0.42	7.03±0.27	<0.5	6.5±0.14
4 dpi	1.75	4.25	6.1±0.91	6.08±0.29	<0.5	7±0.25
5 dpi	1.5	3.5	5.25±0.59	4.17±0.14	<0.5	5.25±0.66
6 dpi	<0.5	1.75	4.05±0.48	1.17±0.58	<0.5	4.25±0.66
7 dpi	<0.5	<0.5	2.15±0.6	<0.5	<0.5	3.25±0.66



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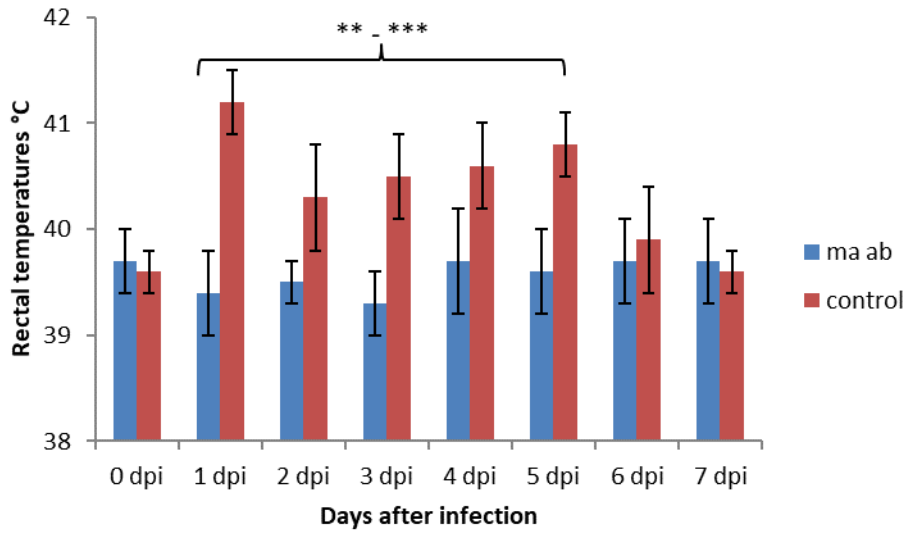

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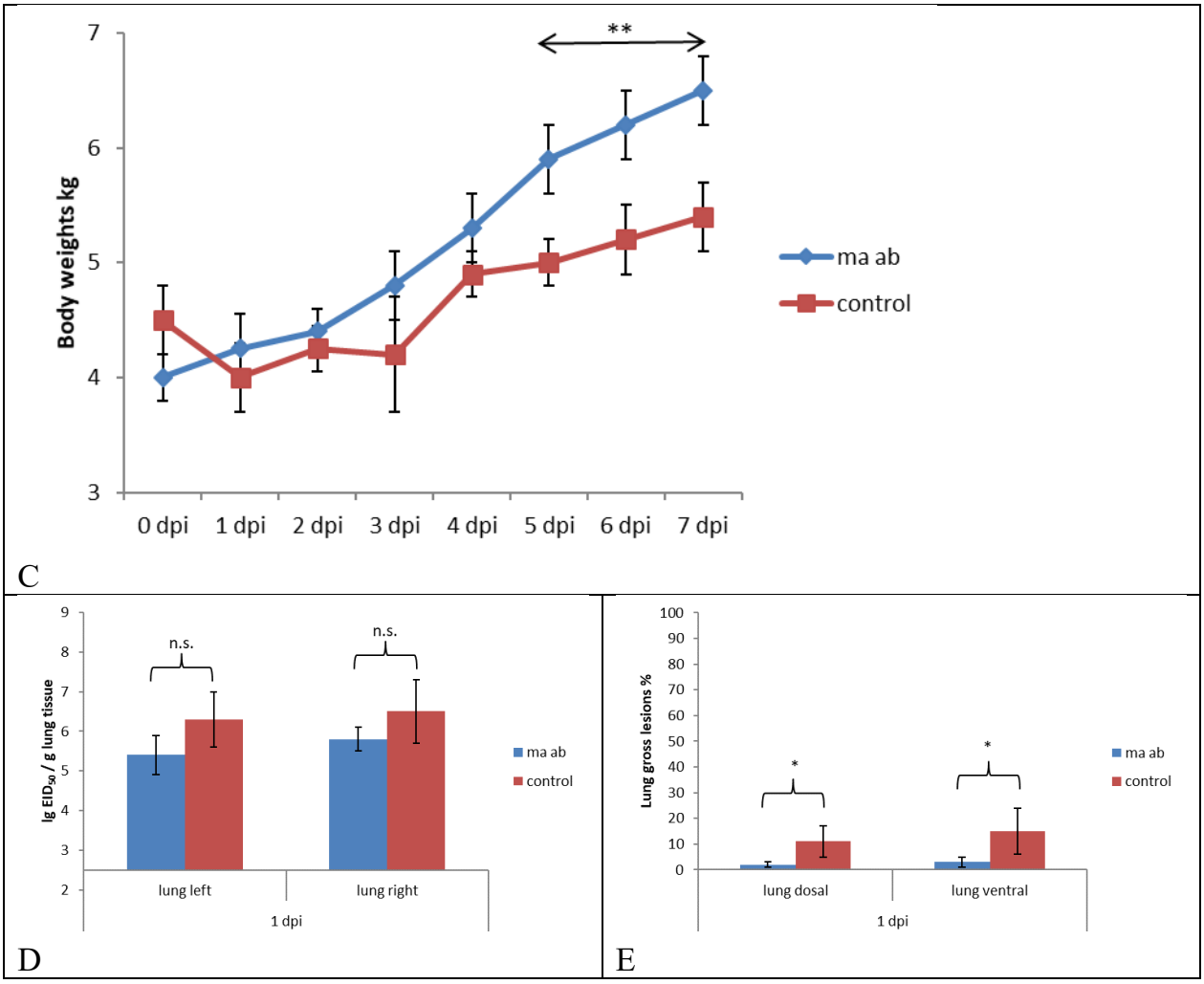


Figure 60: Effects of maternally-derived immunity in piglets infected at an age of 2 weeks

*A, symptoms (dyspnoea score); B, rectal temperatures (°C); C, body weights (kg); D, viral lung load (lg EID_{50}/g); E, lung gross lesions (%); n.s. not significant, * p<0.05 ** p<0.01, ***, p<0.001, comparison of both groups; arithmetic mean with standard deviation*

Short interpretation of results. The most important finding of this study was the severe influence of influenza A virus infection on the not immunised mother sow. This sow showed no respiratory symptoms and no fever. Despite this, the sow refused to eat and gave no milk. This shows that influenza A virus infection can influence reproductive performance of sows without overt respiratory disease. Infectious virus was detected in the milk of the infected sow. In this context, experimental infections of the udder of a dairy cow shortly before drying off are interesting, which showed that H1N1 influenza A virus (A/Puerto Rico/8/1934) replicates to high titres in the udder tissue[281]. Infection of mammary glands of ferrets by $H1_{pdm}N1$ virus was demonstrated in experimental infection indicating susceptibility of this tissue[215]. The mammary gland was not investigated in the sows here, but a potential infection of the udder may have had an impact on lactation as in the H5N1 clade 2.3.4.4b genotype B3.13 infections in dairy cows in the USA in 2024[360]. The long-lasting effect on the body weights of the piglets is most probably caused by the poor

lactation of the not immunised sow because. In the end the average difference between piglets of the immunised sow and that of the not immunised sow were more than 1 kg in body weight. This shows the strong impact of influenza A virus infections on sows despite not having respiratory symptoms.

5.2.1.7 INVESTIGATION OF PIGLETS OF AN AGE OF 4 WEEKS AT INFECTION

Background. The interactions between immunisation and maternally-derived immunity are discussed controversially. Negative interactions are hypothesized because piglets with maternally-derived immunity respond not or only poorly with antibodies to immunisation. High-dose aerosol infection approaches were not carried out on pigs with maternally-derived immunity so far.

Study design. FLUAV-antibody free sows were immunised 5 and 2 weeks before farrowing (inactivated strain FLUAV/Jena/5555/2009 $H1_{pdm}N1$). Other sows remained not immunised.

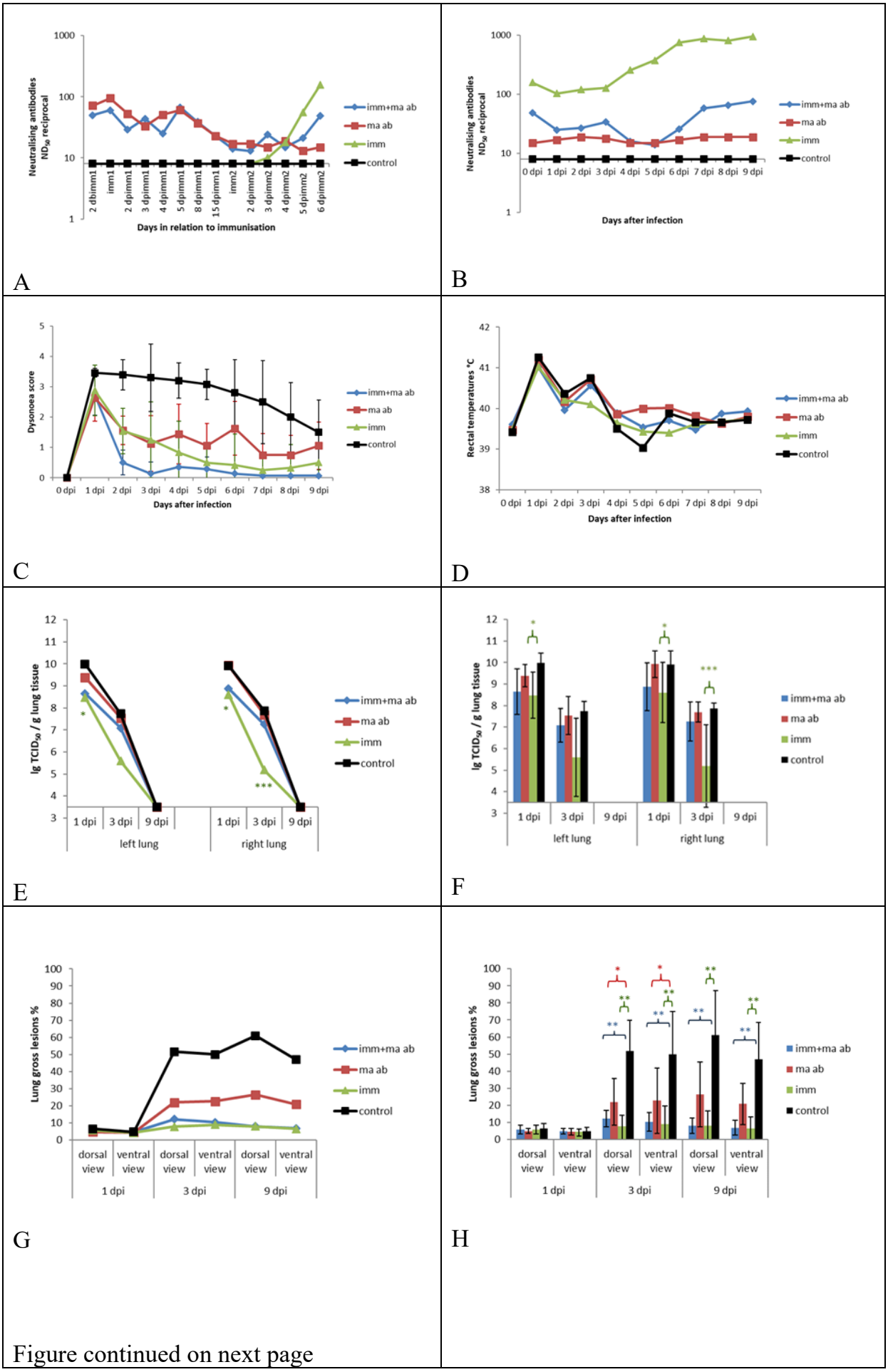
3 and 24 days after birth half of the piglets of immunised and not immunised sows were immunised (inactivated strain FLUAV/Jena/5555/2009 $H1_{pdm}N1$, Table 27). Seven days after second immunisation high-dose aerosol infection was performed using the highly virulent strain FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$) at a dose of 6.17 lg $TCID_{50}/m^3$. One, 3 and 9 dpi lung samples were taken from 5 pigs each group and investigated. Pigs were observed daily for clinical symptoms. Body weights were measured every second day. Blood samples and nasal swabs were withdrawn (nasal swabs daily; blood samples every second day).

Table 27: Trial of the vaccination of piglets at 3 days of age
Overview of the experimental design of the trial investigating effects of high-dose aerosol nebulization of highly virulent FLUAV on immunised and not immunised piglets with and without maternally-derived immunity

Group	Immunity	Abbreviation	Number of pigs included*
1	Maternal antibodies+immunisation	imm+ma ab	19
2	Maternal antibodies	ma ab	20
3	Immunisation	imm	18
4	Control (neither maternal antibodies nor immunisation)	control	18

* there were 20 pigs in each group at the beginning of the trial; the numbers show the pigs available at infection

Results. Antibodies were followed up from the first day after birth onwards (=2dpimm1). Two days later the first immunisation was done (at the 3rd day of life). Maternally-derived neutralising antibodies decreased steadily (Figure 61 A). Immunised pigs without maternally-derived antibodies responded well to the second immunisation with titres of neutralising antibodies in all piglets. The piglets immunised within maternally-derived immunity responded poorly or not in terms of antibodies to immunisation (Figure 61 B). The virus induced severe and long-lasting dyspnoea from 1 dpi onwards in the control group (Figure 61 C). All other groups displayed significantly lower dyspnoea. The group immunised within maternally-derived immunity performed best and had the lowest degree of dyspnoea indicating synergistic effects of both components of immunity. All pigs responded with fever to infection on 1 dpi (Figure 61 D). On 3 dpi there was a second peak of increased temperatures which lacked in the group immunised (Figure 61 D). Five dpi there was a nadir in rectal temperatures in the control group indicating severe disease in individual piglets. Virus titres in the lungs were high on 1 dpi and decreased on 3 dpi (Figure 61 E, F). No virus was detectable anymore in the lungs on 9 dpi. Immunised pigs had significantly lower viral lung loads in their lung in comparison to control pigs. The viral lung load of piglets immunised within maternally-derived immunity and those with maternally-derived immunity differed not significantly from that of the control group. There were strong lung gross lesions in piglets of the control group on 3 and 9 dpi (Figure 61 G, H). The lung lesions in all other groups were significantly lower; the immunised pigs performed best and had the lowest lung gross lesions whereas piglets having only maternally-derived immunity displayed more lung gross lesions. The shedding followed the typical profile in all groups without significant differences between the groups (Figure 61 I, J) but the group with maternally-derived immunity without vaccination shed virus longest (for individual data see Supplement 83, page CLXXI). Immunised piglets and piglets immunised within maternally-derived immunity ceased shedding earlier in comparison to the other groups. The severe outcome of the infection was also reflected by the body weights of the control group which decreased until 4 dpi (Figure 61 K, L). Piglets with maternally-derived antibodies performed similar and reflected also stagnation in the body weights whereas immunised piglets had steady body weight gains. Significant differences between body weights could only be seen on 5 dpi for the group with combined immunisation and maternally derived immunity. This difficulty to ensure significant differences is due to the variation of individual body weights. For individual data it is recommended to look into the supplement (page CLXXI).



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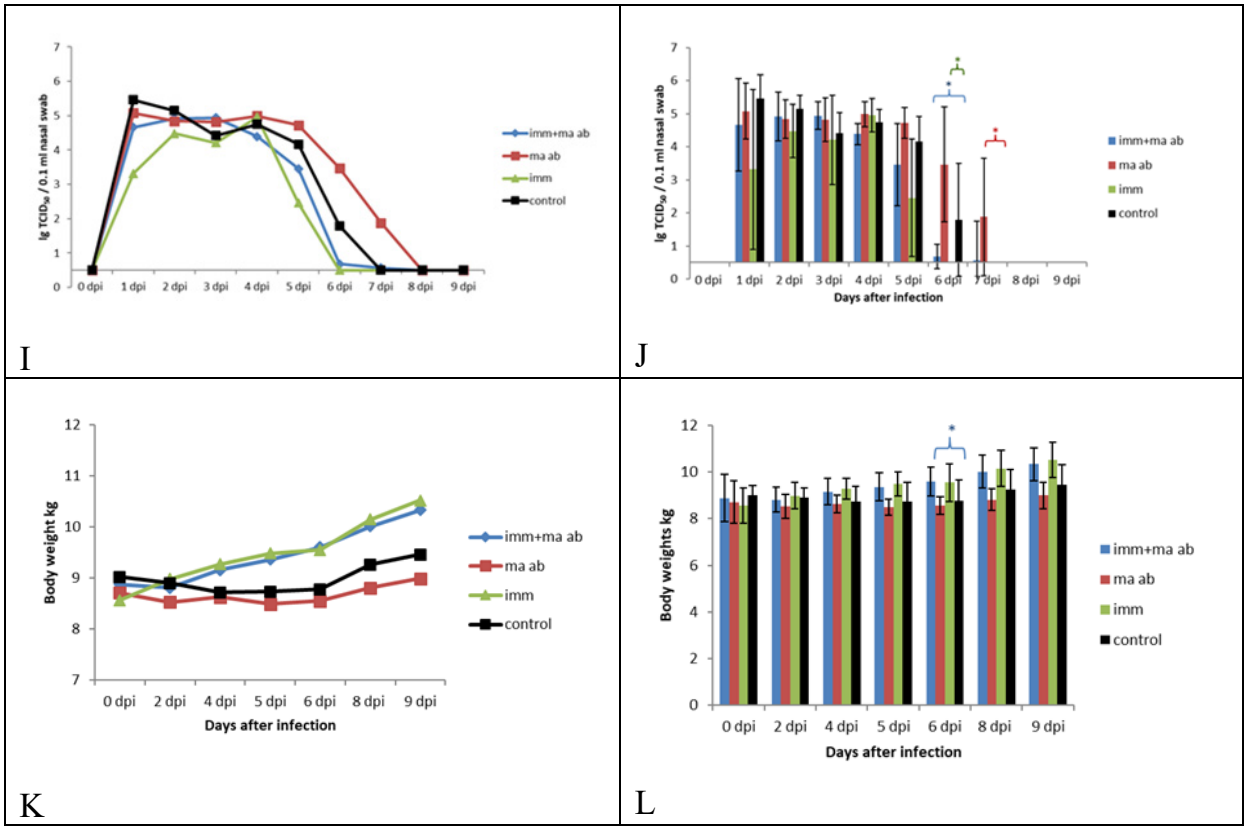

Figure 61: Effects of immunisation into maternally-derived immunity

Results of high-dose aerosol infection on immunised and not immunised piglets with and without maternally-derived immunity after infection with the highly virulent FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$)

A, neutralising antibodies after immunisation (geometric mean); B, neutralising antibodies after infection (geometric mean); C, symptoms (dyspnoea score, arithmetic mean with standard deviation - from 1 dpi onwards significant to highly significant lower symptoms in immunised piglets with maternally-derived immunity, immunised piglets and piglets with maternally derived immunity); D, rectal temperatures (°C, arithmetic mean); E, viral lung load, trends of arithmetic mean; F, viral lung load (lg $TCID_{50}$/g lung tissue, arithmetic mean with standard deviation); G, lung gross lesions (%) trends of arithmetic mean; H, lung gross lesions (%) arithmetic mean with standard deviation; I, virus excretion, arithmetic mean; J, virus excretion (lg $TCID_{50}$/0.1 ml nasal swab), arithmetic mean with standard deviation; K, body weights, trends of arithmetic mean; L, body weights (kg) arithmetic mean with standard deviation

Short interpretation of results. Despite high viral lung loads and virus shedding immunised piglets reflected significantly less dyspnoea and less lung gross lesions. Despite poor antibody induction in pigs immunised within maternally-derived immunity and high replication of virus in these piglets the animals were protected from disease. This indicates that other components of immunity than antibodies are stimulated by immunisation. These components do not prevent viral replication but prevent extension of lung gross lesions. The investigation of these components of immunity maybe of interest for further immunological research. It has to be emphasized that infected piglets have to be regarded as virus shedders. In contrast to immunised older pigs which shed significantly less virus there were no significant differences in immunised pigs in comparison to control pigs in pigs of this young age. The longer shedding in pigs with maternally-derived immunity is of importance because these pigs are able to sustain infection cycles longer than vaccinated piglets or piglets without maternally-derived immunity. The delay caused by unlocking of immunity blockade mechanisms under the umbrella of maternally-derived immunity maybe the reason for prolonged shedding.

6. GENERAL DISCUSSION

6.1 SWINE INFLUENZA PATHOGENESIS

Different infection models for swine influenza were investigated and compared. To date, no suitable methods have been available for studying the virulence of influenza viruses. The aerosol infection method developed in this monograph allows the comparative analysis of different virus infection doses in aerosols. By titrating the initial infective dose in the aerosol infection model, the virulence of influenza A viruses can be determined. High-dose aerosol infections allow the study of the pathogenesis of severe courses of influenza A virus infections. The main results of this part of the study and key discussion points are summarized in Table 28.

Table 28: Key findings and discussion points on the pathogenesis of swine influenza

Findings	Major points of discussion
The swine influenza infection models are linked by the infectious dose and differ in outcome by the initial dose delivered to the lungs	Influenza A virus infections are controlled by a rapid immune response (antibodies appear after 5-7 dpi, but initial effects of virus clearance are evident after 3-5 dpi) associated with local compensatory mechanisms in the respiratory tract (replacement of destroyed lung epithelial cells to maintain respiratory activity; elimination of cellular debris that accumulates and controls bronchioles) (see antibody and virus excretion kinetics in Supplement 18, page LXXXII and histological analyses, for example Supplement 5, page LXIV)
	Influenza is difficult to induce by experimental infections. Only infection models that enable high initial lung burdens are able to mimic the disease (pages 118 - 121)
	Varying initial infectious dose may explain the wide variability of disease; other factors contributing to the initiation of disease include genetic factors and malnutrition (Table 33, page 143)
Influenza A viruses differ in their virulence in pigs during high-dose aerosol infection	Emerging H1N1 viruses such as $H1_{pdm}N1$ are highly virulent and rapidly evolve to very low levels of virulence, while HxN2 viruses remain stable in virulence (pages 121 - 123)
	Virulence is linked to the neuraminidase (pages 123 - 129)
	It was shown in subsequent challenging following immunisation that HA immunogens protect against virus replication and disease, NA immunogens protect against disease, whereas M immunogens do not protect adequately and may exacerbate symptoms (pages 129 - 136)
	Individual differences in the response to infection indicate an influence of genetic host factors on disease severity (page 136)
	Lung lesions reflect a pattern of high inflammatory activity indicative of a local overreaction of innate immunity to infection (pages 136 - 142). The lung lesion is a key to understanding pathogenesis because, at a lower level, it reflects similar histologic patterns to the entire lungs of individuals who have succumbed to the disease (pages 136 - 142)

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Findings	Major points of discussion
Initial infectious viral load, host adaptation of virus and individual as environmental factors determine severity of disease	This chapter summarizes the findings reported above (pages 142 - 144)
Conclusions for future pandemics	This chapter draws implications for future pandemics (pages 144 - 149)
Expression of viral genes in cells outside the respiratory tract indicates systemic infection	In all infection experiments, a wide distribution of viral antigens throughout the body was observed in the pigs, although no infectious virus was detected outside the respiratory tract. This suggests that influenza viruses can spread to and efficiently infect other cells *in vivo*, but cannot complete their replication cycle there due to the lack of appropriate proteases or other factors (pages 149 - 156). Macrophages may play a role in the spread of the virus (Figure 72, page 151)
	Infection of tissues outside the respiratory tract was associated with inflammation (kidney) and cell destruction (heart), indicating pathogenic processes, even in the absence of obvious disease in the pigs (Figure 73, page 153 - Figure 76, page 155)
Immunological reactions to the M protein can enhance lung patholgy	Pigs vaccinated with a vaccine that only protects against internal proteins, such as M protein, had more severe lung lesions than the control pigs (page 129 - page 136)
Oseltamivivir prevents severe disease and lung lesion	Comparative investigations showed that the administration of neuraminidase inhibitors does not prevent virus shedding but can reduce shedding, dyspnoea and stronger lung lesions (page 157)
Immunosuppression prolongs viral shedding	Immunosuppressive treatment enabled a longer time of virus excretion in infected pigs (page 157)

6.1.1 SWINE INFLUENZA INFECTION MODELS ARE LINKED BY THE INFECTIOUS DOSE

An aerosol-mediated challenge model was developed in which infectious material was nebulized via a generator and uniformly distributed into infectious units. The nebulization process caused no loss of infectivity. The infectious dose in $TCID_{50}/m^3$ could be calculated. Aerosol infection provided uniform distribution of infectious virus throughout the respiratory tract of pigs housed in these infection units.

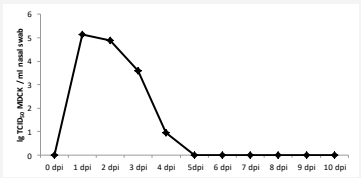
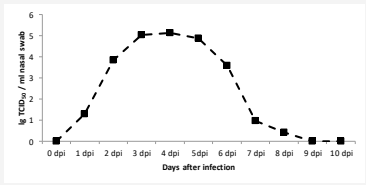
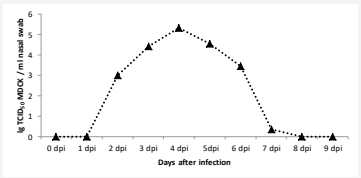
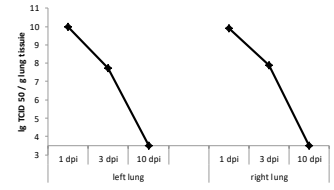
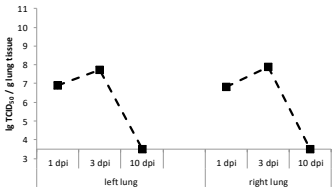
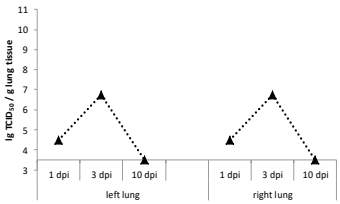
Influenza modelling is highly dependent on the infectious dose. The aerosol infection model allows infection of pigs in a natural way. After adjusting the infection titers, aerosol infection is possible in different variants (Table 29):

i) High dose – The high-dose aerosol infection leads to immediate settlement of large amounts of viruses in the lungs

ii) Medium dose – The high-dose aerosol infection leads to immediate settlement of viruses in the lungs but in contrast to very-high dose not to receptor saturation

iii) Low dose – The low-dose aerosol infection provides only low amounts of viruses which can settle directly in the lungs, it is similar to intranasal, low-dose intratracheal, and direct contact infections and results in almost no disease.

Table 29 (with figures): Overview of swine influenza infection models
All models are linked by infectious dose, which leads to differences in outcome

High dose infection	Medium dose infection	Low dose infection
Infection models:		
● High-dose aerosol	● Medium dose-aerosol ● Medium dose-intratracheal*	● Low dose-aerosol ● Low dose-intratracheal ● Intranasal ● Contact
Virus shedding:		
		
Viral lung load:		
		
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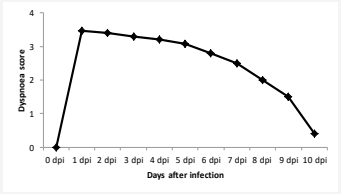	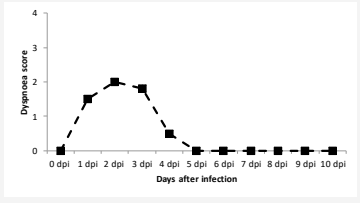	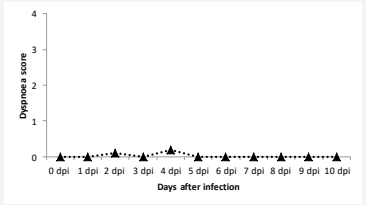

* high-dose infection is difficult to achieve intratracheally without injection of a large volume of suspension (see also Supplement chapter 7 –Aerosol versus intratracheal, page LXXI)

The installation of a high dose can only be achieved by nebulizing a very high and concentrated dose of virus. The virus is placed directly in the lungs. The immediate placement of virus in the lung allows the immune response to be subverted for a short period of time and the true effects of high doses of influenza virus on the respiratory tract to be tested. The immune response can be pronounced, resulting in higher antibody titers and more rapid

clearance of the virus from the lungs, as well as a greater decrease in viral shedding than in natural infection. The induction of disease is strong. Nevertheless, low-virulent viruses induce only moderate symptoms, even with high-dose aerosol infection. This is the reason why this model is the only model to study virulence.

In contrast to nebulization with very high doses, there is no receptor saturation shortly after nebulization of medium doses. This allows the viruses to replicate to higher titers as the infection progresses. In addition, there is no risk of overreaction of innate immunity processes, which can lead to fever and dyspneoa despite the presence of immunity (e.g., homologous vaccination). There is a strong correlation between infectious dose and symptoms. The model is well suited for testing the efficacy of vaccines, antivirals, and other preparations; viral lung load increases from 1 to 3 dpi. Viral excretion is reflected in a bell-shaped curve.

Low dose infection allows viruses to replicate in a normal natural manner. Virus replication in the lungs and virus excretion peak around 4 dpi similar to high dose infection, but due to the lower starting point of the virus dose installed in the lungs, symptoms are moderate or absent. This effect is achieved by different infection models: low-dose aerosol infection, low-dose intratracheal infection, intranasal infection, and contact infection. It should be emphasized that most intratracheal infections reported in the literature are low-dose injections, which means that in principle the difficult intratracheal injection procedure could be saved and the more convenient method of intranasal injection could be used. It is also important to note that, in contrast to aerosol models, the exact dose of material injected is irrelevant in low-dose infections, since the virus must replicate to high titers anyway and the effects are the same within a broad range of installed doses. The method induces no or almost no disease and is suitable for studying all parameters related to viral excretion and natural infection; viral lung load increases from 1 to 3 dpi and the curve of viral excretion is bell-shaped.

The only method that covers all routes of infection, all approaches and all dosages is aerosol nebulization. For all purposes not involving the induction of clinical symptoms, intranasal infection is sufficient. Although the virus may be sneezed out in this intranasal infection, the remaining viral material is always sufficient for infection. Staining experiments with Evans blue after intranasal and aerosol infection showed a fine distribution in the lungs after aerosol infection but a strong staining of the esophagus after intranasal infection, suggesting that a larger portion of the viral material was swallowed after intranasal infection[143].

There is a specific range of infection doses where both can be achieved, disease induction and bell-shaped excretion curves. These models are ideal for studying the efficacy of vaccines, antivirals, and other therapeutic agents.

6.1.2 INFLUENZA A VIRUSES DIFFER IN VIRULENCE AT HIGH-DOSE AEROSOL INFECTION

6.1.2.1 DESCRIPTION OF DIFFERENCES IN VIRULENCE

Calculation of disease indices allowed comparison of the virulence of the different influenza A viruses (Figure 62).

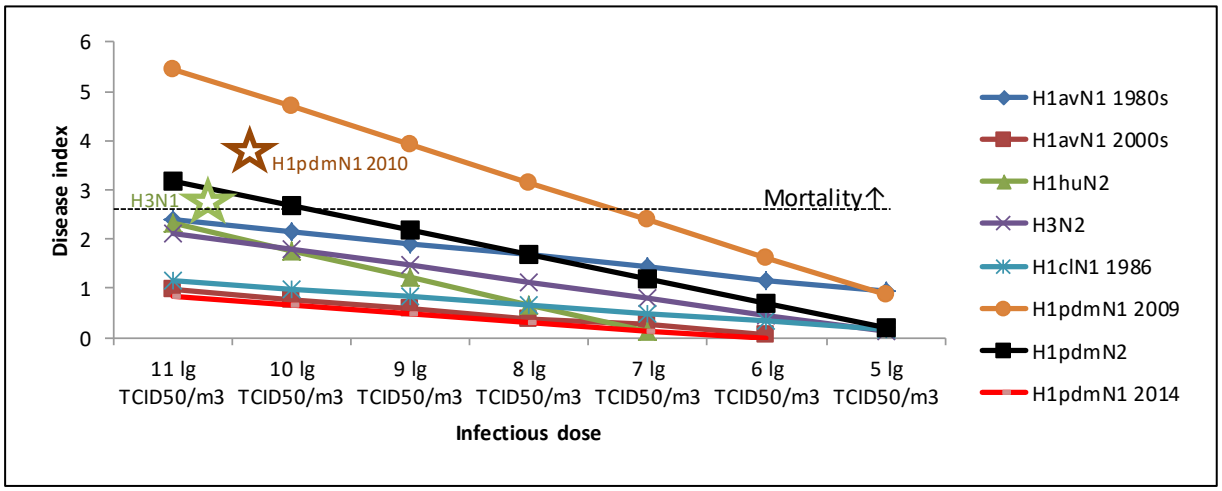

Figure 62: Disease severity index reflects differences in virulence
(disease index calculated for $TCID_{50}$ MDCK infective doses) based on regression analyses of dyspnoea score data obtained after dose titration and evaluation of high-dose aerosol nebulization experiments with influenza A viruses in pigs; $H1_{pdm}N1$ April 2009 virus was the most virulent virus, whereas $H1_{cl}N1$ and $H1_{av}N1$ viruses had the lowest virulence; $H1_{pdm}N1$ viruses lost virulence in the years after 2010 (red line); the brown star indicating $H1_{pdm}N1$ 2010 shows that this evolutionary process toward lower virulence began early; $H1_{av}N1$ 1980s/1990s (HA 1C.1), $H1_{hu}N2$, and H3N2 reflect a similar pattern of virulence at high infectious doses; the newly reassortant $H1_{pdm}N2$ (black line) and H3N1 (green star) viruses showed higher levels of virulence compared with the other viruses, but lower virulence than the April 2009 $H1_{pdm}N1$ virus (the ochre line refers to the results of titration of the infective dose of an $H1_{pdm}N1$ virus isolated in April 2009, strain 1580); all H1N1 viruses approach similar levels of virulence after circulating in humans and/or pigs (compare $H1_{cl}N1$, $H1_{av}N1$ 2000, and $H1_{pdm}N1$ 2014); the dashed line indicates the disease index associated with lethality; the highest observed lethality was 23% (for the April 2009 $H1_{pdm}N1$ virus)

The April 2009 $H1_{pdm}N1$ virus had outstanding virulence. Nineof 52 3-month-old control pigs experimentally infected with high-doses of $H1_{pdm}N1$-April 2009 virus in 2010-2012 died 2-3 dpi in independent experiments (lethality 17.3%). All had severe lung lesions. In contrast, the 2000s $H1_{av}N1$ viruses and the 1986 $H1_{cl}N1$ virus had virtually no virulence, possibly due to host adaptation. Similar observations were made for 2014 and 2015 $H1_{pdm}N1$ viruses, which no longer exhibited the high virulence of 2009 $H1_{pdm}N1$ virus and were within the range of virulence of 1980s/1990s $H1_{av}N1$, $H1_{hu}N2$, and H3N2 viruses at higher infectious doses ($\geq$ 8 $TCID_{50}/m^3$) and within the range of 1986 $H1_{cl}N1$ at lower infectious doses ($\leq$ 7 lg $TCID_{50}/m^3$). An $H1_{pdm}N1$ 2009 virus (strain 1580) isolated in April 2009 was very highly virulent. This virus was used for infectious dose titration experiments and represents the most highly virulent virus in Figure 62. Newly reassortant viruses such as $H1_{pdm}N1$ and H3N1 had higher virulence compared to H3N2, $H1_{hu}N2$, $H1_{av}N1$, and $H1_{cl}N1$ viruses. Virulence in H1Nx viruses changed after reassortment with N1 or N2 neuraminidases. N2 could most likely fix virulence, as H3N2 and $H1_{hu}N2$ viruses did not change their virulence during the observation period.

6.1.2.2 NEWLY EMERGED H1N1 VIRUSES MOVE TOWARDS LOWER VIRULENCE

The investigations show time-dependent differences in virulence between influenza A viruses of the same subtype. Newly reassorted H1N1 viruses show very high virulence and evolve rapidly towards lower virulence. The evolution of $H1_{pdm}N1$ viruses could be followed from April 2009 to March 2015. Already 3-4 months after their emergence, the viruses lost their lethal virulence. After 5 years, they reached the flat level of low virulence, identical to the $H1_{cl}N1$ and $H1_{av}N1$ viruses of the 2000s (Figure 63). Differences in virulence in pigs have also been observed between North American $H1_{pdm}N1$ viruses[99]. Similar differences in virulence of various $H1_{pdm}N1$ viruses were shown by other researchers in pigs and ferrets[361,362]. Some studies of the disease characteristics caused by the $H1_{pdm}N1$ virus in humans suggest that patients in the post-pandemic phase had a more attenuated disease, as reflected by lower rates of acute respiratory illness and lower rates of pneumonia and death[363,364].

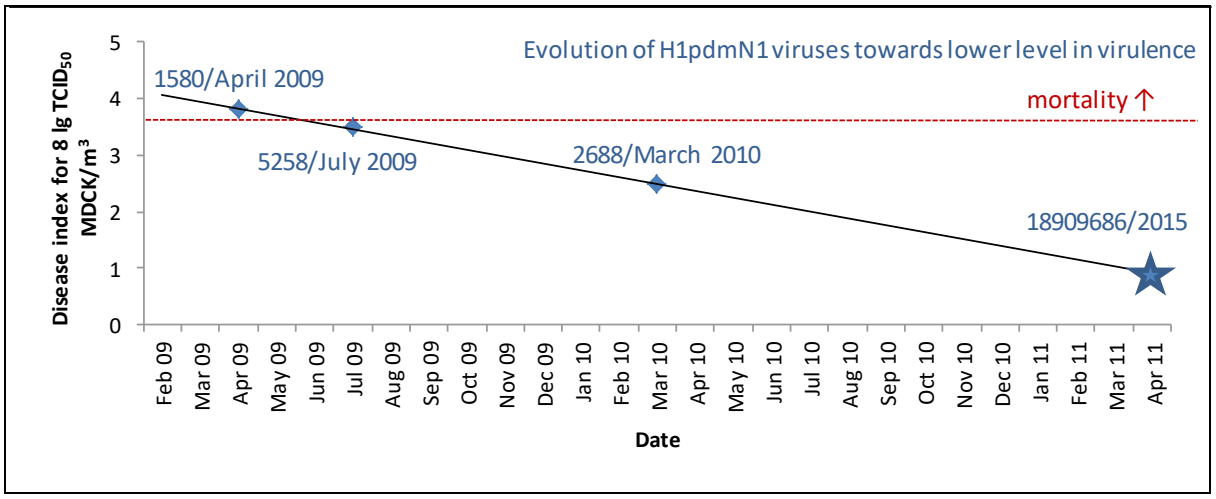

Figure 63: Evolution of $H1_{pdm}N1$ 2009 viruses towards a lower level in virulence
Already 3-4 months after their emergence, these viruses lost their lethality; the trend line indicates that already in April 2011, virulence had reached the level observed for a virus isolated in 2015 (blue star); note that only viruses isolated from human patients are included in this analysis

The flat titration line of $H1_{av}N1$ 1980s/1990s viruses suggests that these viruses were equilibrated at this level of virulence (1C.1 $H1_{av}N1$ viruses according to Anderson et al. 2016[365]) and that the lower virulence of $H1_{av}N1$ 2000s viruses is due to another line of $H1_{av}N1$ viruses (1C.2 $H1_{av}N1$ viruses according to Anderson et al. 2016[365]). A clear change in the virulence of the $H1_{av}N2$ viruses occurred between 1998-2003, with the strain Belzig/02/2001 (an early 1C.2.2 $H1_{av}N1$ virus) showing intermediate virulence. The evolution to low virulence took longer for the $H1_{av}N1$ than for the $H1_{pdm}N1$ viruses, which evolved faster in humans to viruses of low virulence. This could be due to differences in the cell membranes of the two species or the faster achievement of a higher passage level in the larger human population. $H1_{hu}N2$ viruses are virulent but ressortants of these viruses with N1 ($H1_{hu}N1$ viruses) reflected the same low level in virulence as $H1_{av}N1$ viruses. On the other hand, $H1_{av}N2$ viruses were virulent, although the $H1_{av}$ was derived from low

virulence $H1_{av}N1$ viruses. The N2 appears to be a stabiliser in virulence. The data indicate that the NA plays a crucial role in this process.

6.1.3 DIFFERENCES IN VIRULENCE ARE LINKED BY THE NEURAMINIDASE

Current knowledge suggests that dysregulation of the innate immune response plays an important role in the severity of disease following respiratory viral infection. The data presented in this monograph suggest a role for neuraminidase in this process, which may affect innate immunity through interactions with membrane receptors or with factors of the necroptotic cascade (Figure 64).

Much of the scientific literature has focused on finding genetic markers associated with virulence[366]. Alterations in HA receptor binding sites or neighbouring regions can affect virulence, as can masking or unmasking of proteolytic cleavage sites[367,368]. The polymerase basic protein 2 (PB2) may influence virulence by affecting host specificity[369-371]. Mutations in NS1 can support the circumvention of innate immune responses and thereby influence virulence[372,373]. In this analysis, the PA2 K340N mutation was found, which differed between viruses causing mortality and those causing no fatal cases. 340K was found in the highly virulent virus A/Hamburg/NY1580/April 2009, but 340N was found in all later viruses. PA2 K340N is most probably an adaptive mutation in the course of adaptation to human cells. 340K viruses did not cause CPE in MDBK cells whereas 340N viruses did which supports the idea of an adaptive mutation (see Supplement, page CV). Another mutation was found in PA, which distinguishes the highly virulent virus: I118V. Again, this mutation is not known to be a marker of virulence. However, most of the other mutations that differ between virulent and less virulent viruses do not correspond to changes in the functional sites of HA, PB2 and NS1 reported so far. Therefore, other factors may be involved in virulence.

An important determinant of virulence in this study was the NA. N2 was associated with a high level of virulence. In contrast, N1 viruses surpassed N2 viruses in virulence in freshly reassorted viruses, whereas this virulence was gradually lost after longer evolution. This suggests adaptive processes in the balance between HA and NA and the sialoglycan repertoire of the host cell membrane. NA activity has been shown to play an important role in virulence[374-376].

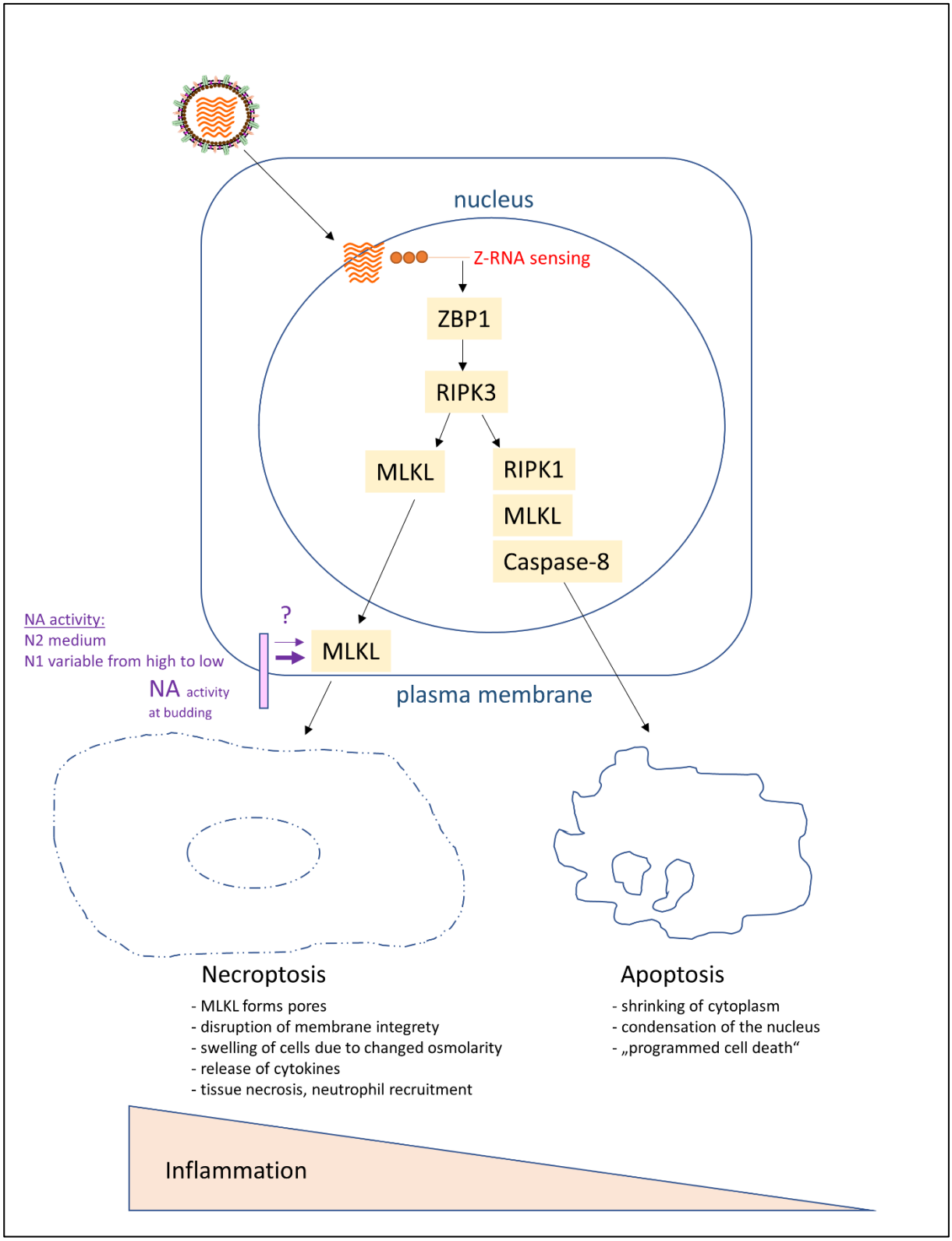

Figure 64: Possible signaling cascades leading to necroptosis supported by neuraminidase activity and/or other viral components capable of sensing receptors after viral entry

Adapted according to Balachandran & Rall (2020)[377] and Zhang et al. (2020)[378], for further details on signaling cascades of innate immunity ses Wu & Metcalf (2020)[379] and Ludwig et al. (2003)[380]; the NA activity here refers to its activity reagarding induction of necroptosis

It is likely that the N1 NA activity in freshly reassorted HxN1 viruses is not yet in equilibrium with the haemagglutinin, the other viral genes and the host cell membrane, and this is

associated with a special activity on budding when virions are detached from the cell. This special activity may influence signaling cascades in infected cells leading to necroptosis or other processes. After adaptation, this special NA activity is lost in HxN1 viruses. The balance between Hx and N2 may require a different activity of neuraminidase and may therefore explain the higher level of virulence of HxN2 viruses in comparison to adapted H1N1 viruses. Recombinant viruses carrying the 1918 pandemic HA and NA in the background of a seasonal human H1N1 virus induced significantly higher levels of chemokines such as IFN-γ, TNF-α, MIP-1α and MIP-2 than the seasonal virus in infected mice[247] which confirms the induction of cytokine pathways by viral gene imbalance.

It is noteworthy that the European swine viruses circulating before 2009 all had a similar genetic background except for HA and NA. The higher virulence of H3N2 viruses with this stable internal gene cassette also supports the hypothesis of an influence of the HA-NA balance within the virus on virulence. The investigations in this monograph have shown that there is a strong evolution of newly reassorted H1N1 viruses towards lower virulence, which is accompanied by genetic changes, but not by unique single genetic markers responsible for the decrease in virulence. An important conclusion from this is that it is not genetic markers but functional properties that are essential for the virulence of influenza viruses. Such functional properties could be influenced by the interaction of newly reassorted segments of FLUAV with membrane components taken up from infected cells during budding. If this is not yet optimised, infection of the target cell results in a different activity profile, leading to different patterns of secretion of biochemical mediators such as cytokines, which determine the further course of the disease. Neuraminidase may play a dominant role in this balance. Thus, determinants on virulence have to be revisited (Figure 65, Figure 66).

There are two processes that need to be analysed. Looking at viruses that have been circulating in their host species for a longer time, HxN2 influenza viruses have a higher virulence than HxN1 influenza viruses. After reassortment of $H1_{av}N1$ viruses with N2, $H1_{av}N2$ influenza viruses had a higher virulence than $H1_{av}N1$ influenza viruses. The reverse was also true. The higher virulence of $H1_{hu}N2$ influenza viruses was reduced after reassortment to $H1_{hu}N1$ (compare with Figure 17, page 54). Because this change occurred within a stable ICG this suggests a crucial role of neuraminidase for virulence. The balance between HA and NA and the shedding process during budding appear to play a role here.

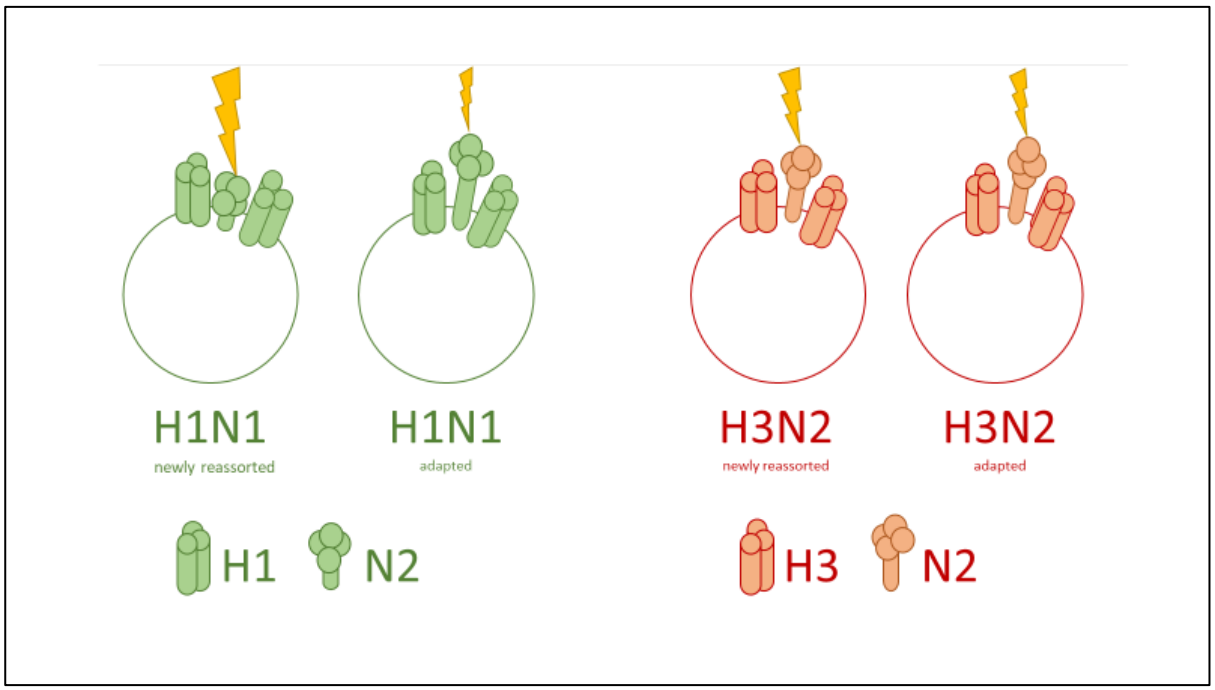

Figure 65: Nongenetic explanation of virulence of influenza viruses
Approach to a working hypothesis
The interaction between haemagglutinin and neuraminidase and their position on the virion membrane determines virulence by influencing neuraminidase activity: lower position requires longer periods of enzymatic activity associated with stronger induction of signaling cascades leading to cytokine storms (this is consistent with the observation that adapted $H1_{av}N1$ viruses induce lower antibody responses in infected pigs, possibly due to neuraminidase coverage of antigenic sites); it is also possible that insufficient neuraminidase activity prolongs the necroptotic process, allowing the cells to release more cytokines; arrows indicate the intensity of necroptosis and release of cytokines (arrows: time periods required for efficient particle release)

A second important aspect is the clear change in the virulence of H1N1 influenza viruses after transmission to a new species, as demonstrated by the example of $H1_{pdm}N1$ viruses. These viruses gradually lost their virulence: the first viruses were associated with 20% mortality in high-dose infection trials in pigs; viruses isolated two months later still made 20% of the pigs severely ill but were no longer lethal; one year after the first appearance of $H1_{pdm}N1$ viruses, pigs were still ill but not severely ill; and viruses isolated even later were similar in virulence to the $H1_{av}N1$ influenza viruses that had been circulating in pigs for a long time.This process suggests that, in addition to the HA-NA balance, a third component plays a role which could be the host cell membrane. Influenza viruses bind loosely to sialic acids of the host cells. These differ slightly from host to host. There were hundreds to thousands of passages between the isolated $H1_{pdm}N1$ viruses in humans, even if they were isolated only two months later. This suggests that the new reassortant influenza viruses initially retain their original binding constellation and gradually adapt to each other and to the new host cell membrane. The mechanism of adaptation is not known; it may be the position of the neuraminidase, the formation of patches or even the inclination of the surface proteins, which must be readjusted. In the initial constellation, the neuraminidase cannot yet react optimally, has to exert more force for the detachment mechanism and therefore possibly induces a stronger response of the innate immunity. This hypothesis is also supported by the unusual behaviour of the H3N1 influenza viruses. These did not

persist in pigs for more than a few transmission cycles in the field and then disappeared. In their initial constellation, they were still infectious, transmissible and highly virulent. During the process of adaptation of H3 and N1 to each other, they then apparently became incompatible and lost their infectivity. The spread of the H3N1 virus from one pig herd to another may not seem like much, but it means that the virus has passed through several hundred pigs. This means that many passages are required to change the virus (in this case towards loss of infectivity). This also emphasises the validity of the data collected here with regard to the cell culture passages of the viruses in canine (MDCK) or bovine cell lines (MDBK) before challenge infection. The one to three passages in these cell lines could not yet influence the configuration of the surface proteins HA and NA in such a way that the original virulence was altered.

Another interesting fact was that the early $H1_{pdm}N1$ viruses became dormant in the first passages in MDBK cells: that is, they infected the cells, replicated in them, expressed antigens but did not escape and did not disrupt the cells, as indicated by the lack of cytopathic effects (Supplementary Table 14, page CV): this may indicate that the virus was not yet adapted to the MDBK cells and that the interaction between the segments of this virus was not yet well balanced; in addition to other factors such as adaptation to ANP32, the neuraminidase function may not have been sufficient to release the virus in some cell types (such as of newly reassorted $H1_{pdm}N1$ virus in MDBK cells). This supports the hypothesis that higher neuraminidase activity is required to release virus from these cells. After further evolution in the human population, the $H1_{pdm}N1$ virus gained the ability to induce cytopathic effects and virus release in MDBK cells. This suggests a changed interaction between the N1 neuraminidase and cell membrane proteins. Cell membrane components within the virion may contribute to improved neuraminidase function, i.e. more efficient virion release requiring less N1 neuraminidase activity after virus passage into cells of the new host, resulting in cytolytic effects on a wider range of infected mammalian cells. Xu et al (2012) showed that the early $H1_{pdm}N1$ viruses had low HA avidity for glycan receptors and weak NA enzymatic activity, which improved as these viruses evolved towards higher HA avidity and higher NA enzymatic activity[381]. The $H1_{pdm}N1$ virus was a multiple reassortant[382], the neuraminidase and the matrix protein were derived from the Eurasian $H1_{av}N1$ influenza viruses and had to adapt to NP, HA and NS of the swine $H1_{cl}N1$ influenza viruses, originally derived from the 1918 $H1_{pdm}N1$ virus, and the polymerase complex of the swine American triple reassortant viruses, which in turn are derived from the human (PB1) and avian (PA, PB2) influenza virus pools. Thus, both the polymerases and the nucleoprotein (replication and synthesis of NA) as well as the matrix protein (M1 plays a role in final packaging at the host membrane) may have contributed to an initially suboptimal neuramidase activity, which later improved in the course of further evolution of $H1_{pdm}N1$.

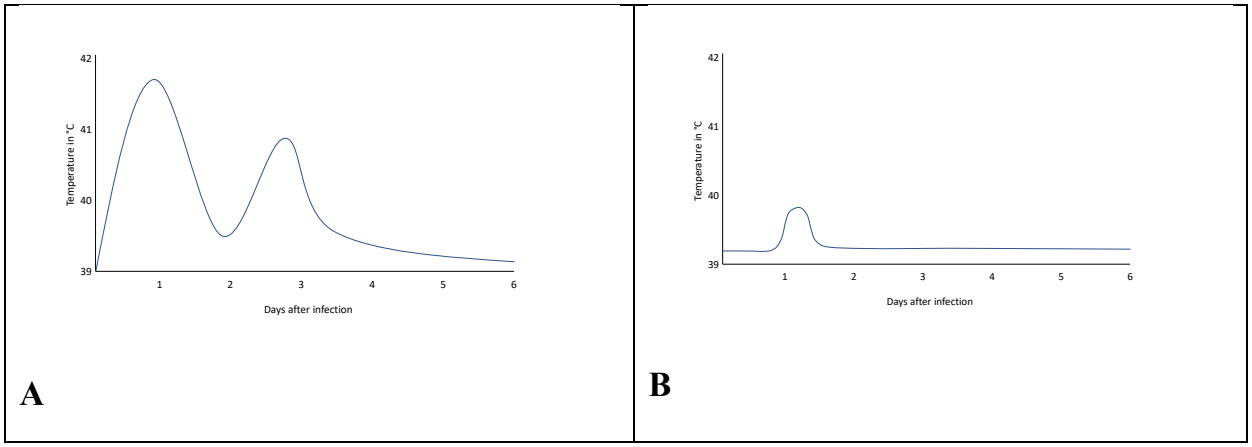

Figure 66: Different fever kinetics caused by influenza A viruses
The different results of the fever kinetics in highly virulent viruses such as $H1_{pdm}N1$ April 2009, HxN2, early $H1_{av}N1$ viruses (A) and low virulent viruses such as later $H1_{pdm}N1$ and $H1_{av}N1$ viruses (B) in the high-dose infection study indicate a different influence of these viruses on the cascades of innate immunity and perhaps a measure to reflect the degree of stimulation of cytokines such as TNF-α and IL-1; the second peak in fever induction occurs at a time when high numbers of viruses are released and may indicate NA activity; the low stimulation of fever and the loss of the second peak may indicate an optimisation of NA activity; thus HxN1 influenza viruses may evolve to lower virulence by undergoing innate immune responses in the course of optimising their NA activity (for fever data see also Supplement 42 A+B, page CXIX , compare individual rectal temperatures of virulent – $H1_{av}N1$ 1C.1 1981 - and low virulent viruses – $H1_{av}N1$ 1C.2.2 2003)

Since the development of molecular methods and the availability of sequence information, much emphasis has been placed on mutations to define virulence markers. The sequencing of the 1918 pandemic influenza viruses was accompanied by the hope of a quick solution to explain the severity of this pandemic. However, there was no quick fix because the viruses were similar to swine influenza viruses and only spare mutations were defined that could have contributed to the virulence of these viruses. The data from the virulence studies in this monograph suggest a more functional reason for virulence. Neuraminidase may play a crucial role in determining virulence. Neuraminidase is a sialidase and functions as a receptor-destroying enzyme. It cleaves sialic acid from cellular glycoproteins and the viral glycoproteins that are expressed in infected cells and assembled into virions; this prevents aggregation of HA and allows viral release[383]. It also has potential roles prior to infection, as well as in the truncation of HA glycosylation and the cleavage of inhibitory components from mucins (for review see[383]). It thus contributes to essential steps within the replication cycle, but also has close contact with the cell membrane and its receptors. The correlation between virulence and neuraminidase activity may suggest that neuraminidase may influence necroptotic pathways through interactions with the cell membrane. These results add neuraminidase activity to the list of major virulence factors (Table 30).

The Ca^{2+} ion at the centre of the NA tetramer is an important determinant of stability[384]. The Ca^{2+} ion is necessary for catalysis. Its binding affinity varies between NAs.

A functional match between haemagglutinin and neuraminidase is required for effective replication in a new host[385].

Table 30: Overview of major virulence factors of influenza viruses and supporting factors

Major virulence factors Factor	 Marker of virulence	Effect
Neuraminidase interactions (haemagglutinin – polymerase – host cell membrane)	Morphological feature Measurable to date only in animal infection models	Cytokine storm in the lung associated with pneumonia and severe lung pathology
Multibasic cleavage site in H5 and H7 viruses	HA 323-330* (R-X-R/K-R)	Systemic lytic infection; at respiratory infection mainly resulting in encephalitis due to vast viral spread via the olfactory nerve
Binding to sialic acids outside of the respiratory tract (or to MHC class II receptor by H17, H18 viruses)	No markers	None or organ failure in individuals with underlying conditions
Supporting virulence factors **Factor**	 **Marker of virulence°**	**Effect**
Higher activity of the viral replication complex	Mutations in PB2, PB1, PB1-F2, PA, NP	Enhanced viral replication
Suppression of host antiviral response	NS1, NS2	Decreased antiviral response in host
Increased virus binding	HA	Increased virus binding to α2,6

* numbering relative to A/Vietnam/1203/2004; ° for details see CDC H5N1 Genetic Changes Inventory: A Tool for Influenza Surveillance and Preparedness https://www.cdc.gov/flu/pdf/avianflu/h5n1-inventory.pdf and Griffin & Tompkins (2023)[382]

This was achieved by the $H1_{pdm}N1$ virus at low levels in early 2009[381]. The early 2009 $H1_{pdm}N1$ viruses showed low HA avidity for glycan and weak NA enzymatic activity, but both increased after circulation in the human population[381]. These findings are consistent with the observations on virulence in this monograph, which hypothesise that NA low enzymatic activity with simultaneous activation of cytokine pathways is associated with longer times for virus detachment from infected cells, giving cells time to activate innate immune mechanisms prior to lytic destruction (cytokine storm), in contrast to cells where virion release is more efficient, resulting in faster lytic death of infected cells.

6.1.4 Influence of immunological response to viral proteins on virulence

Immunisation of pigs with viral vaccines differing in their antigenetic relationship to the infecting strain and subsequent challenge with H3N1 virus revealed different patterns of protection. The HA is the most protective component, indicating protection against both high virus replication and disease. The NA is able to reduce virus shedding and symptoms in infected pigs, but not to prevent the initial entry of virus, as reflected by high viral lung loads under experimental high dose infection. Despite this, it is another important target of vaccine-induced immunity[386]. Immunity to the M gene cannot suppress disease induction. There was evidence that disease was enhanced under conditions where only immunity to the M gene was conferred. This is consistent with the observation of "vaccine-induced enhancement of disease" observed with vaccines that do not contain protective HA+NA against the challenge strain[156,334] and also after immunisation targeted against HA2 (stalk of the hemagglutinin)[159] but not when there was still some cross-reactivity of the infecting

virus to one of the surface glycoproteins either HA or NA[155]. The observations were mainly made on the lung pathology (more extension of lesions)[334]. The effects of this process under very high dose infection conditions have not been reported in the literature. The results of the H3N1 challenge presented here may give an indication of possible interactions between non-homologous HA+NA vaccination and innate immune responses caused by other proteins of influenza viruses. Disease exacerbation has been demonstrated in ferrets infected with seasonal H3N2 virus and subsequently with $H1_{pdm}N1$ 2009 virus[387]. Early life H2N2 influenza virus infection has been shown to increase susceptibility to death in humans after heterosubtypic $H1_{pdm}N1$ influenza infection[388]. Peak mortality due to pandemic 1918 influenza was observed in people around 30 years of age who had been exposed to the 1890 H3Nx influenza virus[389-391]. There are several untested hypotheses aimed to explain this: i) T-cell mediated immunopathology[389,392], ii) antigenic imprinting[389,393], iii) long-term results of *in utero* or neonatal infection[389]. This monograph adds another hypothesis to be tested: induction of strong innnate immune responses in the lung by the M protein. This finding calls for reconsideration of the usefulness of vaccines containing only M protein. Influenza enhancement was also demonstrated for antibodies directed against the conserved stalk region of the HA[394] which is considered to be an approach towards an universal vaccine[395]. These antibodies can support faster and stronger infection of lung cells, inducing significantly higher cytokine levels, increasing pH-dependent HA trypsin sensitivity, disrupting the HA stem domain and promoting virus fusion[394]. The effects of broadly neutralising antibodies against the HA stalk domain, but also the response to the M protein and NP, may be less effective in adults and the elderly than in children because most adults have already been exposed to all seasonal influenza viruses, which prevents replication of FLUAV to high lung titres due to immune priming, allowing a more rapid immune response. Prior exposure to influenza viruses differing in their HA and NA may therefore also contribute to the severe outcome of influenza virus infections. Mortality rates during the 1968 H3N2 pandemic were not elevated in age groups previously exposed to H2N2 viruses, but were elevated in age groups primed by the 1918 heterosubtypic H1N1 viruses[390]. This may reflect the protective effect of the immune response against the neuraminidase which is similar in H2N2 and H3N2 viruses.

Further studies on the topic of antibody-dependent enhancement of disease are listed in the supplement. It was shown that in $H1_{pdm}N1$-immunised pigs that had been infected with $H1_{av}N1$ virus, enhanced lung lesions occurred in those individuals that had only low antibody titres against haemagglutinin and neuramidiase (Supplement chapter 13 – Enhancement of lung pathology within an antigenic supergroup ($H1_{pdm}$ versus $H1_{av}$); page CXXVII). This phenomenon occurred particularly with the use of vaccines that had only a weak adjuvant. This was not observed with vaccines that contained mineral oil adjuvants. However, this should not tempt to prefer vaccines with a strong adjuvant, because these have strong side effects (Supplement chapter 14 – Pyrogenic and virucidal effects of

mineral oils; page CXXXV). The effects of antibody-dependent enhancement of disease manifested themselves primarily in the lungs; clinically, the effects could be largely compensated for by the onset of faster immune responses in immunised pigs (Supplement chapter 13 – Enhancement of lung pathology within an antigenic supergroup ($H1_{pdm}$ versus $H1_{av}$); page CXXVII). However, clinical protection does not generally exist here. It has been shown that peracute progression of disease is possible in vaccinated pigs when the vaccine strain is different from the infection strain, even before lung changes become clearly manifest (Supplement chapter 15 – Stressing the innate immunity; page CXXXVII). On the other hand, such animal studies can also be used to test the accuracy of vaccine matching. When different influenza viruses are antigenically matched to each other, antibody-dependent enhancement of disease does not occur (Supplement chapter 16 – Testing the relationship between $H1_{pdm}N1$ and $H1_{cl}N1$ influenza viruses; page CXLV).

The phenomenon of vaccine-induced disease enhancement is not yet understood. The main basis for disease enhancement is the absence or low level of antibodies against the haemagglutinin head and neuraminidase in the presence of high levels of antibodies against the haemagglutinin stalk or M protein (or most likely other proteins from internal genes). This can also occur under intrasubtype immunisation-infection conditions, when the viruses are more antigenically distant and individuals have low levels of HA and NA antibodies against the infecting strain.

A hypothetical approach to explaining the mechanisms of vaccine-induced disease enhancement is shown in Figure 67. Enhanced disease can lead to severe and fatal cases in situations where the infected person has never been exposed to surface glycoproteins of the infecting virus, but shares an immunological imprint with internal proteins (for example, avian viruses that are not related to seasonal viruses). The best way to avoid vaccine-induced disease enhancement in seasonal influenza is to maintain antibodies against the major surface glycoproteins of circulating influenza viruses

The term "vaccine-induced enhancement of respiratory disease" can be misleading because it focuses on vaccination alone. This situation can occur not only after vaccination, but also after field infection. In fact, it is a general phenomenon independent of vaccination, but vaccination may contribute to it. Immunity is not stable. Usually, after stimulation, antibodies appear, rise to high titres, then titres decrease and remain at a low level or disappear after a certain time, depending on the antigen. After repeated exposure to an antigen, the immune response is higher and antibodies are more stable. As the major surface glycoproteins of influenza viruses are more variable compared to the more conserved structures, this can lead to imbalances in antibodies against the different antigens after repeated exposure to different viruses, as the response to the conserved structures is boosted more frequently than that to HA and NA, as the latter change from time to time. Therefore, the term immune imbalance (II) is more appropriate to characterise this phenomenon. II is a

condition in which antibodies against the conserved structures of influenza viruses are still at a high level, but the strong immunoprotection against HA and NA is absent or inferior. In these circumstances, immune protection against HA and NA is so low that it cannot compensate for the side effects of immunity against the conserved structures. This immunity against conserved structures is not as efficient as that against HA and NA. It targets the antigens expressed on infected cells, leading to irritation of these cells. This can trigger mediators released by the infected cells, which influence the alveolar macrophages and increase the mechanisms of innate immunity. This is particularly important in the first few days after infection. The importance of this mechanism is probably underestimated. It plays a role in seasonal and pandemic influenza. It may have contributed to the severity of the 1918 influenza (priming by H3Nx Russian influenza viruses and infection with H1N1 in middle-aged people). Thus, the severity of influenza is an expression of powerful processes of innate immunity in which host genetics are involved in addition to the degree of adaptation of the virus to the infected cell (IOG, Imbalance between the interaction of influenzaviral genes) and the balance of immunity of the infected individual (II, Imbalance of immune response to antigenically variable and conserved structures of the virus).

Vaccine-induced enhancement of disease (VAERD) = Imbalance of immunoresponse (II) may also occure under conditions of maternally-derived immunity when this immunity suppresses antibody induction to vaccination. Vincent et al., 2012[328], showed that the effects of imbalance of immunity are not observed when live vaccines are administered in contrast to inactivated vaccines.

For other viruses, the mechanisms of antibody-dependent disease enhancement are better understood. For coronaviruses (MERS-CoV), it has been shown that a neutralising antibody can bind to the coronavirus spike protein, triggering a conformational change of the spike and mediating viral entry into IgG Fc receptor-expressing cells through canonical viral receptor-dependent pathways[396]. The authors showed that this process is very complex because it requires a certain level of neutralizing antibodies to shift the balance between entry pathways; only intermediate doses of antibodies cause antibody-dependent enhancement of virus entry[396]. Similar processes contribute to antibody-dependent enhancement of human severe dengue. Here, the risk of severe dengue disease is highest with a narrow range of pre-existing anti-Dengue virus antibody titres[397].

The cell membrane and its receptors play a crucial role in the activation of signalling cascades of innate immunity and necroptosis. Non-specific mechanisms may also contribute to the activation of such cascades.

In addition to the functional activities of viral enzymes, antibodies may also play a role in this process. Antibodies are known to support viral entry via the Fc receptors, leading to antibody-dependent enhancement of disease. The results of this study suggest that the M protein may also be involved in such processes. It was evident that pigs vaccinated with

different vaccines, such as those with only an immune response against the M protein, developed large and severe lung lesions after influenza virus infection. Some pigs died around 5 dpi, one of them with bacteria in their lung tissue. The M protein accumulates near the cell membrane before budding. The ion channel penetrates the envelope and provides opportunities for antibody attack. Around 5 dpi, the antibody response is boosted if there has been a primary contact with the corresponding antigen in the life history of the individual. This is a time when the virus is declining but still active in the lungs after the initial infection. Thus, an increased antibody response to M could also contribute to a non-specific stimulation of cell membrane receptors, which could support signaling cascades leading to necroptosis and imbalances in the control of the local microbiome (Figure 68).

The studies by Kitikoon et al. (2009/2010)[164] appear to contradict the interpretations of a disease-enhancing effect of M2 antibodies, because both the pigs immunised with inactivated vaccine and M2 in combination and the pigs immunised with recombinant M2 alone had fewer lung lesions than the pigs immunised with inactivated vaccine alone. However, the differences between the pigs immunised with the inactivated vaccine alone and those immunised with the inactivated vaccine in combination with recombinant M2 were not significant. In addition, the combined vaccination strategy (inactivated + recombinant M2) appeared to suppress M2 antibody production, as the level was significantly lower in pigs receiving both vaccines compared to pigs receiving the recombinant M2 vaccine alone[164]. Of the total of 6 pigs immunised with recombinant M2 protein alone, one died on day 1 after infection with A/sw/Iowa/1930 ($H1_{cl}N1$) virus. It is possible that this animal did die as a result of antibody-dependent enhancement of disease at a time when severe lung lesions had not yet developed (see also Supplement chapter 15 – Stressing the innate immunity, page CXXXVII), in which a similar peracute course occurred after heterologous infection on 1 dpi). The animal was therefore absent from the assessment of lung lesions on day 5 after infection. In contrast, the study by Heinen et al. (2002)[165] found that after immunisation of pigs with a DNA construct expressing NP and M2 protein, the clinical picture in pigs became more severe after heterologous experimental infection. The authors assume that the cause is the immune response directed only at NP and M2. Jegerlehner et al. (2004)[160] demonstrated that M2-specific antibodies are non-neutralising, but contribute to virus reduction via antibody-dependent natural killer cell activity in the early stages of infection. These could also act on the membranes of infected cells that express M2 and stimulate the induction of cytokines. Expression of M2 protein on the surface of infected cells in the presence of non-neutralising anti-M2 antibodies, but in the absence of HA-neutralising antibodies, may induce cell membrane damage via antibody-dependent cytotoxic cell activity or complement fixation, thereby stressing innate immune responses in the infected cells. In another study, a universal peptide vaccine based on the M2 ectodomain did not protect against H1N1 infection in pigs[398].

Thus, aberrant αM2 immune responses can occur in two stages: i) in the presence of high immunity against M2 as early as 1 dpi, ii) in the presence of low immunity around 5 dpi, when the immune response against M2 increases due to the boost from infection.

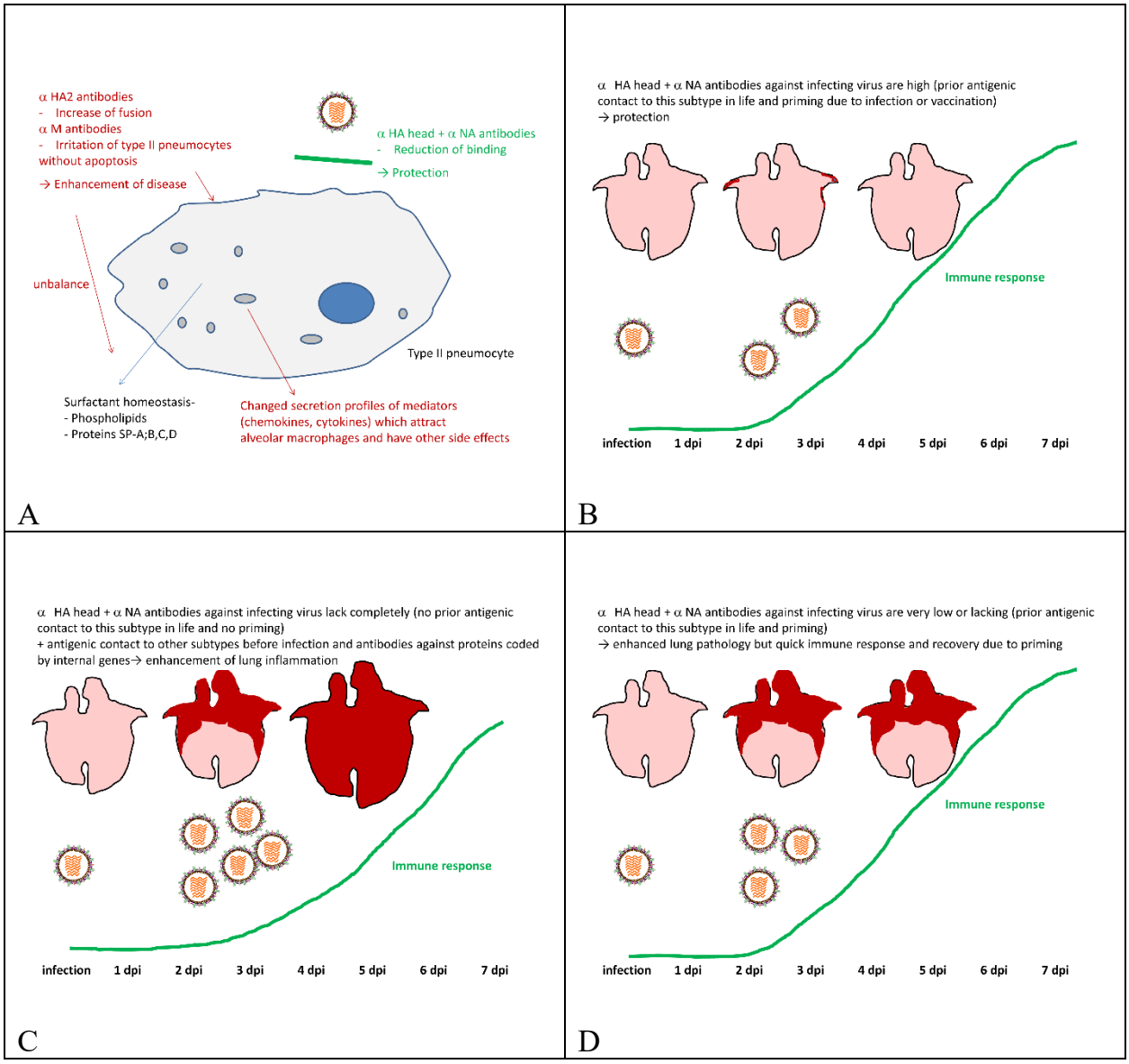

Figure 67: Model of vaccine-induced enhancement of disease

(A) Mechanisms involved in pathogenesis, (B) Protection by vaccination, (C) Severe enhancement of disease in heterosubtypic immunisation-infection conditions (individual had prior contact to influenza virus by field infection or vaccination but was never exposed to the major surface glycoproteins antigens of the infecting virus), (D) Enhancement of disease in homosubtypic immunisation-infection conditions (individual was exposed to influenza virus prior to the infection but has no or low antibody titres against the major glycoproteins of the infecting virus but high titres against HA stalk and/or Matrix protein or other proteins expressed by the internal genes of influenza viruses; the difference between C and D is the later induction of antibodies against HA and NA due to lacking priming resulting in more severe lung pathology as in primed individuals

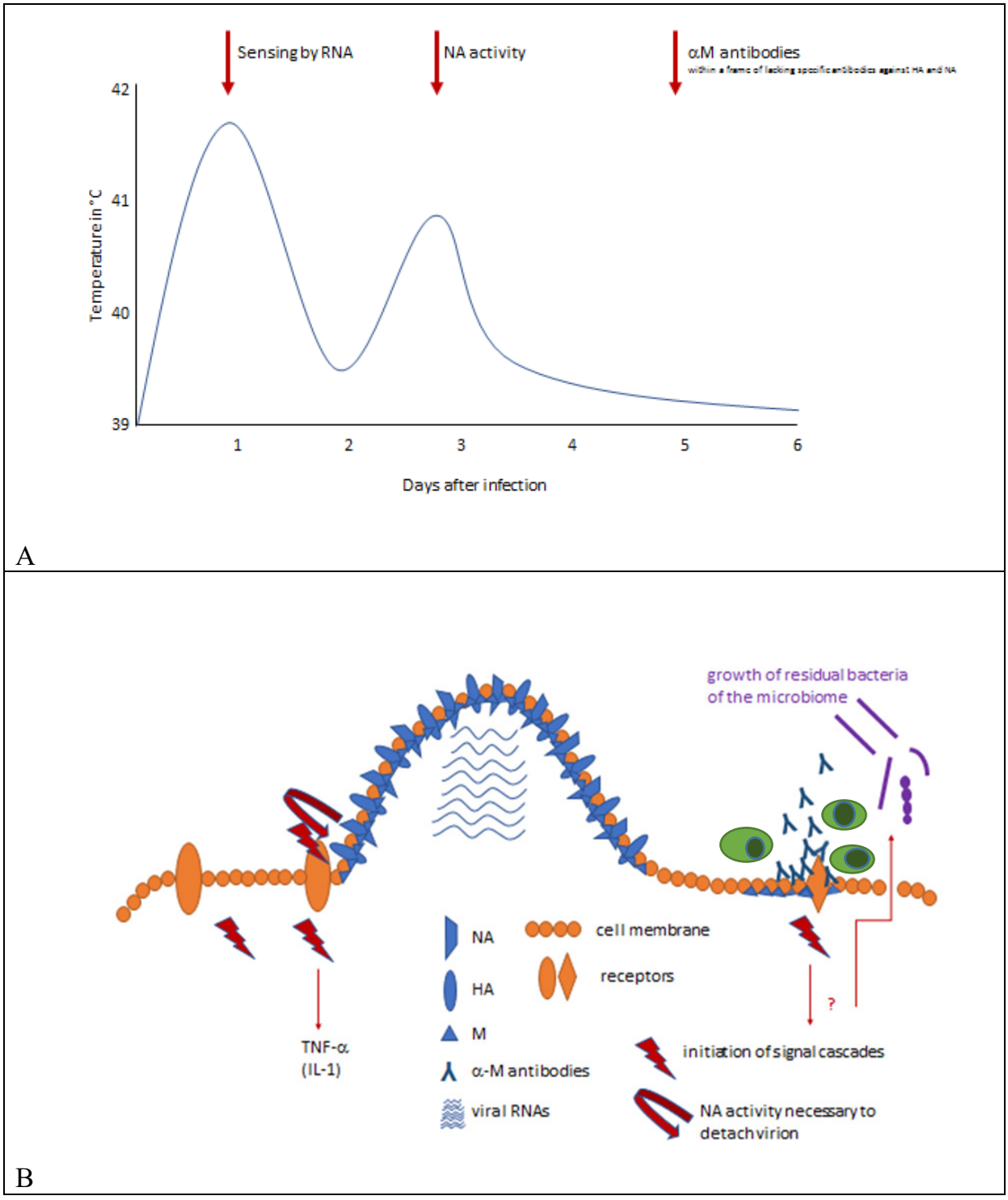

Figure 68: Phases important for induction of severe disease after FLUAV infection

A) There are three phases in the Influenza A virus infection cycle that are important for virulence: i) the first at 1 dpi, when viruses enter cells and their nucleic acids sense innate immunity, ii) at 3 dpi, when virion release from infected cells peaks (neuraminidase activity at virion detachment), and iii) at 5 dpi, when the first antibodies appear (this is only important if protective immunity against the two major glycoproteins, HA and NA, is lacking, but immunity against other proteins such as M is present). B) Hypothetical approach to mechanisms that may support virulence: Phase 2 – NA activity may indirectly stimulate adjacent receptors within the period on detachment, which may initiate signaling cascades of innate immunity and necroptosis at 3 dpi – Phase 3 – Antibodies directed against the M protein may also influence receptors leading to the initiation of cascades of substances that support bacterial growth or apoptosis or by induction of cytolytic T cells (the only occasion on which bacteria were detected in the lungs was in pigs with severe lung pathology due to antibody-mediated disease enhancement; this scenario is similar to observations from the lungs of the 1918 pandemic)

Later, immunity to the surface proteins becomes dominant and the enhancement in disease is compensated. Sudden death of vaccinated pigs after infection with influenza viruses remote from the vaccine virus was observed in the study reported in supplement (page CXXXVII), but also reported by Kitikoon et al. (2009/2010)[164]. Death at 5 dpi was observed in the study reported in section 5.1.6.1, page 76. In general, enhancement of disease is difficult to observe for the following reasons: i) only pigs genetically predisposed to higher innate immune responses are affected (≤25%), ii) high dose infection conditions are required, and iii) the process is quickly compensated by immunity against surface glycoproteins, haemagglutinin and neuraminidase.

These analyses focus on two mechanisms that support virulence: i) the properties of neuraminidase and its influence on immunity and necroptosis at the centre of pathogenesis. The second peak in fever induction at 3 dpi is likely to be associated with virion formation and budding from infected cells and may indicate a functional role for neuramidase activity in virulence by activating innate immunity and necroptosis cascades through non-specific stimulation of membrane receptors. Fever induction by infected macrophages through cytokines (IL-1, TNF-α) was proven in other studies [399,400], ii) immunity against the M protein in the absence of HA and NA antibodies, which may also be able to induce necroptotic processes.

6.1.5 HOST FACTORS CONTRIBUTE TO DIFFERENT OUTCOMES OF INFECTION

A constant proportion of about 20% (14-23%) of pigs with a lethal or severe course in the trials indicates a higher individual susceptibility to influenza virus infections. This proportion is close to that which would be expected according to Mendel's splitting rule for recessive inheritance (approx. 25%). These pigs all came from one farm and had a similar genetic background. The results of the different experiments are therefore comparable. This indicates that there are genetically fixed factors that predispose to an increased susceptibility to severe courses of influenza, presumably through an enhanced response of innate immunity.

6.1.6 THE LUNG LESION – KEY TO DECIPHER PATHOGENESIS

More than 3000 lung samples were examined. Lung lesions developed according to a specific profile (Figure 69). The marginal areas of the cardiac lungs are always affected first in influenza virus infections. The lesion remains confined to this area when low-virulent viruses (such as the $H1_{av}N1$ viruses of the 2000s) infect the lungs. This type of lesion is associated with little induction of respiratory symptoms. Then, lesions may extend toward the center of the medial lobes of the lung and also involve the apical portions of the apical and diaphragmatic lobes. This is associated with the expression of more severe symptoms.

This pattern is mainly caused by influenza A viruses $H1_{hu}N2$ and H3N2. Then, the lesions extend toward the center of the lung and cover almost the entire cardiac and apical lobes of the lung and one-third of the diaphragmatic lung lobe. This is associated with severe disease as caused by $H1_{pdm}N1$ April 2009 and newly reassortant $H1_{pdm}N2$ and H3N1 viruses. At this stage, the infection can be fatal. In certain individuals, it does not stop but quickly covers the entire lung, which is then twice as heavy as a normal lung. This picture is similar to that reported for the 1918 pandemic influenza. In repeated infection experiments with the April 2009 $H1_{pdm}N1$ virus, this picture could be induced by high-dose infection in approximately 20% of pigs.

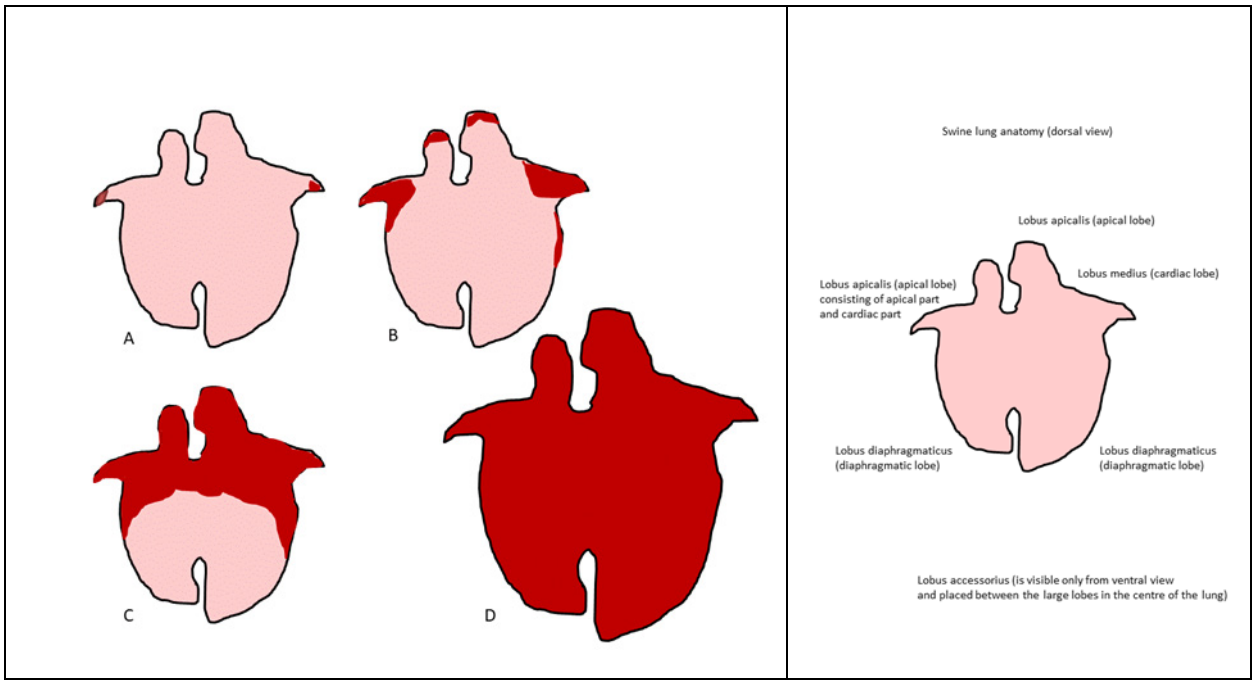

Figure 69: Progression of lung lesion development
left: (A) Always begins at the apical parts of the middle lobes (and stops here when low-virulent viruses infect the lungs), (B) Further expansion (and maximum expansion after infection with high-virulent influenza A viruses such as $H1_{hu}N2$ and H3N2, (C) Further evolution (induced by infection with highly virulent viruses – mostly newly reassortant viruses, (D) Terminal stage (reached in some individuals after infection with highly virulent viruses such as $H1_{pdm}N1$ April 2009 virus); Right: Anatomy of the porcine lung

Histologically, the lesion is characterized by large areas of mononuclear infiltrates composed mainly of aleveolar macrophages but also neutrophils. In these lung lesions, there is increased release of cytokines and other mediators, most likely by type II pneumocytes and activated immune cells. Higher levels of TNF-α can be measured in the lesion compared with adjacent regions without lesion. Oedema and vascular leakage are observed. When examining lesions of vaccinated pigs, the studies presented here demonstrated that in the marginal regions of the cardiac lobe of the lung, viral replication is barely reduced by vaccination compared with other lung regions. This allows the hypothesis that blood flow in these areas is insufficient to provide sufficient antibodies for local protection. Consequently, there are undersupplied areas of the lung where not only antibodies but also

nutrients are inadequately provided. Such undersupplied cells tend to increase activation of pathways of innate immunity during infections. Local inflammation leads to destruction of epithelial layers, oedema, and in severe cases, erythrocyte infiltration. The intensity of the inflammatory process within the first hours and days is crucial for the further course. Rapid progression of inflammation can lead to lung failure.

The higher susceptibility of the apical part of the lung lobes, especially the cardiac lobe, needs further investigation. The difference in viral load of individual vaccinated pigs in the lesions and in the adjacent area is striking. In the latter, despite the viral load in the adjacent region with lesion, almost no virus is detectable. In contrast, there is almost no difference in viral load between regions with and without lesion in unvaccinated pigs (Table 31).

Table 31: Analysis of differences between viral load in lung lesions of vaccinated and not vaccinated pigs

Vaccination status	Lesion	Neighboured region without lesion	Connecting link	Explanation
Vaccinated	High viral load	Low viral load	Circulation	Insufficient provision of antibodies in the apical parts of the lung lobe
Not vaccinated	High viral load	Viral load equal	Circulation	A lower supply of nutrients in the apical lobes of the lung leads to local stress conditions that sensitize innate immunity pathways

Therefore, sampling from regions with or without lesions does not make a difference in unvaccinated pigs but may be important in studying efficacy in vaccinated pigs. The relationship explaining the spread of lesions from the edges to the center could be a lower supply of nutrients, antibodies, and other components through the capillary network. Poor local supply of nutrients could make type II pneumocytes more vulnerable to stressors. Increased cytokines are released during infections, leading to infiltration of large numbers of mononuclear cells. The higher level of cytokines such as TNF-α in lung lesions compared to lung areas without lesion was demonstrated in this study. Local differences within the lung have also been found in other investigations[135].

TNF-α secretion and other cytokines were found to be significantly higher in lung areas with lung lesions than in areas without lesions. Lesions were larger in lungs infected by the more virulent strains, and the more virulent viruses induced a second peak in fever, which may be related to neuraminidase activity because it coincides with the time expected for budding in the infection cycle. Thus, the investigations of this study add two factors to the common knowledge: i) the virulence of influenza viruses is determined by their degree

of adaptation of neuraminidase to the host cell and to haemagglutinin, and ii) this process is quantitative in nature. An attempt was made to apply this knowledge to the epithelial cells of the upper respiratory tract, where loss of cilia was observed *in vitro*[401]. Histological examination of experimentally infected pigs showed loss of cilia only in small areas of the tracheal epithelium, but the large accumulation of debris in the bronchioli and in bronchi suggests a high degree of necroptosis of infected cells and a severely altered function of the epithelial layer. Bronchioli obstruction may also contribute to the severity of the disease and the difficulty in obtaining sufficient oxygen, as reflected by dsypnoea. Porcine airway epithelial cells revealed a different pattern of loss of cilial cells when $H1_{pdm}N1$ 2009 and 2010 viruses were used for infection in comparison to $H1_{pdm}N1$ viruses of 2014 and 2015[401]. The basis for this loss is still unknown. To reconcile this observation with the results of the animal studies, a hypothesis was developed (Figure 70). Influenza A viruses replicate in bronchial and alveolar epithelial cells and infection spreads to resident alveolar macrophages. The process of inflammatory leukocyte recruitment is initiated by the release of cytokines (TNF-α, IL-1) and chemokines (CCL2, CCL3/4, CCL5, CXCL8, CXCL10) from epithelial cells and alveolar macrophages[80,402-410] of which CCL2 and CCL5 are major monocyte chemoattractants[411,412]. It was shown by Herold et al (2006) that alveolar epithelia cells direct monocyte transepithelial migration after influenza A virus infection and that the interaction between alveolar epithelial cells and resident alveolar macrophages enhances transepithelial monocyte migration by TNF-α secretion of alveolar macrophages[413]. Therefore, in this work the focus was put on the investigation on TNF-α.

Proinflammatory cytokine levels correlate with the severity of highly virulent influenza virus infection in animals and humans[414-416]. A strong proinflammatory cytokine response may contribute to fatal outcomes[417]. Experimental infections with swine influenza viruses demonstrated significant correlations between the levels of cytokines like TNF-α/IL-6 and clinical signs[81] (Table 32). Interleukin-1β (IL-1β), interleukin-1α (IL-1α), and tumor necrosis factor alpha (TNF-α) are the main proinflammatory cytokines that mediate the host response to infection[418,419]. Investigations on TNF-α-receptor- and IL-1-receptor-knockout mice revealed that tumor necrosis factor receptor (TNFR) signaling increased the severity of $H1_{pdm}N1$ 1918 influenza virus infection whereas IL-1R1 signaling was largely protective indicating that TNF-α signaling may be a key regulator of pathogenesis[251]. Imbalances in the signaling cascades including the suppression of downregulation of TNF-α to promote a balanced anti-inflammatory state contribute to severe outcomes of infection[251]. It is possible that the as yet not well-characterized signaling cascades triggered by neuraminidase activity support such imbalances toward TNF-α. It is also possible that the neuraminidase activity only affects the TNFR and that the compensatory countermeasures induced by the IL-1 receptor are missing. Alveolar epithelial cells are sensitive to T-cell-triggered, TNF-α-mediated apoptosis[420]. In a fatal pneumonic mouse model of influenza TNF-α was implicated in lung inflammation and gross lung lesions, weight loss and mortality[421,422]. In

human volunteers experimentally infected with influenza virus TNF-α and IL-6 were most strongly associated with symptoms[423,424].

Table 32: Selected references reporting activation of major cytokines in pigs after experimental infection with European swine influenza viruses

Cyto-kine	Function[300]	Described in pigs	Infection route	Virus subtype
TNF-α	- essential mediator of inflammation - activates and attracts mast cells, macrophages, lymphocytes, neutrophils, vascular endothelium - stimulating effects on neutrophils - triggers changes in vascular endothelial cells (microvascular thrombosis, capillary leakage) - involved in apoptosis - alters lipid metabolism - activates adhesion molecules - induces acute phase proteins - activates procoagulants	Van Reeth et al. (1999)[79]	Intratracheally	$H1_{av}N1$ 1983
		Deblanc et al. (2020)[425]	Intratracheally	$H1_{hu}N2$
		Van Reeth et al. (2002)[81]	Intratracheally	$H1_{av}N1$ 1998, $H1_{hu}N2$, H3N2
		Barbé et al. (2011)[426]	Intratracheally	$H1_{av}N1$
		Pomorska-Mól et al. (2014)[135]	Intratracheally	$H1_{av}N1$ 2004°
			Intratracheally	
			Intratracheally	
IL-1	- acts on vascular endothelial cells to make them adhesive for neutrophils - escapes into the bloodstream during severe infection and causes fever, lethargy, malaise, loss of appetite by acting on the brain, mobilization of amino acids causing pain and fatigue by acting on muscle cells, induces acute phase proteins by acting on liver	Van Reeth et al. (1999)[79]	Intratracheally	$H1_{av}N1$ 1983
		Van Reeth et al. (2002)[81]	Intratracheally	$H1_{av}N1$ 1998, $H1_{hu}N2$, H3N2
		Pomorska-Mól et al. (2014)[135]	Intratracheally	$H1_{av}N1$ 2014
IL-6	- is stimulated by TNF-α, IL-1 or bacterial endotoxins and is a major mediator of acute-phase reaction and septic shock - increases mucin secretion in goblet cells	Van Reeth et al. (2002)[81]	Intratracheally	$H1_{av}N1$ 1998, $H1_{hu}N2$, H3N2
		Pomorska-Mól et al. (2014)[135]	Intratracheally	$H1_{av}N1$ 2004
		Deblanc et al. (2020)[425]	Intratracheally	$H1_{hu}N2$
IFN-α	- type I interferon; glycoprotein with antiviral activity - enhances the neutrophil respiratory burst	Van Reeth et al. (1999)[79]	Intratacheally	$H1_{av}N1$ 1983
		Deblanc et al. (2020)[425]	Intratracheally	$H1_{hu}N2$

° the year was provided here because $H1_{av}N1$ viruses of the 2000s are less virulent than earlier $H1_{av}N1$ viruses; following aspects of the publications are of interest: Van Reeth et al. (2002) showed a signification correlation between cytokines and severity of influenza in pigs; Pomorska-Mól et al. (2014) demonstrated differences in cytokine expression within the lung lobes (more in the medial lobe); and Deblanc et al. (2002) found higher amounts of TNF-α in pigs infected with a new variant of $H1_{hu}N2$ virus

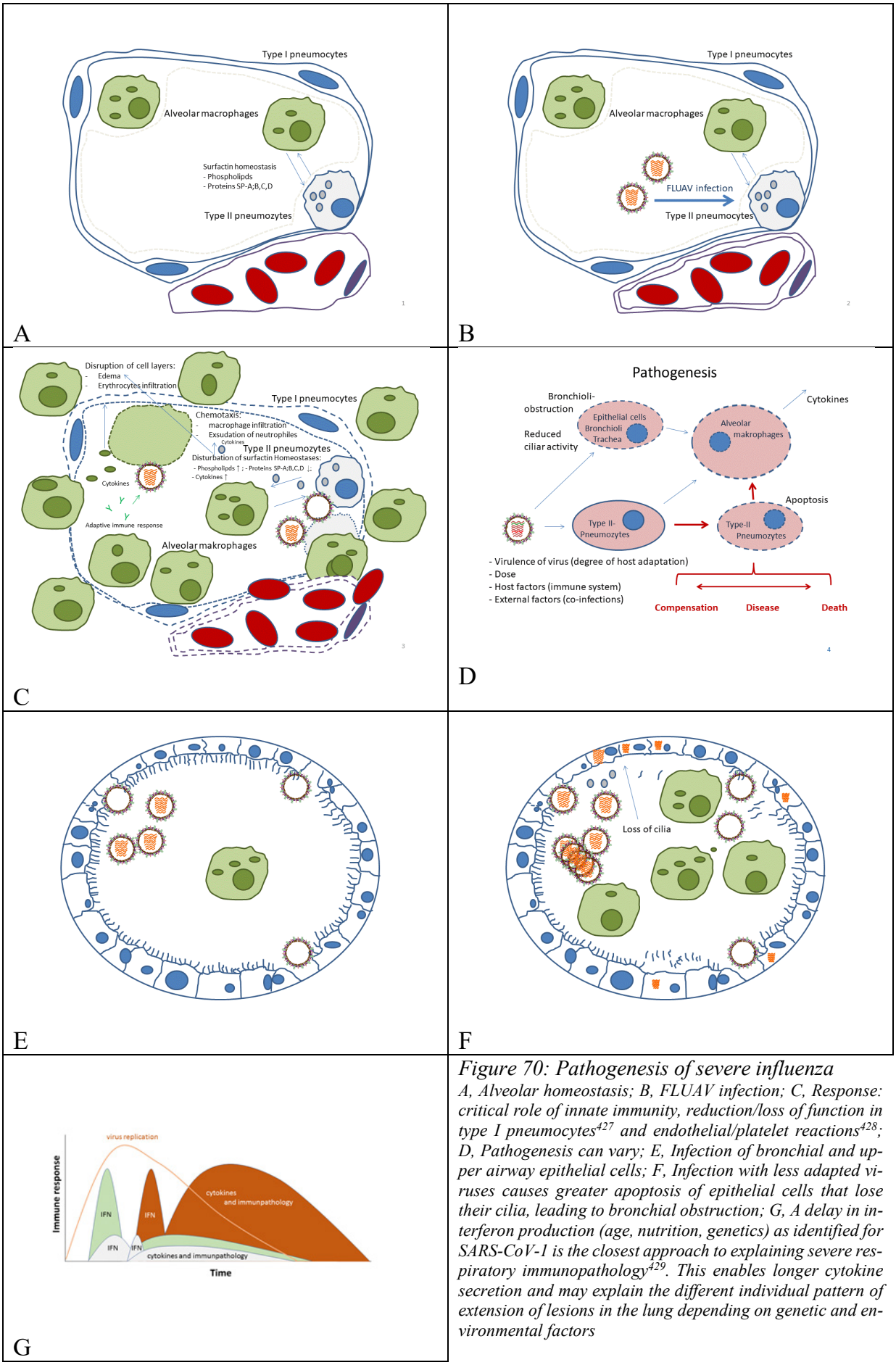

Figure 70: Pathogenesis of severe influenza

A, Alveolar homeostasis; B, FLUAV infection; C, Response: critical role of innate immunity, reduction/loss of function in type I pneumocytes[427] and endothelial/platelet reactions[428]; D, Pathogenesis can vary; E, Infection of bronchial and upper airway epithelial cells; F, Infection with less adapted viruses causes greater apoptosis of epithelial cells that lose their cilia, leading to bronchial obstruction; G, A delay in interferon production (age, nutrition, genetics) as identified for SARS-CoV-1 is the closest approach to explaining severe respiratory immunopathology[429]. This enables longer cytokine secretion and may explain the different individual pattern of extension of lesions in the lung depending on genetic and environmental factors

The fact that in vaccinated animals there are clear differences in the viral load between unchanged areas of the lung and areas with macroscopically visible lesions, but not in unvaccinated animals, while both groups tend to have lesions in the apical areas of the lungs, especially in the cardiac lobes, points to anatomical peculiarities of these regions. The apical location suggests special features in the supply of these areas. An increasingly poorer supply of antibodies and nutrients to distal areas could reflect similarities between the two groups. The supply status of the cells could have an influence on their cell metabolism and on greater sensitisation to the activation of mechanisms of innate immunity. Delayed IFN-I signaling has been shown to influence disease severity after SARS-CoV-2 infection in mice[429]. This was because delayed IFN-I signaling allowed viruses to replicate to higher titres, and subsequently the delayed peak in IFN-I led to the accumulation of pathogenic monocytes and macrophages, resulting in lung immunopathology, vascular leakage and inadequate T-cell responses[429]. Delayed IFN-I signaling may also be the reason for the peripheral emergence of lung lesions in the course of changed cell metabolism due to local supply bottlenecks (Figure 70 G).

6.1.7 INITIAL INFECTIOUS VIRAL LOAD, HOST ADAPTATION OF VIRUS AND INDIVIDUAL AS WELL AS ENVIRONMENTAL FACTORS DETERMINES SEVERITY OF DISEASE

In the previous chapters, the crucial role of the initial infective dose in the lung (6.1.1), differences in virulence between influenza viruses (6.1.2), and the influence of individual and environmental factors (6.1.3 and 6.1.4) were presented. The severity of the disease is mainly associated with quantitative factors resulting from the overreaction of infected cells and secretion of biochemical substances such as cytokines due to high viral replication. However, viral and host factors also contribute to the severity of influenza. The following major factors have been identified as contributing to the severity of the disease: infectious dose, virulence of the virus, and host-related as well as environmental factors (Table 33).

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Table 33: Overview of factors that contribute to the severity of illness after influenza virus infection

Factor	Reason
Infectious dose	Numerous dose titration studies have clearly shown that there is a linear relationship between the amount of virus in the airways and the severity of disease.
Virulence of virus	In particular, reassorted HxN1 viruses were more virulent but rapidly evolved to lower virulence, whereas HxN2 viruses were more stable in virulence, in agreement with *ex vivo* studies: The replication pattern of $H1_{pdm}N1$ viruses differed between highly virulent and less virulent viruses: the highly virulent viruses grew to higher titres in precision-cut pig lung slices[287,401]. Highly virulent viruses caused greater loss of ciliated cells (reduced thickness of the epithelial layer) and reduced ciliary activity in the epithelial layers of bronchial cells in precision-cut porcine lung slices compared to low virulent viruses, indicating stronger effects on apoptosis[287,401]. Virulent viruses such as H3N2 induced an increase in RIG-I, IFN, IFN1, Mx1, OAS1, PKR, IL-6, SOCS1 in porcine precision-cut lung slices, indicating activation of the interferon signaling cascades[288]. These data suggest that the interferon signaling cascades are sensed differently by HxN2 and the newly reassorted HxN1 viruses, with the inflammatory and apoptotic pathways being sensed more intensively, while HxN1 viruses are able to adapt to less intense sensing of these cascades. NA activity of FLUAVs correlates with the ability to induce IFNα and INFβ[374]. NA treatment of cells with recombinant NA induces production of IL-1 and TNF-α[375]. NA converts transforming growth factor-β (TGF-β) from the latent form to an active form sufficient to induce apoptosis[376]. Although the molecular mechanisms and pathways involved in NA-induced signaling are not known, these data underline the importance of NA in the induction of cascades leading to necroptosis[380]. Only a few mutations were identified when comparing highly virulent to less virulent $H1_{pdm}N1$ viruses, but no unique mutation was found to be responsible for changes in virulence (see chapter Genetic characterisation of influenza A viruses differing in virulence in the supplement, page CX). This suggests that other factors may also be involved in stimulating innate immune responses, such as NA activity, which may be influenced by morphological factors such as position on the virion and interaction with HA.
Host factors	Approximately 20% of pigs died or developed more severe disease than their littermates after infection with highly virulent influenza viruses in independent studies, suggesting a genetic background to this increased susceptibility to infection (which could be due to a different pattern of innate immune response). The individual immune status can influence the course of influenza, especially if there is no immunity to the surface glycoproteins, but immunity to internal proteins such as M is present, it can lead to increased symptoms in some individuals.
Environmental factors	Comparative investigation of lung lesions and neighboured lung regions without lesion revealed that additional factors other than viral load might influence inflammation; the fact that apical lung regions are more frequently altered hints to limitations in supply and supports the hypothesis that nutrition may influence cell metabolism and the reaction pattern of innate immunity to infections (stronger in cells with deficiencies).

In simple terms, there are four factors at work in the induction of influenza which are interrelated (Figure 71). The initial infectious dose is a crucial factor here. High initial exposure can lead to the development of virulence if host and/or environmental factors favour an overreaction of the innate immunity. This shows that the interplay of all components is very complex and explains the difficulties in understanding the pathogenesis of influenza, especially that of severe influenza (such as the 1918 influenza). On the other hand, it also shows that effective preventive measures include preventing high initial exposure to influenza viruses. The importance of the initial infection dose was extensively elaborated in this study.

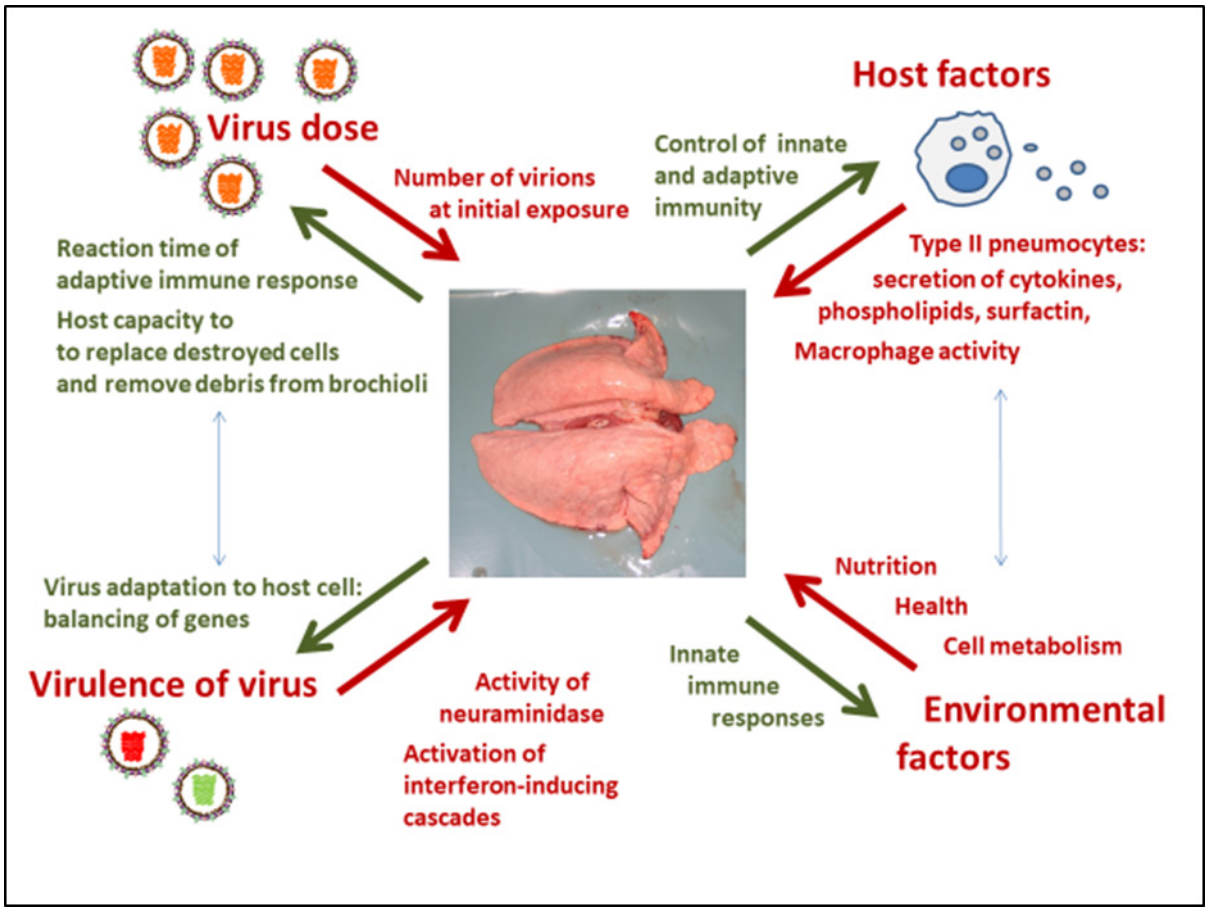

Figure 71: Simplified scheme of the factors contributing to the severity of influenza
Viral and counteracting host factors are involved; an initial high viral load in the lung is crucial for induction of severe influenza; i.e. in the absence of a high initial load there is no severe disease, but in the presence of a high initial viral load the combination of one of the other factors can lead to severe disease (either virulent virus or host cell overreaction); note that type II pneumocyte reactivity may also be influenced by neuraminidase activity of the virus and environmental factors like nutritional deficiency and other factors influencing cell metabolism

6.1.8 Conclusions for the analysis of pandemics

The differences in disease outcome between different doses of highly virulent viruses were as strong as those between highly and low virulent viruses in high-dose infection when high doses were compared with low doses (especially at 4 lg $TCID_{50}/m^3$ there are not many differences between the viruses, compare with Figure 62, page 121). This means that the initial dose into the respiratory tract is crucial for the clinical outcome and can have an impact on the fatal or non-fatal course of the disease. Even highly virulent viruses were unable to induce severe influenza when low doses were used for infection. The quantitative nature of this process indicates that the host is able to counteract initial low-dose infections. The rapid action of the immune system (the first effective countermeasures are detectable as early as 3 dpi, as indicated by a decrease in lung viral loads in the respiratory tract from 3 dpi) limits the time available for efficient replication of influenza viruses. This means that respiratory viruses can only escape host acquired immunity by evolving into rapid replication patterns. The exponential growth of viruses means that the initial infectious dose critically influences the viral load that respiratory viruses can establish within the

short replication period available. This was particularly evident during the SARS-CoV-2 pandemic in 2000/2021, when countermeasures such as the wearing of face masks, general hygiene and improvements in air hygiene (better ventilation, keeping distances) significantly reduced the burden of respiratory infections in the human population[430]. The immune system responds immediately to exposure to antigen, regardless of the infectious dose. Exposure to low doses of virus at first infection stimulates an immune response that is strong enough before viruses can replicate to high viral loads in the respiratory tract that can cause disease. A correlation between infectious dose and disease severity as well as cytokine induction has been described in previous studies in pigs experimentally infected with influenza virus[82].

To reduce the opportunities for high initial replication, prophylaxis of severe influenza should focus mainly on reducing this process, which can be achieved by i) vaccination, ii) early antiviral treatment, and iii) air hygiene. The latter is often neglected. Sitting in overcrowded lecture theatres, attention to the lecture is often reduced due to lack of oxygen. Forgetting to open the windows from time to time also means that high viral loads can be generated when infected people are crowded together. It is therefore advisable to open the windows frequently, both in hospital rooms and in meeting rooms.

Similarities to severe $H1_{pdm}N1$ 2009 influenza observed in pigs in this study have been seen in human patients after H5N1 virus infection[431]. Autopsy samples of such patients revealed diffuse alveolar damage[432]. High viral replication was associated with high levels of chemokines and cytokines[414]. Elevated levels of proinflammatory cytokines were seen in human alveolar and bronchiolar epithelial cells[433-435]. Experimental infection of non-human primates with H5N1 virus led to severe disease targeting type II pneumocytes and macrophages associated with secretion of high levels of inflammatory cytokines[436,437].

To this day, it is difficult to assess the potential of influenza viruses in terms of disease severity. Why was the 1918 influenza so damaging? Why was the 2009 influenza only mild in humans? This knowledge would be important in assessing the potential of viruses to cause human epidemics and pandemics. Based on the studies reported in this monograph, three main factors have been identified (see Table 34, page 146): i) The virus must be able to replicate efficiently in the respiratory tract of its respective host to provide high-dose infection conditions and basic immunity in the population infected is low. This mechanism may explain the severity of the 2017/18 B/Yamagata influenza. Influenza virus circulation is mainly dominated by A viruses. B viruses often follow in the circulation after FLUAV circulation has declined. FLUBVs thus cause a second, usually lower, peak in the season. In 2017, a situation arose where there was still strong baseline immunity to both influenza A viruses ($H1_{pdm}N1$ and H3N2) in the human population at the beginning of the season, which prevented their circulation. As a result, FLUBVs had free rein and were able to circulate rapidly. At that time, there was little basic immunity to B viruses in the human

population. Both of these factors support rapid and high infestation associated with high-dose infection conditions. This may explain the high excess consultation rate observed in in 2017/18 in Germany[438].

Table 34: Major factors that could be involved in severity of influenza (pandemic and seasonal)

Factor[1]	Pig	Human	Human	Human	Human	Human
	Experi-mental H1N1 Pan-demic April 2009	Moderate H1N1 Pandemic 2009	Severe H1N1 Pandemic 1918	Severe H2N2 Pandemic 1957	Severe H3N2 Pandemic 1968	Severe B/Yama-gata season 2017/18
Excess mortality*	-	2.9	598.0	40.6	16.9	25.4
Malnutrition	-	-	+	-	-	-
Imbalance of genes of a newly reassorted FLUAV	+	+	+	+	+	-
Imbalance of immunity (no antibodies against HA +NA but to other proteins)	-	**(-)**	++	+	**(-)**	-
High-dose infection conditions due to rapid spread of the virus	+	**(+)°**	+	+	+	+

* per 10000 persons/year according to Morens et al. (2009)[439], Dawood et al. (2012)[440], Nielsen et al. (2019)[441]; excess mortality for pigs is not available, in experimental trials mortality in pigs was 20% which is very high; the pigs were immunologically naïve, thus, no imbalance of immunity could occur; ° this was less important in the Northern hemisphere because the virus emerged in spring which limited the spread of the highly virulent spring virus; in the autumn wave the virus had already adapted and exhibited lower virulence; [1] there are adaptive mutations in PB2 (supporting transmission between birds and mammals as well as mutations in NP which can evade host interferon responses but these are no specific markers of virulence)

ii) A major contributor to severity is the functional imbalance of viral genes resulting from reassortment of influenza viruses because it can lead to altered functional patterns, such as changed neuraminidase activity, which redirect innate immune signaling cascades in a more TNF-α dominated direction leading to necroptosis. This imbalance may differ between reassorted viruses. Several reassortment events have been observed in the evolutionary history of human seasonal influenza viruses[442]. Human influenza pandemics and seasonal epidemic events reflected different excess deaths from any cause, which were high after the emergence of new viruses or intrasubtypic reassortment[439]. Both factors, the ability to replicate in the new host and the functional imbalance due to insufficient adaptation, were present in the $H1_{pdm}N1$ April 2009 virus used in the experimental aerosol porcine model and led to a mortality of about 20% in infected pigs. It is likely that the virus was not yet adapted to human and porcine cells and required increased activity of its neuraminidase to release virions from the new membrane. The further evolution allowed the virus to improve the functional balance of its genes and host membrane components after lots of passages in the new host and to reduce its virulence. Why seemed the $H1_{pdm}N1$ virus to be less virulent than the 1918 virus? It may be that a critical factor in the severity of the disease

was excluded because most people had previous exposure to seasonal H1N1 viruses, which may have some cross-reactivity with the $H1_{pdm}N1$ neuraminidase and affect neuraminidase activity, although too low to affect infection. This cross-reactivity could also have prevented antibody-mediated enhancement of disease. In addition, the virus was facing a much denser human population, which allowed it to pass through and adapt more quickly. And the general conditions in the human population were much better than in the post-war years 1918-1920. The $H1_{pdm}N1$ 2009 virus did not initially spread through the pig population. Due to the circulation of viruses with similar surface glycoproteins, there was still immunity in parts of the pig population. The risk of an imbalance in immunity was low, which is the third factor involved in the severity of the pandemic. iii) The imbalance in immunity in humans with respect to the $H1_{pdm}N1$ 2009 virus is difficult to assess. It is possible that there was still immunity to N1 neuraminidase in humans that could have prevented the negative effects of this imbalance. Immunity to more conserved regions of the virus, in the absence of the protective effects of antibodies against the HA head and NA, targets and stresses infected FLUAV antigen-presenting cells, leading to overreactions in innate immunity.

The 1918 pandemic was susceptible to all of these factors. Recent analyses have shown that human H1 emerged from an avian source around 1901 (1895-1907) but before the most recent common ancestor of the pandemic and seasonal lineages around 1907 (1903-1910)[391]. The latter virus was the ancestor of at least two lineages: i) the 1918 pandemic H1N1 viruses ($H1_{pdm}N1$ 1918) and the classical swine influenza viruses that resulted from the transmission of $H1_{pdm}N1$ 1918 viruses to pigs, and ii) the pre-pandemic H1 virus (probably H1N8), the HA of which was later incorporated into the seasonal H1N1 viruses of humans[391]. The HA-encoding segment is the only one in the $H1_{pdm}N1$ 1918 virus genome with uracil content in the human range indicating that the H1 was already adapted to human cells[391]. All other segments of the $H1_{pdm}N1$ 1918 viruses were derived from avian viruses shortly before 1918[391]. By the 1910s, several H1 viruses were circulating in humans. They had already adapted to replicate strongly in the human upper respiratory tract. Adults aged 20 to 40 years in 1918-19 who had experienced the H3N8 pandemic of 1898-1893 but had never been exposed to H1N1 may have had immunity against proteins of the ICG like the M protein but not to H1N1[391]. In case of H1N1 infection this could lead to antibody-mediated enhancement of disease due to imbalance of immunity. The $H1_{pdm}N1$ 1918 virus most likely also reflected an imbalance of genes due to recent reassortment, although the timing of this reassortment event is difficult to prove. According to the results of this study, such reassortment should have occurred between the spring and autumn influenza waves of 1918, because the spring wave was moderate but the autumn wave was very severe, indicating the emergence of a virus with an imbalance in its genes. Worobey et al. (2014)[391] calculated that the reassortment between pre-pandemic H1 viruses circulating in humans and avian viruses, which provide the other seven segments in addition to HA, occurred

shortly before 1918. Due to the lack of viruses from this period, it is impossible to determine the exact nature and timing of this reassortment.

The aerosol model allows evaluation of the virulence of influenza viruses via infection-dosistitration series. Viruses such as the 2009 $H1_{pdm}N1$ virus are among the most virulent mammalian influenza viruses, causing more severe dyspnoea, pulmonary lesions, and a higher mortality rate than the other influenza A viruses circulating in pigs. These viruses, although mild in most cases of infection, can reach their full virulence potential when they reach extremely high titers in the lungs. Three conditions can lead to high lung titers: i) high initial infectious doses, ii) delayed immune responses that allow expansion of otherwise very limited replication of the viruses in the lungs, iii) coinfection with other pathogens and resulting synergistic effects. Scenarios with these conditions have been discussed for the 1918 pandemic: a high dose may have been possible in crowded army camps[443,444], on navy ships[445] in diamond mines[446] but also in inuit houses. The particular situation during World War I and shortly thereafter may have supported the weakening of the immune system due to exhaustion, injury, malnutrition, and other factors[443,447]. Bacterial co-infections were found frequently[448-450]. Pregnant women as well as immunocompromised patients were most vulnerable in both pandemics[451-453]. The studies show that 2009 $H1_{pdm}N1$ viruses are special in terms of virulence. The 1918 and 2009 pandemics were both caused by H1N1 viruses. After circulation in the population, pandemic H1N1 viruses have lost virulence, as shown by infection trials with $H1_{pdm}N1$ viruses isolated in 2014/2015. The high virulence of the 2009/2010 $H1_{pdm}N1$ viruses upon high-dose aerosol infection and the loss of virulence after a few years of their circulation in humans and pigs suggest that this virus was truly novel to humans and pigs and only later had adaptation of these reassortants to their hosts occurred. One explanation could be that the gene segments of the newly reassortant viruses were not yet balanced and therefore not yet very well adapted to the host cell systems. The viral neuraminidase seems to play a special role in this process. A necroptotic pathway is known, although not yet investigated, to be activated by influenza viral neuraminidase, leading to enhanced secretion of cytokines[454]. Different conformations of the surface proteins of freshly reassortant H1N1 viruses may allow for different patterns of neuraminidase activity compared to adapted viruses. It is known that parts of the viral envelope originate from the host cell[455]. These could have an impact on the anchoring of surface proteins and explain adaptive changes[456]. In this regard, there appear to be marked differences in the flexibility of N1 and N2 neuraminidase, because HxN2 viruses are more fixed in their virulence and do not show as large adaptive differences between early and late viruses as HxN1 viruses. Differences in virulence of $H1_{pdm}N1$ 2009 influenza A viruses have also been observed in ferrets. Ferrets infected with early $H1_{pdm}N1$ viruses from Mexico expressed more cytokines, severe disease, and pathology compared with viruses isolated later[457]. Adaptation of $H1_{pdm}N1$ viruses in the short period between 2009 and 2010 was also confirmed in infecting mice[364].

Although infection with FLUAV is usually benign, the consequences of high initial exposure can be catastrophic. Understanding these differences is important in terms of pathogenesis and prevention. Paulo et al. (2010) hypothesised that the influenza infectious dose may explain the high mortality of the second and third wave of the 1918-1919 influenza pandemic[458]. A high viral load in the lungs can lead to severe disease, as demonstrated by the 2009 $H1_{pdm}N1$ virus infection trials in pigs presented here. Infections of pigs with the $H1_{pdm}N1$ 1918 virus reported by Weingartl et al. (2009) did not lead to severe disease[73] because no high-dose infection was performed. The higher virulence of the 1918 and 2009 $H1_{pdm}N1$ viruses and the H5N1 viruses has been confirmed in several studies[91,105,205,247,440,459-461].

It is possible that 1918 $H1_{pdm}N1$ viruses do not differ from early 2009 $H1_{pdm}N1$ viruses in terms of virulence. The differences described in humans, and in particular the higher lethality in the 20-40 age group in the 1918 pandemic, may be due to antibody-dependent enhancement of disease in the course of immune priming by a virus that contained a matrix protein related to the 1918 $H1_{pdm}N1$ virus circulating before 1918 and that did not share any of the surface glycoproteins of the 1918 $H1_{pdm}N1$ virus. As in the H3N1 infection trial in pigs, this may have induced more severe pulmonary lesions. It could be that the antibody-dependent effects target matrix proteins accumulated in the infected cell membrane prior to budding, stimulating pathways that lead to potent cytokine release. In 2009, the population had already had contact with H1N1 viruses, and some baseline immunity was present, particularly to N1. The global population was much larger in 2009. Thus, the $H1_{pdm}N1$ 2009 virus was able to adapt more rapidly than in 1918.

In this study a higher virulence was demonstrated for three newly emerged viruses: $H1_{pdm}N1$ of 2009, $H1_{pdm}N2$ of 2010, and H3N1. Imbalance of surface glycoproteins resulting from fresh reassortment and the virus envelope which contains host components of a new host could be drivers in virulence.

6.1.9 VIRUS GENE EXPRESSION IN CELLS OUTSIDE OF THE RESPIRATORY TRACT

The activity of alveolar macrophages in the lung is enormous and continues after the period of active infection (Figure 72).

During the course of influenza virus infection, mononuclear cells distribute viral components throughout the body. As a result, viral antigen may be expressed in most tissues of the body. This is the reason for irregular PCR-positive reactions in other organs outside the respiratory tract such as the brain, heart, and others when diagnostics are used to examine organs other than the respiratory tract. The distribution of influenza virus components in different tissues can have pathological consequences if the inflammatory reactions against the antigens expressed there are too strong (encephalitis, myocardial infarction, myositis,

rhabdomyolysis, nephritis[462-465]). Although wide-spread cell lysis does not occur outside respiratory tract because the appropriate proteases are not present in these cells to cleave haemagglutinin efficiently and thus the viral replication cycle cannot be completed, some viral proteins can obviously be expressed there.

Ocular inoculation of influenza viruses can also spread the viruses throughout the body and induce respiratory disease, but the disease is milder because the immunological responses occur before the viruses can replicate to high titres in the respiratory tract[219,220,466]. Unlike seasonal viruses, ocular inoculation of highly virulent influenza viruses (H5N1) can cause fatal disease in ferrets[221].

Studies by *in situ* hybridization confirmed the restriction of the nucleoprotein to the respiratory tract. Viral RNA was found in bronchial and alveolar epithelial cells and in some macrophages within small foci of inflammation after 1 dpi. Three days after infection, viral load was lower in bronchial epithelium and almost absent in alveoli, whereas inflammation in the lungs apparently increased. At later stages (14 dpi), no signals against NP but considerable inflammation were present in the lungs. No signal with probes directed against NP was detected in the brain or other organs at any time point.

In contrast, antigen expression was found in cells of almost all organs. Most signs of antigen expression were associated with cells of the immune system, but antigen expression was also observed in other cells: neurons, epithelial cells of the intestinal tract and glands, and the kidney (Figure 25, page 63, Figure 73) and the heart (Figure 74, Figure 75). A polyclonal antibody was used for immunohistological staining. Thus, it can only be concluded that influenza virus genes were expressed in these cells, but not which ones. The following morphological changes were associated with the corresponding organs: brain - perivascular infiltrates and leptomeningitis, spleen - hyperemia and nephritis, intestine - mucosa-associated lymphoid tissue hyperplasia and edema of gastrointestinal epithelial cells, tonsils - hyperplasia of germinal centres and atrophy of the parafollicular area, liver - activation of Kupffer cells and multifocal endothelial nodules, lymphnodes - increased mitotic index, heart - infiltration and destruction of cardiomyocytes. No symptoms associated with these histological findings were observed in the pigs.

Despite this expression of antigens there was no staining by *in situ*-hybridization (probe directed against NP). No infectious virus was found in the corresponding tissues. This suggests infection of the cells, but incomplete replication within the cells. Macrophages from lung lesions could be a transport vehicle for viral elements (Figure 76).

One possibility would be that only fragments of the surface proteins bind to the cells, thereby triggering immune reactions. On the other hand, genetic material is also sometimes detected in infected tissues outside the respiratory tract, which is more in favour of the uptake of genetic material from the virus. Another possibility is that after internalisation,

influenza viruses are trafficked to late endosomes but the process stops here. In addition, the uniform expression of antigen in histological images of such cells is more likely to indicate active expression of influenza virus genes by cells outside the respiratory tract.

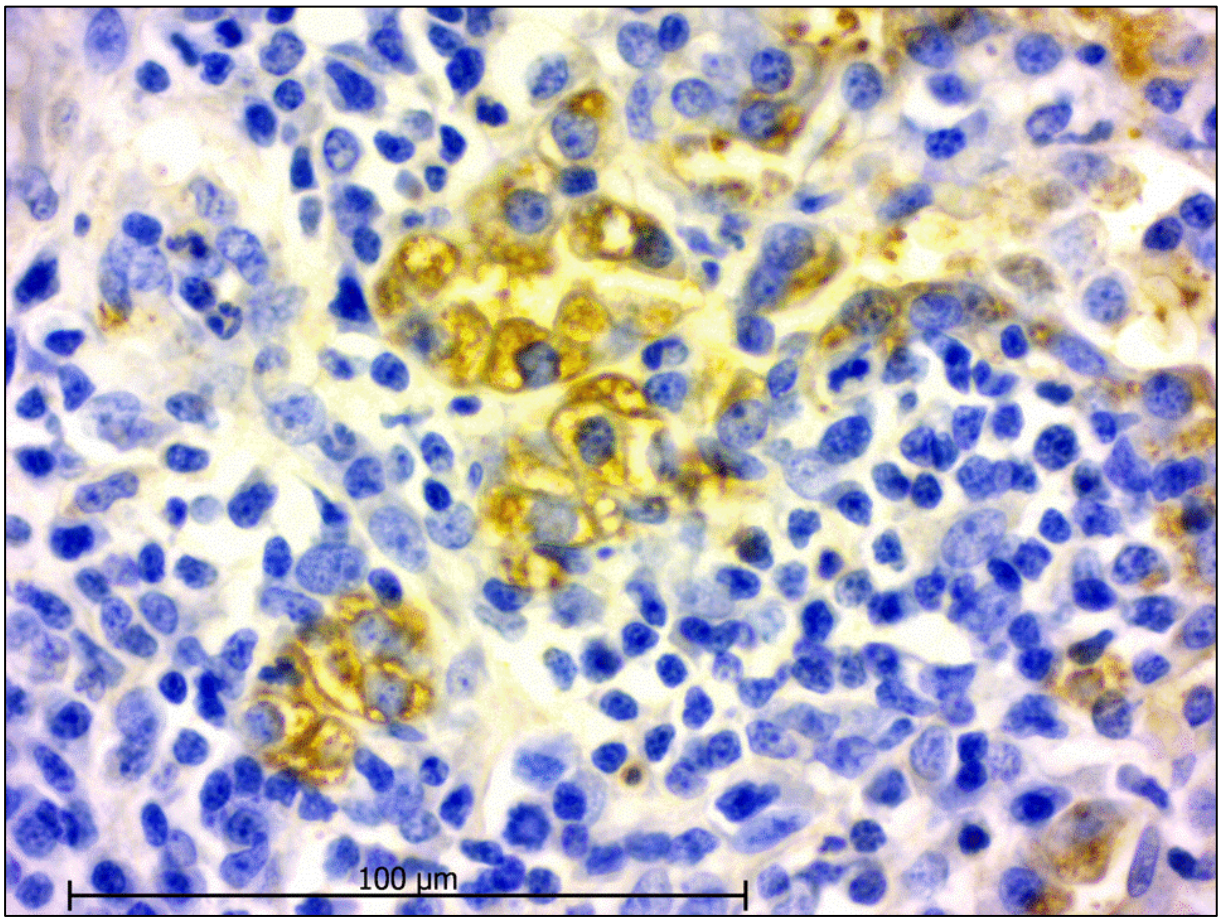

Figure 72: Role of macrophages in the immunopathology of influenza
Large numbers of mononuclear cells accumulate in the lung interstitium; influenza virus-specific staining (SABC) of alveolar macrophages (brown) indicates uptake of viral antigens and the enlargement of macrophages indicates phagocytic acitivty; Oedema is seen around the macrophages: oedema is reflecting membrane lesions due to high cytokine activity; lung of pig 9 dpi after infection with FLUAV A/Hamburg/NY1580/2009 $H1_{pdm}N1$; at this time (9 dpi) there is no infectious virus any more in the lung; thus, the virus-specific staining indicates residual viral antigen

The binding of FLUAV to cells requires the presence of receptors. Avian FLUAVs bind stronger to sialic acid α2,3-galactose linked receptors whereas human FLUAVs prefer sialic acid α2,6-galactose receptors[467,468]. Both receptors are present in porcine organs such as trachea, lung, heart, skeletal muscle, brain, liver, kidney, spleen and intestinal tract[147,148]. The distribution of sialic acid receptors is similar in pigs and humans: the sialic acid α2,6-receptor is the predominant receptor throughout the respiratory tract whereas sialic acid α2,3-receptors are not detected in the upper respiratory tract but in epithelial cells of bronchioli and alveoli at lower level than α-2,6[148]. The wide distribution of these receptors within the body indicates that a lot of tissues are susceptible for virus entry. Sialic acids and glycans are found on almost all cells. It is possible that influenza viruses bind to all sialic acids, but more efficiently to α-2.3 or α-2.6, depending on the virus. For viral infectivity and spread, proteolytic activation of HA is essential. The mature hemagglutinin

consists of two subunits: the globular subunit HA1 (head) and transmembrane unit HA2 (stalk). HA1 facilitates membrane binding to sialic acid receptors whereas HA2 mediates membrane fusion. In order to get this membrane fusion potential the precursor hemagglutinin HA0 has to be cleaved by cellular membrane-bound proteases[469-472]. This can take place in different compartments: in the trans-Golgi network or at the cell surface. It can also occur at different points in the viral life cycle: during transport of HA to the plasma membrane, during budding, or during attachment and entry into a new cell. The HA0 of LPAIV and mammalian FLUAVS can be cleaved *en route* to the cell membrane (proteases such as TTSPs, transmembrane serin-like proteases such as TMPRSS2, TMPRSS4) or upon insertion into the cell membrane (by human airway trypsin-like protease HAT). Cleavage can also occur after incorporation of HA0 into virions by soluble proteases such as plasmin or by serine proteases in endosomal vesicles of infected cells (for review see Bertram et al. (2010)[473]). Proteases like HAT are present in cells outside of the respiratory tract like in the brain[474,475]. An alternative cleavage mechanism has also been discussed[476]. The cleavage of the FLUAV hemagglutinin by host cell proteases is essential for virus infectivity[473]. The HA0 of HPAIV is cleaved in the Golgi apparatus by proteases of the subtilisin family like furin or PV6.

Proteases that allow the cleavage of HA with a monobasic cleavage site are TMPRSS2, TMPRSS4 and HAT. While TMPRSS2+4 are found in various organs, HAT is common in the respiratory tract (for details see Böttcher-Friebertshäuser et al. (2013)[471]). Infection of cells without suited proteases can result in progeny with uncleaved HA[471]. This progeny is unable to infect cells expressing only TMPRRS2. Bacterial soluble proteases can help to activate HA proteolytically, but can also augment cellular proteases[471].

The expression of antiviral antigens outside the respiratory tract indicates that influenza viruses can easily overcome some hurdles, that they can bind to glycans at will, and that they can also achieve, albeit inefficient, cleavage of haemagglutinin by host cell proteases. Lack of nucleoprotein synthesis or low levels of nucleoprotein formation below the detection limit indicate blockages in the subsequent replication process (see also Figure 80 C in volume 1 of this monograph[1]). Myxoprotein, which can inhibit the formation of ribonuclein complexes, may play a role in this. There may be differences in Mx1/MxA activity between airway cells and other tissues.

Many other host- and virus-related factors contribute to virus replication[477,478]. The process of FLUAV replication outside the respiratory tract is not well understood and requires further investigation. Viral genetic material and antigens were observed outside the respiratory tract in humans infected with H5N1 influenza virus and viral antigens were observed in pigs infected with influenza viruses, consistent with the distribution of sialic acid receptors in these organs (Table 35, page 156).

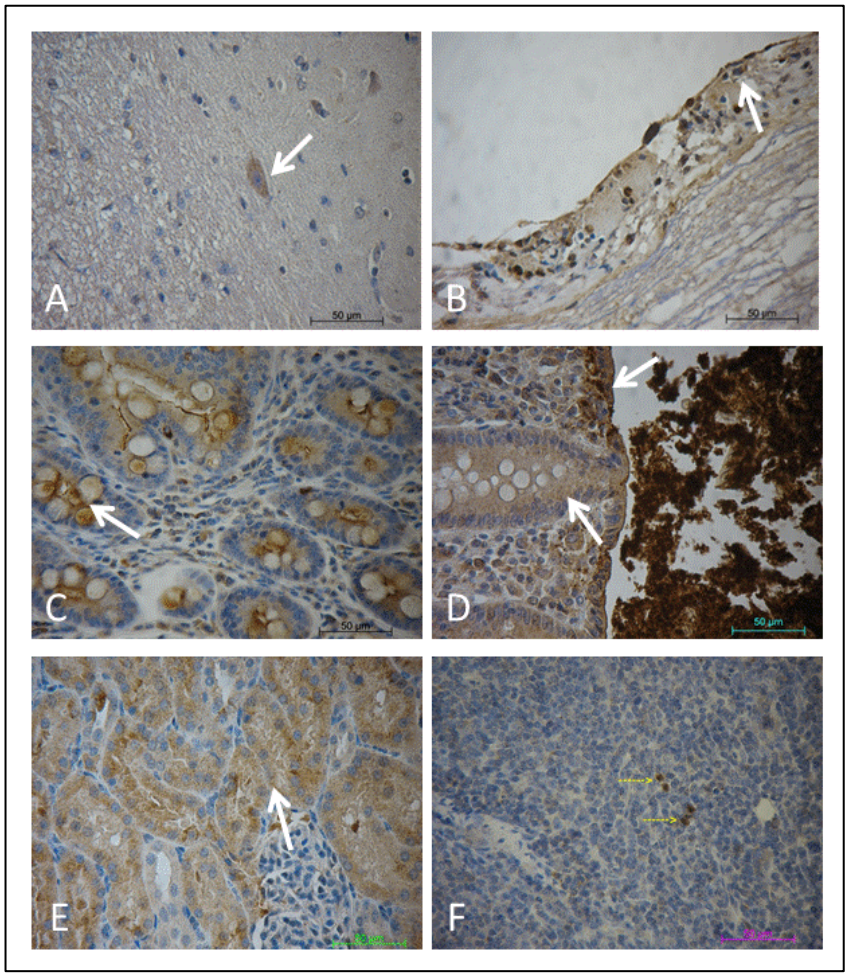

Figure 73: Expression of viral genes (arrows) outside of the respiratory tract after high-dose $H1_{pdm}N1$ infection of pigs, SABC method

(A) Cornu ammonis: specific staining of neurons, (B) Spinal cord: leptomeningitis and virus-specific staining of microglia cells, (C) Specific staining of Lieberkühn glands in the small intestine, (D) Specific staining of epithelial cells of large intestine, (E) Specific staning of epithelial cells in kidney tubuli, (F) Specific staining of macrophages in the lung lymphnode

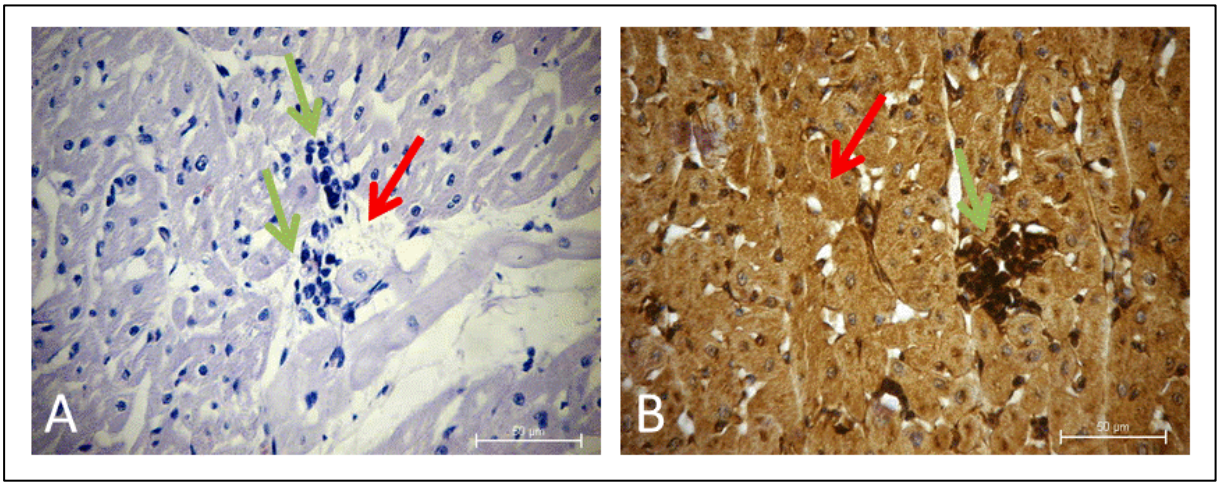

Figure 74: Multifocal cellular infiltration between cardiomyocytes of pigs caused by G1 H1N2 virus

(A, green arrows) and destruction of cardiomyocytes (A, red arrow); virus-specific staining of cardiomyocytes (B, red arrow) and macrophages (B, green arrow) 3 days after infection of a pig with influenza virus A/Ploufragan/0113/2005 (G2 H1N2); A, HE staining; B, SABC staining

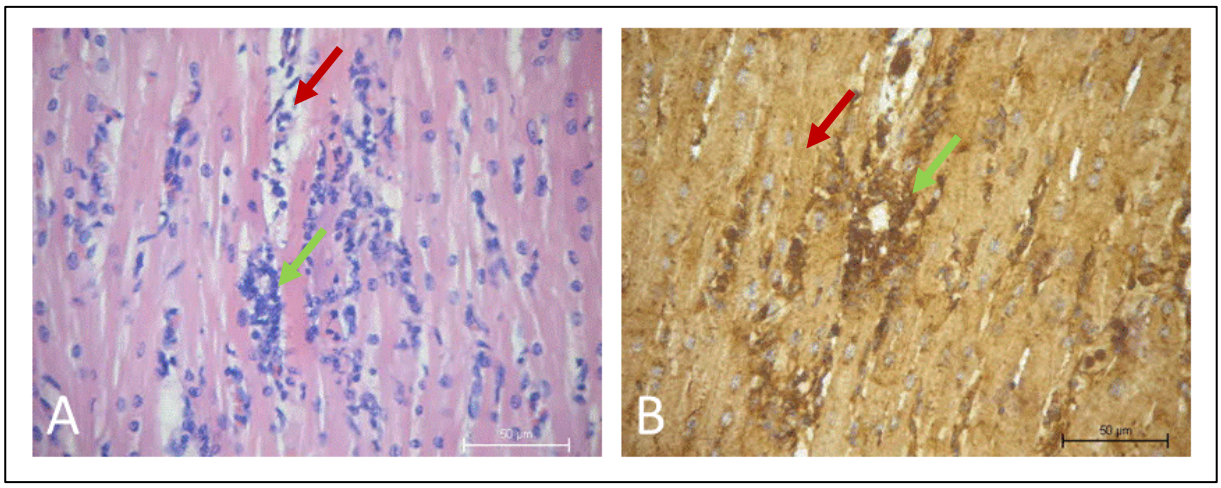

Figure 75: Multifocal cellular infiltration between cardiomyocytes caused by $H1_{av}N1$ viruses
(A, green arrows) and loss of striation and destruction of cardiomyocytes (A, red arrow); virus-specific staining of cardiomyocytes (B, red arrow) and macrophages (B, green arrow) 3 days after infection of a pig with influenza virus A/Ploufragan/0070/2005 ($H1_{av}N1$); A, HE staining; B, SABC staining

For influenza A and/or B viruses, persistent infection of human lung cells and cell cultures (MDCK, MDBK, HeLa cells) *in vitro* has been reported[479-481]. This has been achieved by infection with low doses of influenza virus (multiplicity of infection <1)[481], by selection of unlysed cells within the infected cell population)[479], or by the use of defective and temperature-sensitive viruses[480]. The majority of these persistently infected cells contained the complete viral genome and expressed viral antigens[480]. It can be assumed that the systemic distribution of influenza virus in the body corresponds to a low-dose infection in the body. The possibility of dormancy due to persistence of viral genes in infected cells was discussed in part 1 of this monograph[1].

Striking differences in synthesis profiles of avian and mammalian influenza viruses were described by Bogdanow et al. (2019)[482]. In these studies, the matrix protein M1 was inefficiently produced during non-permissive infection due to excessive splicing of the avian influenza virus M1 to alternative transcripts[482]. The results of this unbiased proteomic analysis indicated that differences between permissive and non-permissive influenza virus infection are due to differences in viral synthesis[482]. It is possible that similar mechanisms are at work at the level of the host cell in those cells that are thought to be non-permissive for influenza outside of the respiratory tract. These cells allow viral replication in a non-permissive manner, stopping replication at a step that does not lead to the release of infectious virions but to the synthesis of viral products.

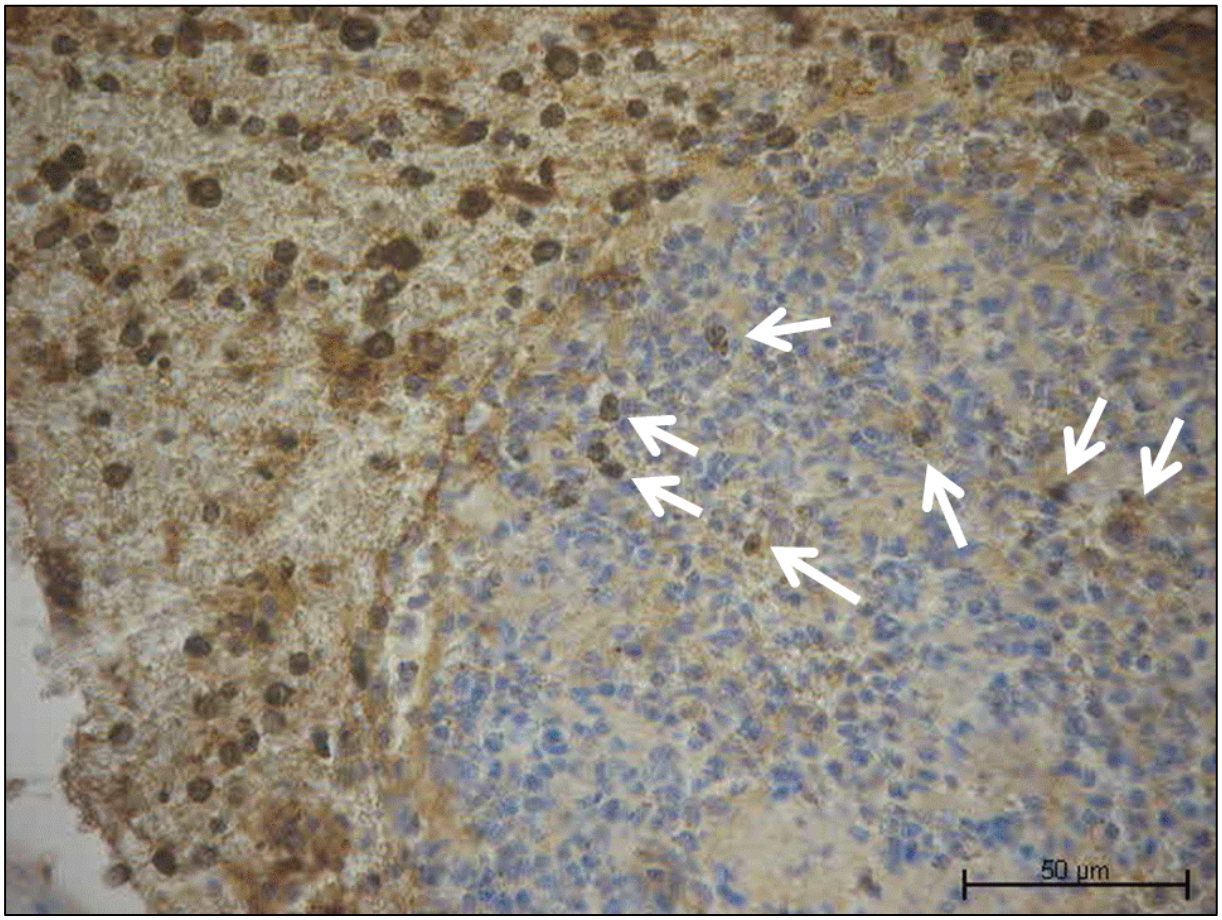

Figure 76: Markant virus-specific staining of macrophages in a capillary of a kidney of a pig
infected with influenzavirus A/Ploufragan/0214/2006 (G1 H1N2) demonstrates that macrophages can be a vehicle for transportation of influenza viral elements through the body

Influenza virus infections are systemic infections without viremia that invade many organs throughout the body and cause multiple disorders, although the virus replicates efficiently only in the respiratory tract, where it can cause overt respiratory disease (Figure 77). It maybe possible that this ability to infect many cell types allows viruses to gain access to cells (i.e. lymphoid cells) where they can remain dormant and replicate after the host's immune system is compromised, following stimulation of the cells by other infections, or following reinfection.

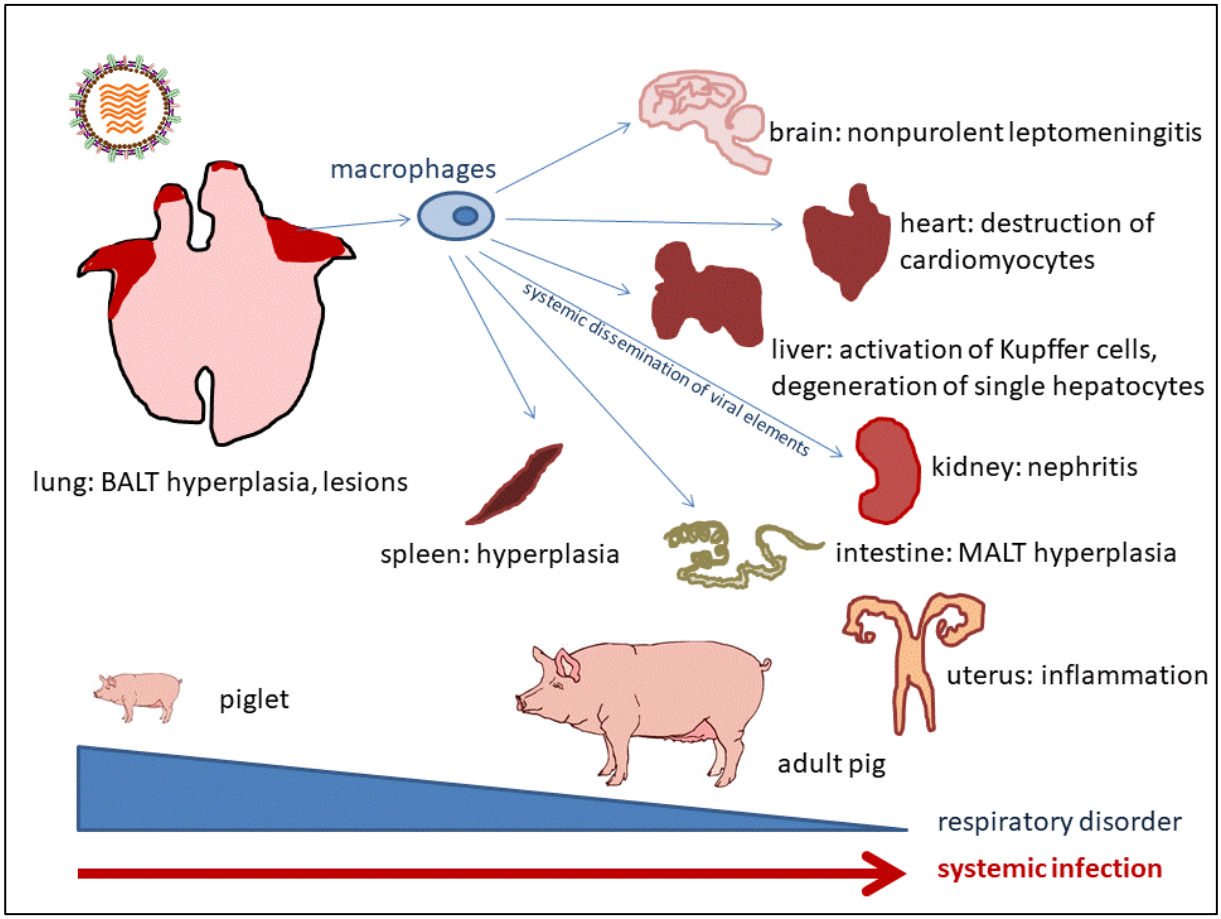

Figure 77: Influenza viruses cause systemic reactions although the virus only replicates efficiently in the respiratory tract

Table 35: Organ distrubition of influenza virus-specific receptors and influenza viruses described in humans and pigs

Refe-rence	Technique	Respirat. tract	Heart or skelatal muscle	Spleen	Liver	Kid-ney	Lnn.	Intestine	Brain	Placen ta	Fetus
Gu et al. 2007[432]	*In situ* hybr. (HA + NP)	+	-	-	-	-	-	+	+	+	+‘
H5N1 in humans	IHC (HA + NP)°	+	-	-	-	-	-	-	+	+	+’
	PCR H5	+	+	+	+	+	+	+	+	+	n.d
	Histology	+	-	-	-	-	-	-	-	-	-
Nelli et al. 2010[147]	IHC (SNA lectin) SAa2,6-Gal	+	+	+	+	+	n.d.	+	+	n.d.	n.d.
Sialic acid re-ceptors in pigs	IHC (MAA II lec-tin) SAa2,3-Gal	+	+	+	+	+	n.d.	+	+	n.d.	n.d.
this study $H1_{pdm}N1$ in pigs	IHC[1]	+	+	+	+	+	+	+	+	n.d.	n.d.
	In situ hybr. (NP)	+	-	-	-	-	-	-	-	n.d.	n.d.

+ positive for influenza viral signals, - negative, *In situ* hybr., *In situ* hybridization, ° NP was mainly detected in the nucleus and HA in the cytoplasm, ‘ in the respiratory tract of the fetus, [1] anti $H1_{pdm}N1$ polyclonal rabbit serum, Lnn. Lymphnodes

6.1.10 INVESTIGATION OF ANTIVIRALS (NEURAMINIDASE INHIBITORS) – REVEALS THE HIGH-DOSE PIG MODEL AS AN IDEAL METHOD

The data provided here adds to the knowledge already published[88]. The data show that neuraminidase inhibitors can reduce viral shedding, lung lesions and mortality in infections with highly virulent viruses such as the $H1_{pdm}N1$ viruses isolated before May 2009. Neuraminidase inhibitors are most effective shortly after infection because of this effect on viral replication kinetics. In patients who are not immunocompromised, it is not necessary to use antivirals for more than 7 days because antibodies to the infectious virus appear about 7 days after infection and eliminate the virus.

6.1.11 IMMUNOSUPPRESSIVE TREATMENT – PROLONGS VIRAL SHEDDING

Despite individual impairments in symptoms, lung inflammation and body weight development, immunosuppressive treatment did not promote severe influenza in pigs, but inhibited antibody formation and prolonged virus shedding. The immunosuppressive treatment in the pig study was done as a pre-exposure approach, meaning that the treatment was started one day before the experimental infection.

The phenomenon of prolonged influenza virus shedding is frequently observed in human patients[483]. These patients recover from influenza but shed virus, suggesting that despite late induction of antibodies, the virus is unable to induce the high viral load effects that would be expected if viral replication was not hampered by immune responses[483].

6.2 ANALYSIS OF COMPONENTS OF MATERNALLY-DERIVED IMMUNITY

The results are summarized in Table 36. Maternal immunity suppresses the serological response to immunization long after the disappearance of detectable maternal HI antibodies; despite this interaction, the immune system of piglets with maternal immunity is primed by immunization, resulting in a rapid immune response to infection regardless of the absence of antibodies; in pigs immunized in the presence of maternal antibodies, synergistic effects of immunization and maternal immunity on disease suppression are observed, although virus replication is not prevented.

Table 36: Overview of major findings on maternally-derived immunity

Findings	Major points of discussion
Maternal immunity and prevention of disease	Maternally derived antibodies decline steadily after birth and persist for 4-12 weeks, depending on colostral uptake, immune status of the sow and virus subtype (Figure 54, page 102) Effective disease prevention through maternal immunity has been demonstrated for for H3N2, $H1_{hu}N2$ and $H1_{av}N1$ influenza viruses (page 160) Despite protection, virus shedding is not limited by maternally derived immunity; therefore, infected pigs can shed high levels of virus in the absence of symptoms (Figure 61, page 116 I)
Interactions of maternal immunity with antibody response to immunisation	Vaccine-induced antibodies can be blocked by maternal immunity in individual pigs up to 8 months after birth (Figure 55, page 103, pages 161 - 162) In spite of this blockade, vaccination primes the immune system, resulting in a more rapid response to infection compared to animals that are not vaccinated (Figure 59, page 108) The blockage is released by an infection, results in quick antibody response and recovery from disease (Figure 59, page 108)
Simultaneous effects of vaccination and maternal immunity	Piglets can be immunised shortly after birth irrespective of the presence of maternal immunity (pages 113 - 116, pages 160 - 162) There are synergistic effects between vaccination and maternal immunity (Figure 61, page 116)

6.2.1 MATERNAL IMMUNITY AND PREVENTION OF DISEASE

Disease induction is necessary to demonstrate the efficacy of maternally derived immunity after infection. The best effects are seen when aerosol infection is tuned towards a very high dose leading to disease induction. The limited availability of suitable infection models has limited the study of the protective effects of maternal immunity.

The protective character of maternally-derived antibodies has been investigated in several studies[31,33-35,63,316,317,484]. Piglets with maternal antibodies had significantly fewer symptoms and lower viral lung loads, which is consistent with the results of the studies presented here. Kitikoon et al. (2006) did not observe effects in a group vaccinated late in maternal immunity[317]. The pigs had been vaccinated in the 3rd and 5th week of life and were infected intratracheally in the 7th week of life. The profile of this group suggests that maternal immunity was exhausted at the time of infection. Pigs responded with a large increase in antibody to infection, confirming the priming effect of vaccination. Loeffen et al. (2003) observed clinical protection in piglets with maternal antibodies, but discussed negative effects of maternal antibodies on growth performance[65]. However, the authors ignored the fact that there were already significant differences in body weight between the groups at the start of the study. Loving et al. (2014) reported a vaccine-associated enhancement of disease in pigs vaccinated to maternal immunity following challenge with a virus heterologous to the vaccine antigen[330].

The results of this study include:

i) Maternally-derived antibodies protect piglets against disease,

ii) Immunisation into maternally-derived immunity primes the immune system from 3 days after birth onwards and this priming leads to a rapid and high serological response following infection,

iii) Immunisation and maternally-derived antibodies act synergistically,

iv) Pigs that do not respond to vaccination with antibodies are partially protected despite the lack of seroconversion: they do not perform as well as vaccinated pigs with antibodies, but better than unvaccinated controls,

v) Maternal immunity interferes with the antibody response to immunisation for much longer than maternal HI antibodies are detectable.

6.2.2 INTERACTIONS OF MATERNAL IMMUNITY WITH ANTIBODY RESPONSE TO IMMUNISATION

The interference of maternally derived immunity with seroconversion in the absence of measurable antibodies has already been the subject of discussion by Menšik and Pokorny (1971)[34]. The authors observed that the inhibition of antibody production was still present at 15 and 22 weeks after birth, at which time maternal antibodies were no longer serologically detectable. The data from the studies reported here show that this inhibitory effect can persist for half a year after birth (up to eight months after birth in some individuals). It is important to note that the priming of the immune system and the generation of memory cells are not affected by this inhibition (Figure 78). Although more sensitive methods may be able to detect antibodies for longer, it is unlikely that it is antibodies that inhibit the serological response to influenza virus antigens under maternal immunity because this interaction takes a really long time and the antibodies have disappeared by then (Figure 78).

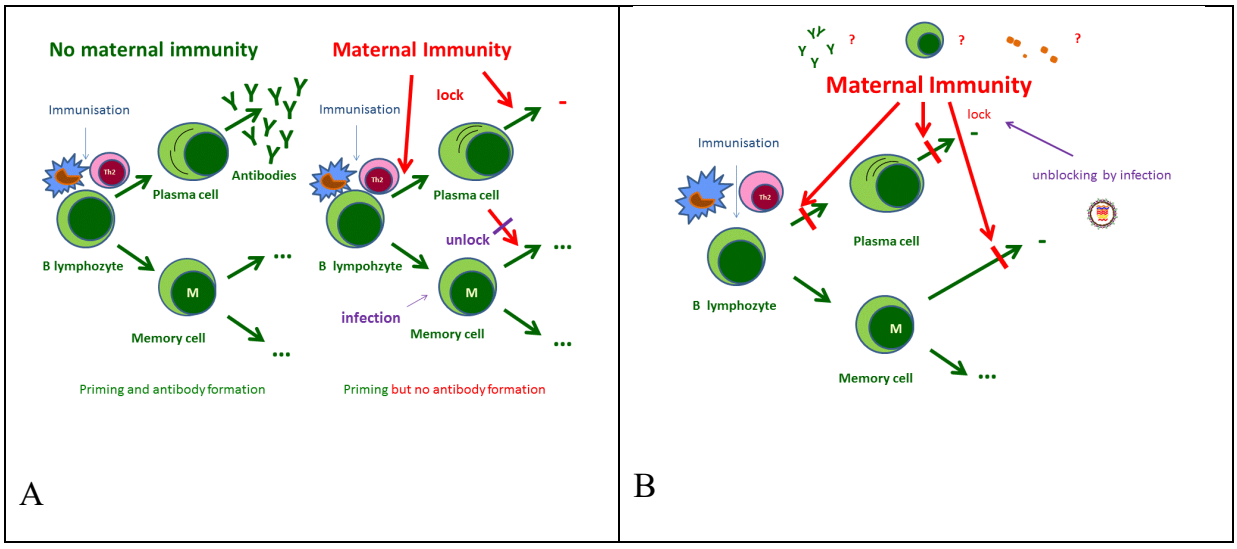

Figure 78: Influence of maternal immunity on B-cell responses
A, in contrast to the responses of pigs without maternal immunity to antigens, the serological response (plasma cell proliferation and/or immunoglobulin secretion) but not priming and memory cell formation is blocked in piglets with maternal immunity; B, the sites and components involved in blocking antibody formation are still unknown; memory cell formation is not blocked

Menšik and Pokorny (1971) discussed immunotolerance as a possible reason for interference with maternally-derived immunity and antibody production[34]. The hypothesis was that virus enters the uterus and and thus induced immune tolerance in the fetus. However, this is unlikely because this effect is also observed after immunisation, when no active virus enters the body of the sow. Figure 79 summarises some other mechanisms that could be involved in the blockade of antibody formation, which are discussed in literature[332]. Complete neutralisation of the virus can be ruled out because the virus replicates under maternally derived immunity and the virus is shed in large quantities. Negative feedback of colostral antibodies via certain receptors may be a possible route, but it is uncertain whether these antibodies persist as long as the blocking interference with maternally

derived immunity is observed. In this sense, maternally-derived immunity must be distinguished from maternally-derived antibodies and defined as immunity achieved by transfer of all immunologically active components via colostrum. This includes maternally-derived antibodies, but probably also other components. It may be possible that other components of colostrum circulate in the pig for a longer period than the maternally derived antibodies. Masking of epitopes by antibodies has also been discussed, but this can be ruled out as there would be no priming. It is known that cytokines can be transferred via colostrum. Such small molecules could be transferred in large numbers and have a longer half-life. It is also possible that there may be other unknown components or other processes involved in this blockade. The reason for this blockade may be to conserve the piglets' resources. Piglets are confronted with many antigens in their environment. In order to cope with all these antigens and to save resources for more important actions, the formation of antibodies against the specific antigens that the sows have already covered by immune transfer via colostrum is blocked. This blockage is lifted when an acute infection signals danger. Immunised pigs can then respond quickly due to the priming already in place.

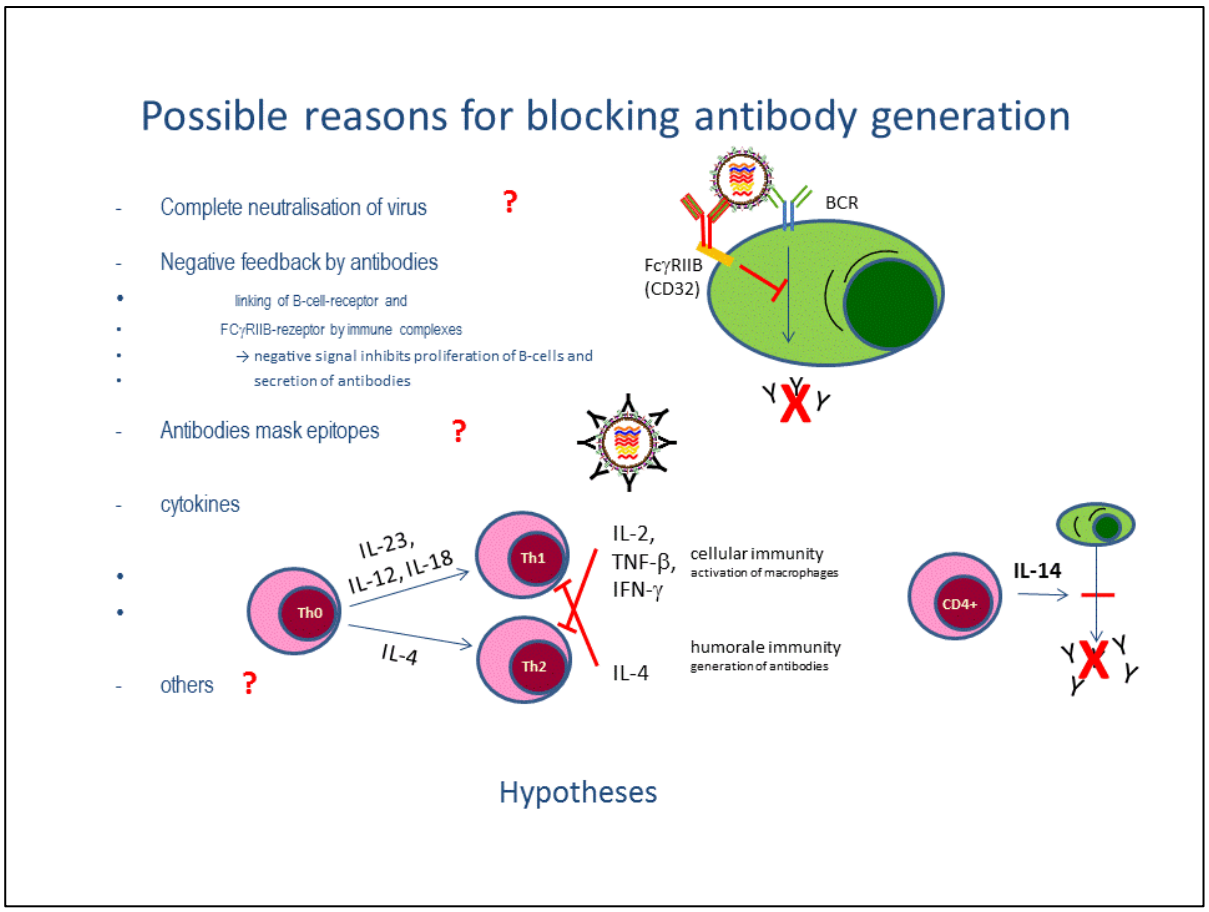

Figure 79: Possible mechanisms involved in the blockade of antibody formation under the control of maternally-derived immunity
(modified after Niewiesk, 2014[332] and Tizard, 2004[300])

6.2.3 VIRUS SHEDDING IN PIGLETS WITH MATERNAL IMMUNITY

During the first (at least four) weeks after birth, virus shedding is not under control, despite protection of the corresponding piglets by maternal immunity or vaccination. Virus shedding in young piglets is unaffected by maternally derived immunity and/or vaccination, in contrast to vaccination studies in older pigs where vaccinated pigs reflect significantly reduced virus shedding. This fact may be important for the evaluation of surveillance data and the control of swine influenza. Similar data have been found by other groups investigating maternally-derived immunity[319]. Ryt-Hansen et al. (2019)[325] demonstrated an early infection and persistence of influenza A viruses in Danish grower pigs despite the presence of maternally-derived antibodies. Even in pigs as young as 3 days – an age at which high titres of maternally-derived antibodies circulate – virus shedding was observed[325]. This is consistent with the results of the shedding observed in piglets during the first four weeks of life in this monograph. The observed shedding does not suggest that maternal immunity is not protective against disease during the first four weeks after birth.

Maternal immunity does not prevent viral shedding at infection. This virus excretion under the clinical protection of maternal immunity and the blockade mechanism of humoral immunity have consequences for the practical application of immunoprophylaxis. In piglets with maternal immunity, virus excretion takes longer than in piglets without maternal immunity (by 1-2 days, because the blockade mechanism must first be lifted by the infection; however, the immunity that is then built up neutralises the virus with delay). Thus, in vaccinated sow herds, there are clinically unrecognisable virus foci in piglets, which can have effects after transmission to the flat deck. It therefore makes sense to vaccinate piglets in vaccinated sow herds as early as the first week of life. This leads to a lifting of the blockade of humoral immunity. The piglets can therefore produce their own antibodies more quickly when infected and eliminate the virus faster (virus excretion is stopped 1-2 days earlier compared to piglets with maternal immunity). A single vaccination of the piglets may interfere with the blockade mechanism. However, a second vaccination would be better to break the blockade, especially if the vaccine does not contain a strong adjuvant. Despite this, vaccination during this time of maternal immunity does not induce antibody formation in most individuals. This then only takes place upon infection.

Despite the lack of antibody response, it is reasonable to immunise piglets that have maternally derived immunity. The interference of maternally-derived immunity with the serological response to immunisation is also of great importance when analysing scientific data. This lack of antibody formation can lead to misinterpretation in cases where piglets from sows with a different history of exposure to antigens are involved. Wang et al., 2014[485] provided data suggesting that the immune response in one of the treated groups differed from that in the other groups, but did not consider maternal immunity. Their HI data indicate the presence of antibodies to $H1_{av}N1$ virus in a few individuals of unvaccinated pigs; unfortunately, the authors did not present the complete results of the more

sensitive NP ELISA for the critical days when more positive reagents would be expected. This led to erroneous conclusions in this study.

Investigations by Rose et al. (2013)[320] based on active surveillance of piglets in French pig farms where sows had been vaccinated, showed that the earliest time of signs of influenza in their piglets was the 5th week of life, which corresponds to the time of clinical protection determined in this monograph (see volume 1 of this monograph[1], protection until day 33 of life). The data indicate that the control of influenza by inactivated vaccines in pigs within the period of maternal immunity is difficult in terms of reducing virus shedding, but clinical protection is achieved. Here, live vaccines[328] may be an alternative that can overcome the blockade of antibody responses following clostral transfer of maternal immunity. Cador et al. (2016)[486] demonstrated that maternally derived immunity prolongs the persistence of swine influenza viruses in pig herds. As shown in this monograph, piglets with colostral maternal immunity excrete influenza virus 1-2 days longer than piglets without maternal or vaccinated piglets (Figure 61 I+J, page 116). This may be due to the fact that the blockade mechanisms of maternal immunity must first be released.

Negative effects of vaccinating piglets with maternal immunity were reported by Andraud et al. (2023)[487]. They studied piglets from vaccinated and unvaccinated sows. The piglets were vaccinated once with RESPIPIPORC® FLU3 at 4 weeks of age and infected 17 days later through direct and indirect contact with sows. What the authors do not take into account is the generally longer period of shedding in infected piglets with maternal immunity. Here, the blockade of the immune response must first be overcome. This results in longer shedding because immunity develops with a delay of 1-2 days. As the sows with maternal immunity excrete for longer, contact animals are also exposed for longer. The prolonged shedding is therefore not an effect of vaccination but of maternal immunity. The vaccination itself broke the block in some animals. This led to a slight shortening of the excretion time compared to animals with maternal immunity that had not been vaccinated. A second dose of vaccine would have to be given to make these effects even more pronounced. There is no negative interference from vaccination with maternal immunity. Rather, there are synergistic effects.

Tests on sows indicate that influenza viruses can also infect the udder. Although only one unvaccinated sow was used in the trial reported in this study, high levels of virus were detectable in the milk. The sow's mammary glands were hardened and sore, indicating mastitis. The milk was also darker in colour than that of the unvaccinated sow. Due to the high viral load resulting from the nebulisation of high doses of virus during aerosol nebulisation, the virus was probably introduced into the mammary gland when the piglets suckled. The effects of mastitis on the piglets were considerable; the body weights of piglets from the vaccinated sow were on average 1 kg higher than those of piglets from the unvaccinated sow after one week. Also in connection with field studies, in which high viral loads

were detected on the teats, it appears that influenza-induced mastitis could also play a role in the field[488,489]. Immunoprophylaxis by vaccination is very important in sows. Sow vaccination is essential to protect sows from mastitis and to protect piglets from disease during the first 3-4 weeks of life. On the other hand, if sow vaccination is carried out, early vaccination of piglets is also necessary to unlock the blockade of maternal immunity before natural infection occurs, as otherwise prolonged shedding of infected piglets could occur, supporting virus transmission within the pig herd.

7. SUMMARY

In 1918 and 2009, there were two pandemics of H1N1 influenza A viruses caused by agents with a haemagglutinin related to that of classical swine influenza A viruses, which differed in terms of disease pattern from other pandemics and seasonal influenza viruses[440,490,491]. Pigs were involved in both pandemics and the viruses were easily transmitted between humans and pigs[492,493 74,494]. These epidemiological implications favour the use of pigs as model animals for influenza and the study of infection dynamics in pigs. Here, the development of an aerosol-mediated infection model is reported and its comparative analysis in 3,131 pigs using 50 different strains of European swine influenza A viruses, pandemic (H1N1) 2009 viruses and avian influenza viruses. Pigs were readily infected by all respiratory routes of infection, even at the lowest dose, but showed no signs of disease at low doses. The rapid immune response, indicated by the appearance of antibodies as early as 5-7 days post infection (dpi), inhibited high viral replication in the lung and prevented disease. Only high doses of infectious virus, which immediately resulted in extremely high lung viral loads, were able to induce clinical signs and thus evade this rapid immune response for a short period of time. Influenza viruses differ in their virulence at high dose infection. Swine avian-like H1N1 viruses of the 2000s reflected the lowest virulence by causing only mild symptoms, whereas avian-like H1N1 viruses of the 1980s/1990s, human-like H1N2 viruses and human-like H3N2 viruses induced typical signs of influenza. The early H1N1 virus of the 2009 pandemic was the most virulent, resulting in an experimental case fatality rate of 14-23% in repeated experiments. These viruses lost their lethal virulence within 3-4 months of emergence. Within the first five years after their introduction into the human and pig populations, they became very low virulent and reflected the same pattern as other H1N1 viruses that circulated longer in pigs. These data are the first to show that newly emerged H1N1 viruses evolve towards lower virulence. In contrast, HxN2 viruses remain stable in virulence for years. It is likely that neuraminidase activity, influenced by the position of this glycoprotein on the virion and its interaction with haemagglutinin and the cell membrane, plays an important role in virulence by triggering strong innate immune signalling cascades, especially as no clear genetic markers have been identified to explain differences in virulence. The high-dose aerosol infection model was validated and investigated in detail. It was applied to the investigation of vaccines, antivirals, immunosuppressive effects and maternally-derived immunity. The data show that exposure to high initial viral doses is important for influenza pathogenesis. Therefore, prevention of primary (initial high-dose exposure resulting in high viral lung load) and secondary high-dose conditions (influenza virus replicates to high titres in the lungs; this can

occur, for example, with immunosuppression, malnutrition and bacterial co-infections) is essential for influenza control: Primary high-dose illness can be prevented by air hygiene, vaccination and early antiviral treatment. Secondary high dose conditions can be prevented by vaccination and prevention of co-infections.

Expression of viral antigens was observed in numerous tissues outside the respiratory tract, although no infectious virus and no NP could be detected in these. It can therefore be assumed that virus components are widely distributed throughout the organism via macrophages. The nature of the expressed proteins and the mechanisms of their replication are still unknown. However, these proteins can trigger inflammation and play a role in the pathogenesis of systemic influenza, even if they were tolerated by the pigs in the infection studies without any clinical symptoms.

Another mechanism that led to an intensification of disease symptoms in some individuals in the infection experiments, sometimes with fatal consequences, was the presence of immunity to the matrix protein (or other internal proteins) in the absence of immunity to the haemagglutinin and neuraminidase of the infecting virus. Again, the mechanisms leading to the onset of these severe disease courses are not yet known. However, an overreaction of the innate immune system is suggested by both the peracute fatal course shortly after infection and the severe lung lesions around 5 days observed in individual pigs after experimental infection.

There is a long period of interference with maternal immunity to antibody responses to vaccination, i.e. no or insufficient antibodies are induced by vaccination during this long period of almost 8 months. Nevertheless, immunisation can be given shortly after birth and primes the immune system, giving an advantage over unimmunised offspring and protection against lethal influenza.

To sum up the results of both parts of the monograph are summarised here:

1. In short:

i) One of the longest surveillance activities for swine influenza was initiated and continued for 13 years, providing new insights into the evolution of swine influenza viruses in Germany (volume I of this monograph[1])

ii) An aerosol infection model for swine was validated and compared with other infection models. The initial dose of infection was shown to be critical for pathogenesis. The pattern of lung lesion development was investigated and its key role in pathogenesis was assessed. The A(H1N1)pdm April 2009 virus induced severe 1918 influenza-like illness in pigs using a high-dose aerosol infection approach. The evolution of $H1_{pdm}N1$ viruses towards lower virulence was demonstrated. Vaccine-induced enhancement of disease was shown for the matrix protein, suggesting that immune responses against internal genes can

stimulate cytokine expression in infected cells when an immune response against the corresponding haemagglutinin and neuraminidase is insufficient

iii) The duration of maternal immunity has been determined and shown to last up to eight months after birth, although protection against disease by maternal antibodies is limited to a period of 4-5 weeks after farrowing. Protection by vaccination of piglets with maternal immunity has been demonstrated, reflecting synergistic effects of maternal and vaccine-induced immunity already in the first week of life, but also prevention of severe disease and death due to faster immune responses to vaccine-induced priming in the period after the first month of life.

2. In more detail:

i) The epidemiology of swine influenza has changed with changes in farm structure. While the H1N1 and H3N2 subtypes dominated in Europe until the early 2000s, the circulation of H3N2 influenza viruses decreased with the reduction in the number of pig farms and the expansion of sow vaccination following the introduction of safe vaccines and with the increase in farm size and specialisation in pig farming. These H3N2 viruses dominated reassortments, i.e. in mixed infections H3N2 viruses remained dominant and did not allow reassortments. With the decline of H3N2 viruses and the entry of $H1_{pdm}N1$ viruses into the pig population, the way was open for reassortments of swine influenza viruses. As a result, the number of newly reassorted viruses in the pig population increased in the 2010s. H1N1 ($H1_{av}$, $H1_{pdm}$ as well as $H1_{hu}$) viruses are particularly compatible with N2, but reassortment also leads to changes in the haemagglutinin antigenic sites, resulting in new viruses that are not or only weakly cross-reactive with the circulating H1N1 viruses and the vaccine strains of the authorized vaccines. Even if there is still a protective effect via neuraminidase, this increases the risk of disease enhancement in vaccinated animals due to antibodies against the internal proteins, especially M, via antibody-dependent enhancement mechanisms. Therefore, pigs should be vaccinated at least three times to ensure sufficient baseline levels against HA and NA. The $H1_{av}N1$ viruses are true swine viruses; they induce only weak immunity in pigs and can therefore infect pigs several times. They also induce only mild symptoms in pigs. Nevertheless, these viruses are not insignificant, especially in co-infected pigs. These viruses will persist in the pig population also in future. Despite genetic evolution, there is little antigenic drift in H1N1 viruses, but drift is suddenly forced by reassortment with HxN2 viruses, and $H1_{av}N1$ 1C.2 viruses are prone to this. Because of their weak antigenicity, these viruses are also a problem for vaccine development. Instead of working with strong adjuvants, it is possible to achieve broad immunity in this antigenic H1 group by combining different viruses of an antigenic supergroup. Such immunity can also be achieved by triple vaccination, if the last vaccination is not too close to the second. In this work, the combination of immune and hyperimmune sera was used for the first time for antigenic characterisation. Hyperimmune sera reflect the maximum cross-reactivity

achievable. All viruses that react against a hyperimmune serum belong to an antigenic supergroup. All viruses in an antigenic supergroup share at least one epitope and therefore react to infection with another virus in the supergroup, even if it is not recognised by an immune serum, as if it were a booster with a broad immune response. The immune system works economically. The production of many immune cells following stimulation by an antigen consumes energy. This is why, after the peak of the response around 10 days after vaccination, the immune system regulates itself back down again. This means that the protection to infection provided by the vaccine is only short-lived and limited to a short period within the first month after vaccination. The immune system relies more on memory cells and rapid reactivity than on lasting protection against infection. As a result, chains of infection from pathogens that multiply rapidly - such as respiratory viruses - can only be broken by vaccinating the entire population at the same time. Swine influenza viruses circulate all year round due to swine stabling without access to sunlight. They pose a zoonotic risk due to their year-round circulation as well as their readiness to reassort and the sudden change in their antigenicity after certain reassortments. The zoonotic transmissions of swine influenza viruses to humans detected during the observation period showed that the greatest risk exists in children and young adults. Transmission from person to person occurred in some cases, but no stable chains of infection developed in humans. Closed vaccination of children is the best preventive measure against zoonotic infections from the pig population because there is remote cross-protectivity against many swine influenza viruses and because this reduces the risk of reassortment with human seasonal influenza viruses.

ii) An infection model for airborne infection of pigs was developed and used for investigations on an unprecedented scale. It has been shown that young pigs (up to six months of age) can be used to model severe influenza and to determine the virulence of influenza viruses. Virulence can be determined by titrating differently diluted doses of virus in pigs or other animals. A steep regression curve is typical for highly virulent viruses and a weak one for less virulent viruses. There are differences in virulence between viruses. Freshly reassorted HxN1 viruses are highly virulent, but evolve into low virulence viruses after a short time of circulation in the population. The spring 2009 $H1_{pdm}N1$ virus caused symptoms similar to those of the 1918 influenza in pigs. HxN2 viruses have stable virulence and are less virulent than freshly reassorted HxN1 viruses. Reassortment changes virulence: low virulent HxN1 viruses that reassort with N2 become more virulent, high virulent HxN1 viruses lose some of their virulence by absorbing N2, as do H1N2 viruses that reassort back with N1, that is, to an N1 to which the H1 had previously adapted. The high-dose infection model is also suitable for studies of antibody-dependent enhancement of disease after vaccination. It was confirmed that the lack or low antibodies against HA and NA in the presence of antibodies against M supports stronger lung lesions in individual vaccinated pigs if the infection strain differs antigenetically to a certain degree from the vaccine virus. It has been possible to confirm the involvement of the M protein in triggering severe lung

changes, providing further evidence that immune responses to the internal proteins of influenza viruses contribute to this mechanism.

iii) Numerous studies have also been carried out on maternal immunity. There is an interference in seroconversion to vaccination that lasts up to 8 months in some individuals. However, the development of immunity is not suppressed by maternal antibodies, but synergistic effects of maternal immunity and vaccination have been demonstrated. The blockade of immune system reactivity to antigenic stimuli by maternal immunity can be reversed by antigenic exposure such as vaccination or infection. In general, the immune system of piglets is not yet mature enough for the clearance of viruses after infection to be reduced by existing immunity; on the contrary, it is even longer in piglets with maternal immunity than in piglets without. Therefore, in vaccinated sow herds, infection can lead to herd infections in the sow compartment and flat deck, which contribute significantly to the maintenance of herd infection cycles. It is therefore advisable to vaccinate piglets in the first week of life.

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SUPPLEMENTARY MATERIAL

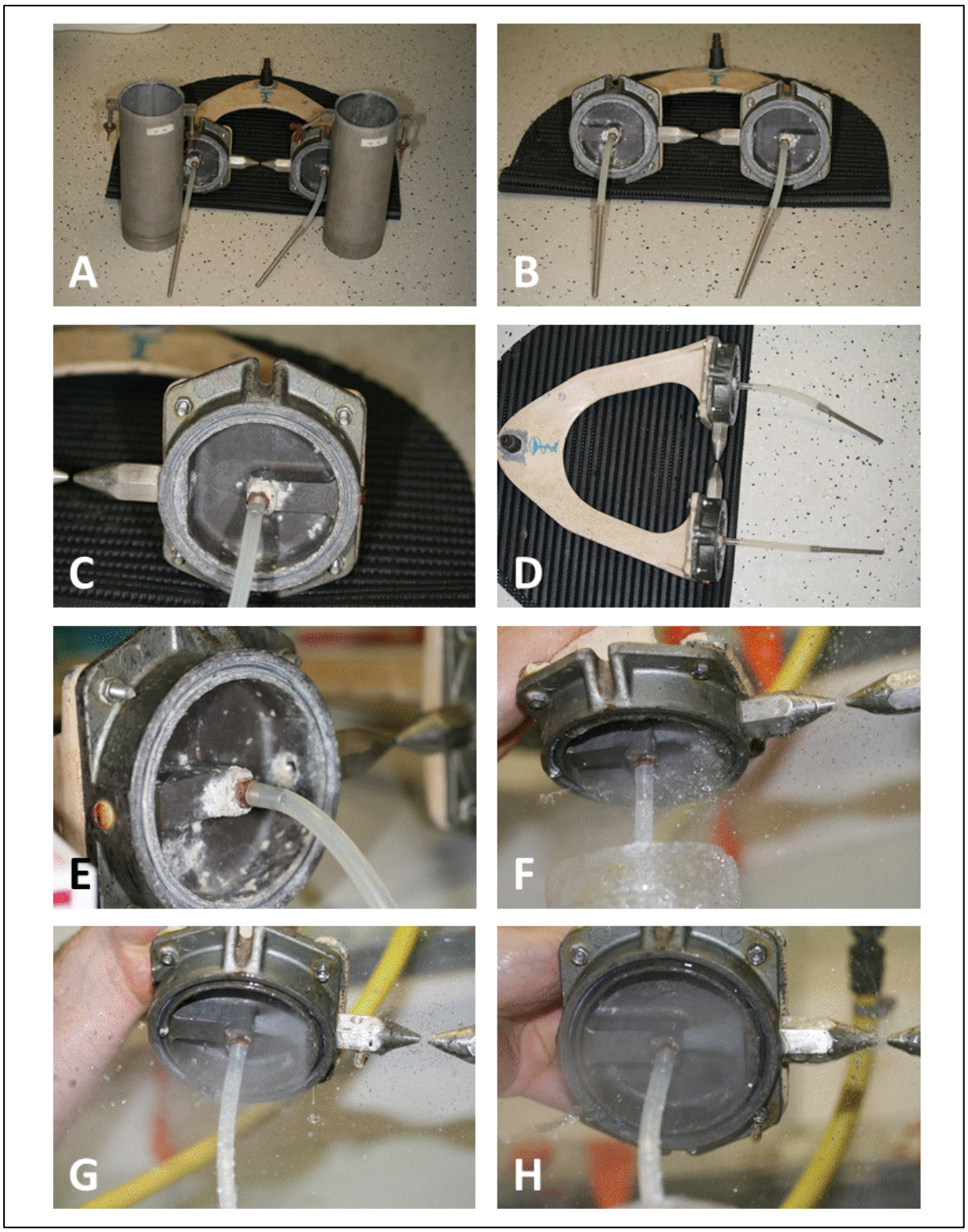
SUPPLEMENT CHAPTER 1 – OVERVIEW OF VIRUSES USED

Supplementary Table 1: Overview of infection strains

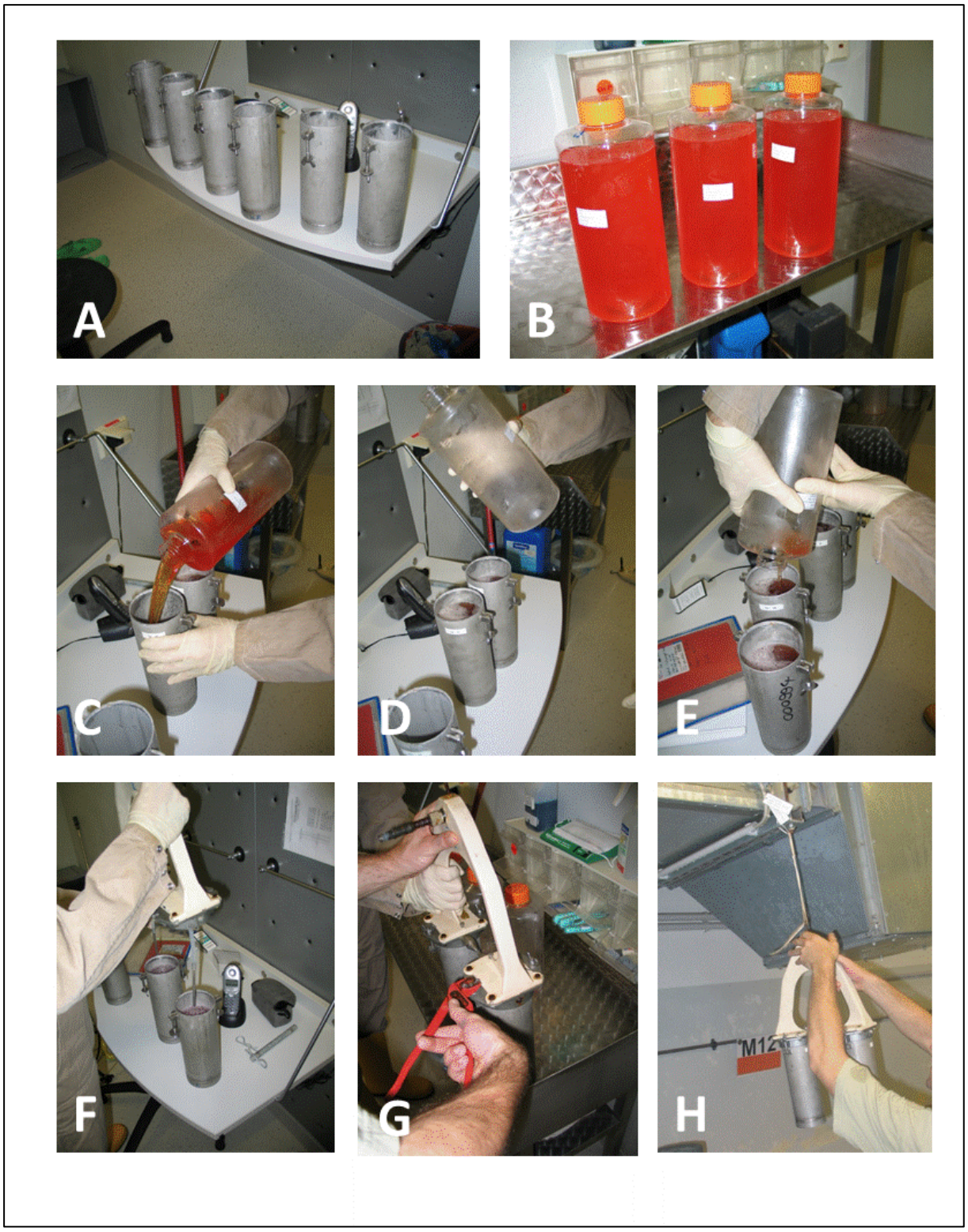
Group	Virus
Avian viruses	duck/Potsdam/2216-4/84 (H5N6)
	turkey/Garrel/2000 (H9N2)
classical swine	sw/England/117316/1986 ($H1_{cl}N1$)*
avian like swine H1N1	sw/Potsdam/15/1981 ($H1_{av}N1$)*
	sw/Schwerin/103/1989 ($H1_{av}N1$)*
	sw/Ghent/V196/1992 ($H1_{av}N1$)
	sw/Bakum/3543/1998 ($H1_{av}N1$)*
	sw/Belzig/02/2001 ($H1_{av}N1$)*
	sw/Haselünne/2617/2003 ($H1_{av}N1$)*
	sw/Harlebach/2998/2004 ($H1_{av}N1$)*
	sw/Vechta/2623/2003 ($H1_{av}N1$)*
	sw/Greven/2889/2004 ($H1_{av}N1$)*
	sw/Bad Rothenfelde/4255/2005 ($H1_{av}N1$)*
	sw/Ploufragan/0070/2005 ($H1_{av}N1$)
	sw/Melle/5003/2006 ($H1_{av}N1$)*
	sw/Bad Griesbach/5604/2006 ($H1_{av}N1$)*
	sw/Ennigerloh/5803/2006 ($H1_{av}N1$)*
	sw/Freren/8297/2009 ($H1_{av}N1$)*
Reassortant H1N1	sw/Ploufragan/0190/2006 (rH1N1)
H3N2	sw/Ghent/1/1984 (H3N2)*
	sw/Bakum/1769/2003 (H3N2)*
	sw/Nettetal/1870/2003 (H3N2)
	sw/Bissendorf/1864/2003 (H3N2)*
	sw/Melle/4312/2005 (H3N2)
	sw/Melle/5706/2006 (H3N2)*
	sw/Damme/5673/2006 (H3N2)*
	sw/Hamstrup/5445/2006 (H3N2)*
	sw/Bondelum/5959/2007 (H3N2)*
	sw/Ostenfeld/8082/09 (H3N2)*
H3N1	sw/Coesfeld/19499/2014 (H3N1)*
human-like swine H1N2	sw/England/17394/1996 ($H1_{hu}N2$)”
	sw/Ghent/7625/1999 ($H1_{hu}N2$)*
	sw/Bakum/1832/2000 ($H1_{hu}N2$)*
	sw/Granstedt/3435/2003 ($H1_{hu}N2$)*
	sw/Dötlingen/4735/2005 ($rH1_{hu}N2$)*
	sw/Cloppenburg/4777/2005 ($rH1_{hu}N2$)*
	sw/Ploufragan/0214/2006 (G1 H1N2)
	sw/Kitzen/6142/2007 ($H1_{hu}N2$)*
	sw/Groitzsch/6016/2007 ($H1_{hu}N2$)*
	sw/Ghent/102/2007 ($H1_{hu}N2$)
	sw/Bottrop/8644/2009 ($H1_{hu}N2$)*
reassortant $H1_{av}N2$	sw/Denmark/12687/2003 (rH1N2)
pandemic H1N1 of humans	Regensburg/06/2009 ($H1_{pdm}N1$)°
	Hamburg/NY1580/2009 ($H1_{pdm}N1$)*
	Jena/VI5258/2009 ($H1_{pdm}N1$)*
	Jena/VI2688/2010 ($H1_{pdm}N1$)*
	Kiel/18909686/2015 ($H1_{pdm}N1$)*
Pandemic H1N1 of swine	Schallern/19989/2014 ($H1_{pdm}N1$)*
	sw/Tesp/2110/2015 ($H1_{pdm}N1$)
Pandemic H1N2 of swine	sw/Papenburg/12653/2010 ($H1_{pdm}N2$)*

* for these viruses the complete sequence information is available: for accession numbers see supplement of volume 1[1]
° GenBank accession numbers of A/Regensburg/06/2009 ($H1_{pdm}N1$): FN401574-FN401581
“ A/England/17394/1996 ($H1_{hu}N2$) is identical to A/sw/Poland/T2/2008

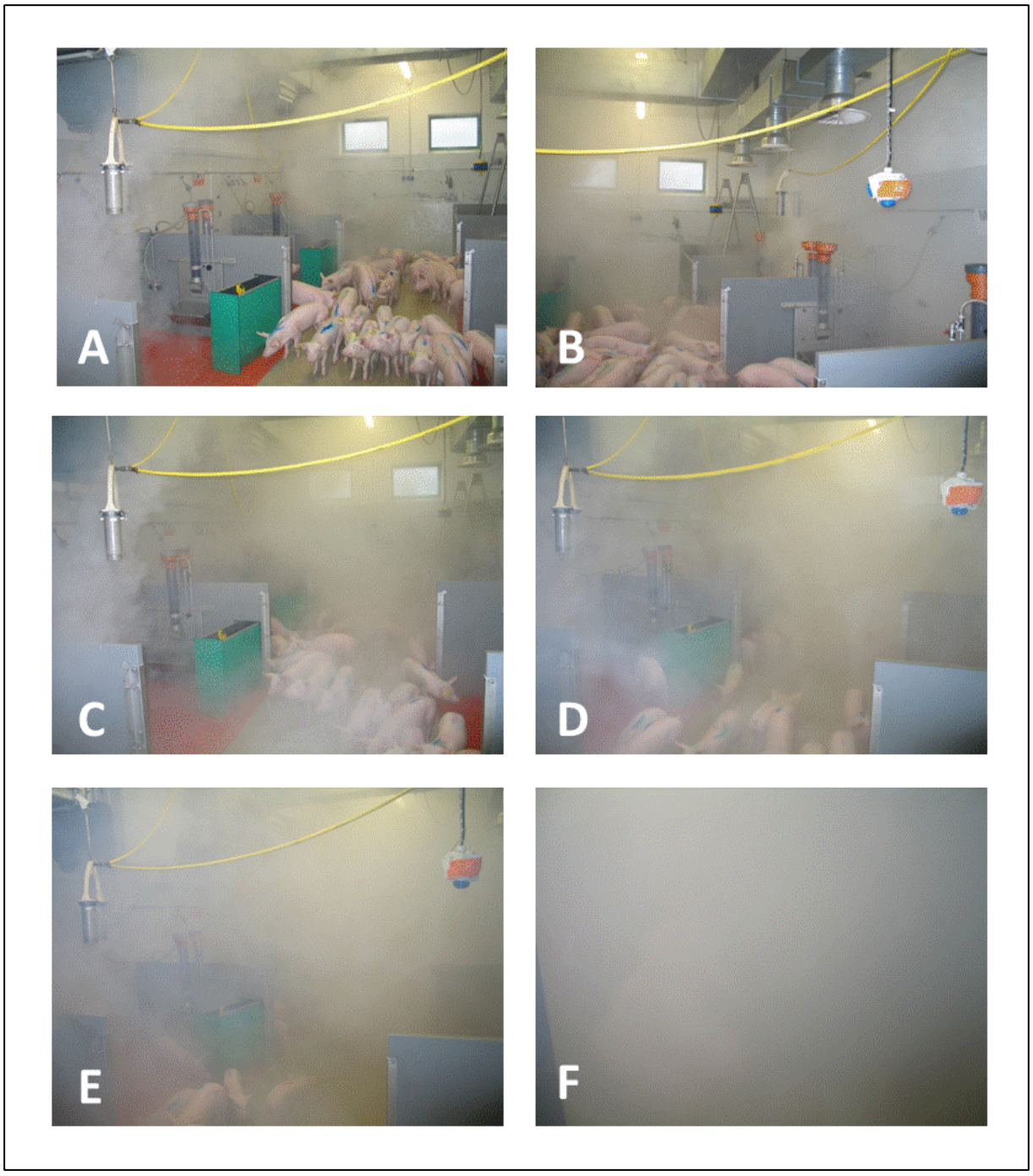
SUPPLEMENT CHAPTER 2 – VIUSUALISATION OF INFECTION METHODS



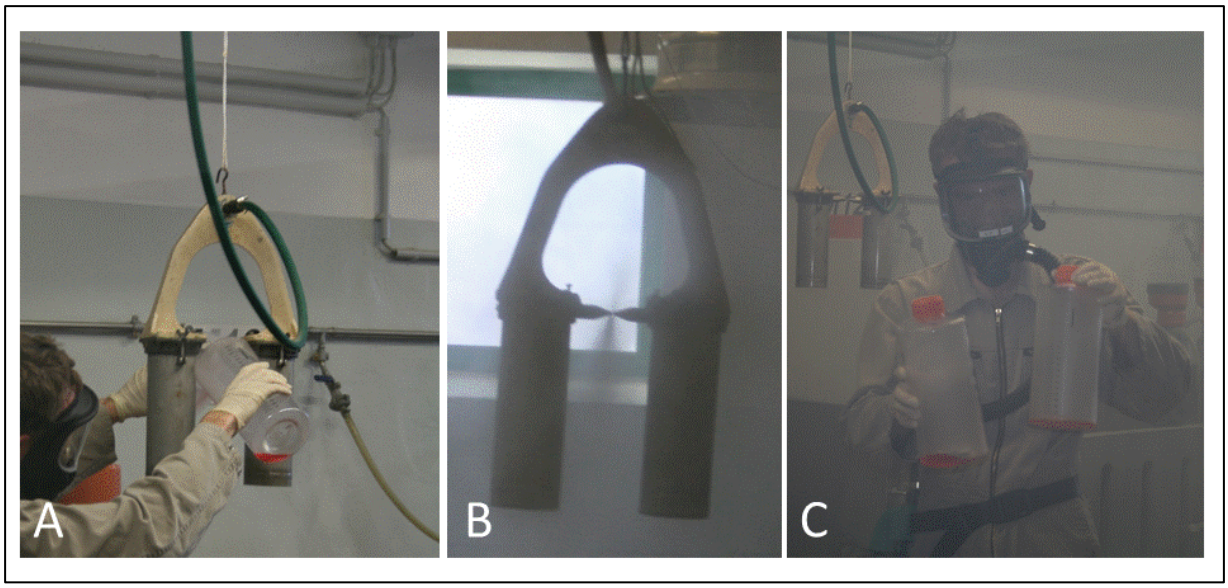
Supplement 1: Technical details of aerosol generator SAG-1: A, B, composition; C, D, E, interior equipment; F, G, H, generator in action: the low pressure generated sucks the liquid infectious material trough the flexible tubes into the airstream which is directed to the jets through the open space of the container; on exiting the jets both currents impinge on each other and create a highly dispersed aerosol



Supplement 2: Preparation of the nebulisation procedure: A, containers; B, infectious material in roller bottles (here 6 litres); C, D, E, filling of containers; F, G, attaching and screwing of the containers to end pieces of the generator; H, fixing the generator below the ceiling of the infection unit

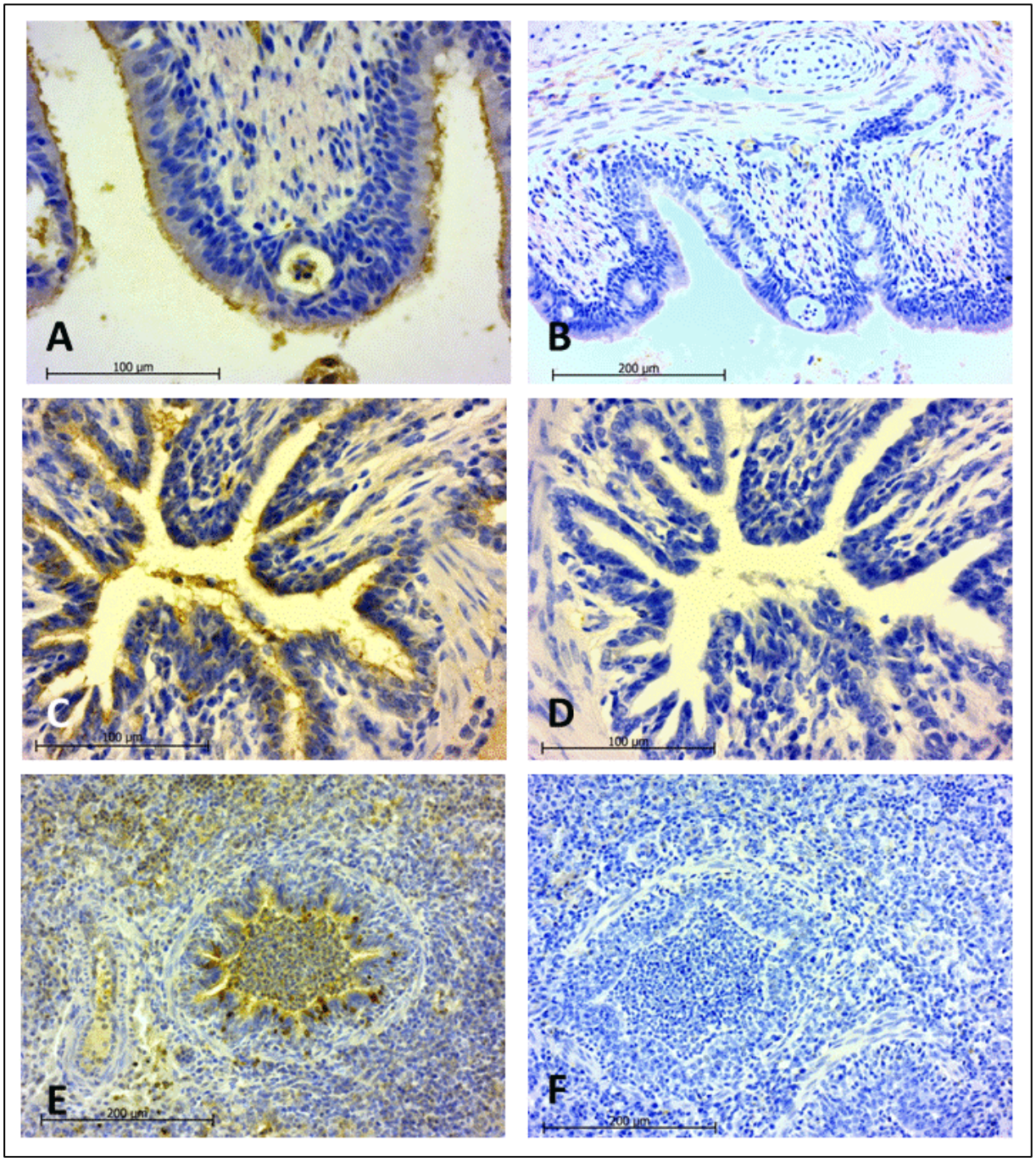


Supplement 3: Aerosol generation: procedure of aerosol nebulisation; A, before nebulisation, B, after 30 s; C, after 1 min; D, after 2 min; E, after 3 min; F, after 5 min (dense aerosol)



Supplement 4: Collection of nebulised material; A, collection of aerosol in roller bottles during nebulisation; B, nebulisation procedure; C, collected material for back titration of nebulised virus

SUPPLEMENT CHAPTER 3 – SPECIFICITY OF SABC STAINING



Supplement 5: Proof of specificity of SABC staining, A, C, E, use of anti-influenza-virus specific polyclonal rabbit serum as primary antibody; B, D, F, use of not immunised rabbit antiserum as primary antibody; the picture show structures in the respiratory tract of pigs after infection with A/Hamburg/NY1580/2009 H1$_{pdm}$N1 virus

SUPPLEMENT CHAPTER 4 – INTRAMUSCULAR INFECTION

Background. Influenza A viruses require host enzymes to replicate into infectious viruses inside infected cells. In mammals, these enzymes can only be provided by cells of the respiratory tract. Therefore, infection of other tissues should not support the generation of infectious particles and their distribution within the organism.

Study design. Pigs were examined at 8 weeks of age. Intramuscular infection was performed with 2 ml virus suspension containing a total of $10^{8.8}$ $TCID_{50}$ of strain A/sw/Haselünne/2617/2003 ($H1_{av}N1$). 5 pigs received the injection, 5 pigs were kept as contact control in the same pen of the infection unit. Rectal temperatures, clinical signs and virus shedding were measured. Viral signal distribution was measured by PCR amplification using primers encoding the M segment in one pig from each group at 1, 4, 7, 11 and 18 days post infection (dpi).

Results. There were no changes in rectal temperature and no clinical signs in either the injected pigs or the contact controls (Supplementary Table 2, Supplementary Table 3). No virus was shed or detected in organ samples from pigs injected intramuscularly with active $H1_{av}N1$ virus or from contact controls (Supplementary Table 4, Supplementary Table 5). The injected pigs developed HI antibodies with titres ranging from 1:64 to 1:128, while the contact control pigs remained negative.

Short interpretation of results. The data show that intramuscular injection of influenza viruses does not cause infection. This observation is consistent with the fact that the haemagglutinin of influenza A viruses must be cleaved by enzymes to produce infectious virions. These enzymes are not present in muscle cells.

Supplementary Table 2: Kinetics of rectal temperatures (°C) in 5 pigs after intramuscular injection with $10^{8.8}$ $TCID_{50}$ H1N1 virus

Pig ID	0	2 hpi	4 hpi	6 hpi	8 hpi	10 hpi	12 hpi	14 dpi	16 hpi	18 hpi	20 hpi	22 hpi	24 hpi	26 hpi
383	39.8	39.9	39.6	39.7	39.6	39.7	39.7	39.7	39.5	39.7	39.6	39.4	39.3	39.1
384	39.7	39.4	39.8	39.9	39.3	39.8	40.0	39.7	39.9	39.9	39.5	39.4	39.5	39.4
385	39.3	39.5	39.7	39.4	39.5	39.4	39.6	39.4	39.5	39.6	39.4	39.5	39.3	39.4
386	39.7	39.7	39.8	39.2	39.4	39.5	39.5	39.6	39.8	39.7	39.3	39.4	39.3	39.3
387	39.3	39.5	39.8	39.5	39.6	39.4	39.4	39.3	39.5	39.4	39.5	39.7	39.4	39.4
Pig ID	28 hpi	30 hpi	32 hpi	34 hpi	48 hpi	50 hpi	52 hpi	54 dpi	56 hpi	58 hpi	72 hpi	74 hpi	76 hpi	78 hpi
383	39.1	39.5	-d	-	-	-	-	-	-	-	-	-	-	-
384	39.3	39.5	39.9	39.7	39.1	39.4	39.6	39.6	39.8	39.9	39.9	39.8	39.9	39.8
385	39.1	39.3	39.0	39.3	39.4	39.5	39.3	39.5	39.5	39.3	39.7	39.3	39.4	39.5
386	39.7	39.6	39.6	39.6	39.6	39.3	39.6	39.5	39.6	39.7	39.3	39.5	39.5	40.0
387	39.3	39.5	40.0	39.7	39.5	39.5	39.6	39.7	39.7	39.6	39.6	39.5	39.7	39.5
Pig ID	80 hpi	82 hpi	96 hpi	98 hpi	100 hpi	102 hpi	104 hpi	106 hpi						
384	39.9	39.8	40.0	39.9	39.8	40.1	40.0	40.4						
385	39.4	39.6	39.5	39.3	39.4	39.1	39.3	39.3						
386	39.9	39.9	39.4	39.6	39.7	39.5	39.6	39.7						
387	39.7	39.7	39.4	39.5	39.6	-d	-	-						

5 contact pigs kept together with the i.m. infected pigs did not show increased rectal body temperatures (data not shown); -d, pig removed for investigation of organ samples

Supplementary Table 3: Clinical symptoms after intramuscular injection of H1N1 virus in pigs

Pig ID	0	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	8 dpi	9 dpi	10 dpi	11 dpi	14 dpi	15 dpi	16 dpi	17 dpi	18 dpi
383	0	0	-d	-	-	-	-	-	-	-	-	-	-	-	-
384	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
385	0	0	0	0	0	0	0	0	0	0	-d	-	-	-	-
386	0	0	0	0	0	0	-d	-	-	-	-	-	-	-	-
387	0	0	0	0	0	-d	-	-	-	-	-	-	-	-	-

5 contact pigs kept together with the i.m. infected pigs did not have any symptoms (data not shown); -d, pig removed for investigation of organ samples; dyspnoea was assessed as follows: 0 = breathing unaffected; 1 = increased respiratory frequency and moderate flank movement; 2 = marked breathing difficulty and severe flank movement; 3 = laboured breathing affecting the entire body together with pronounced flank movement and substantial movements of the snout, 4 = severe breathing reflecting substantial lack of oxygen

Supplementary Table 4: Virus excretion in pigs injected intramuscularly with H1N1 virus

Pig ID	0	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	8 dpi	9 dpi	10 dpi	11 dpi	14 dpi	15 dpi	16 dpi	17 dpi	18 dpi
383	∅	∅	-d	-	-	-	-	-	-	-	-	-	-	-	-
384	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅
385	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	-d	-	-	-	-
386	∅	∅	∅	∅	∅	∅	-d	-	-	-	-	-	-	-	-
387	∅	∅	∅	∅	∅	-d	-	-	-	-	-	-	-	-	-

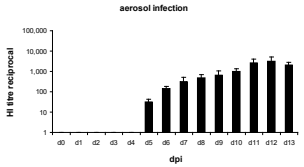
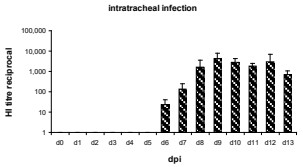
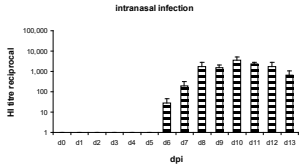
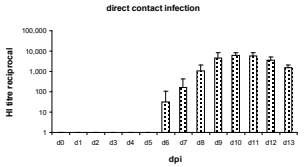
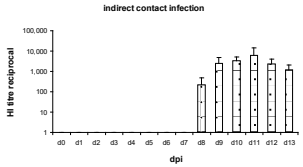
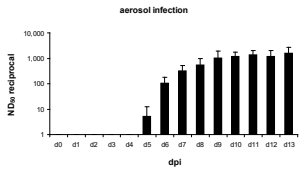
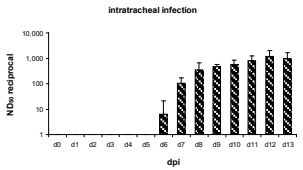
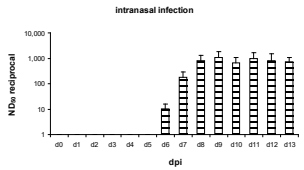
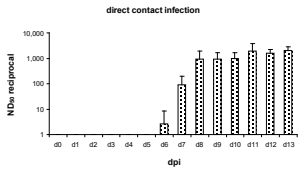
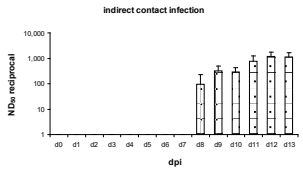
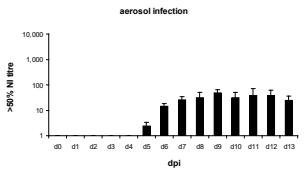
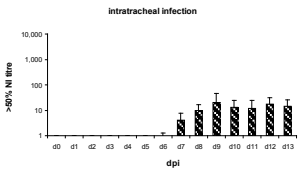
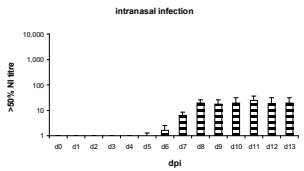
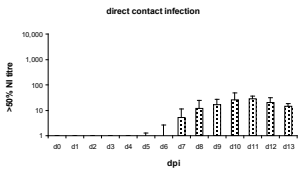
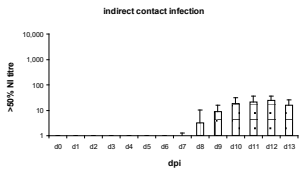
Ø, negative by PCR; -d, pig removed for investigation of organ samples; - , not investigated; 5 additional pigs housed together with the 5 i.m. infected pigs did not shed virus (data not shown)

Supplementary Table 5: Virus detection in organ samples adter intramuscular injection of H1N1 virus in pigs

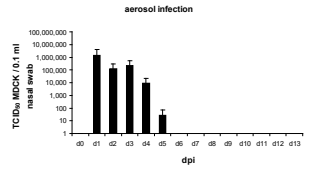
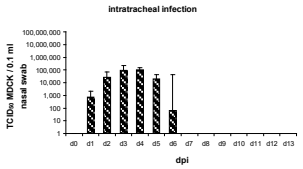
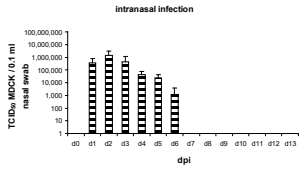
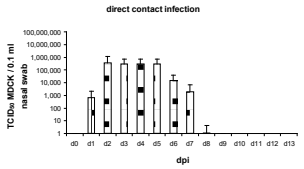
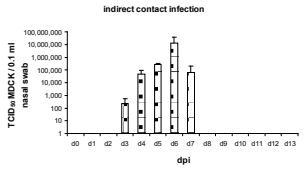
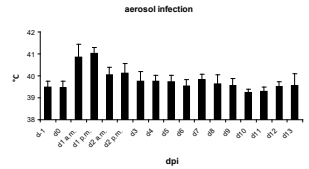
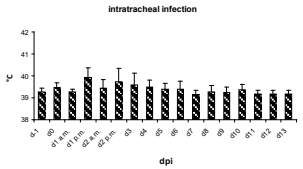
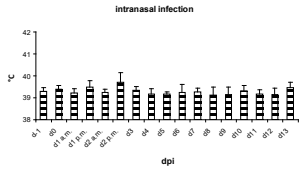
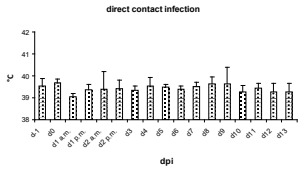
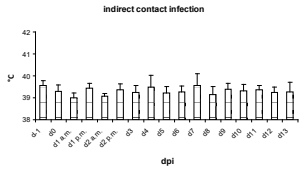
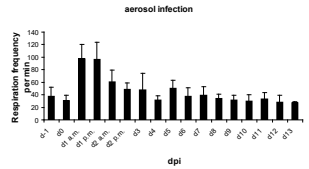
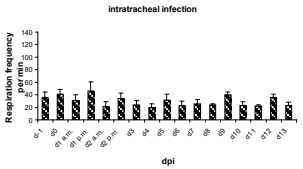
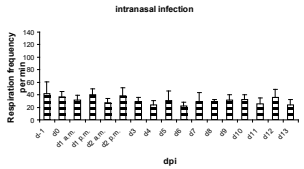
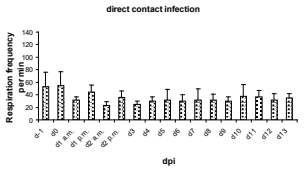
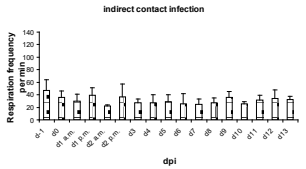
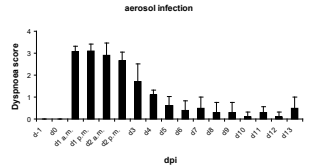
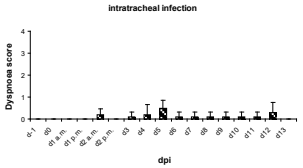
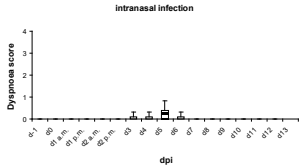
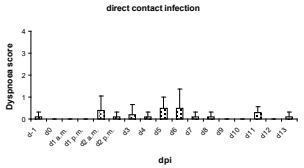
	1dpi Pig 383	4dpi Pig 387	7dpi Pig 386	11dpi Pig 385	18dpi Pig 284
buffy coat*	∅	∅	∅	∅	∅
injection site	∅	∅	∅	∅	∅
bone marrow	∅	∅	∅	∅	∅
spinal cord	∅	∅	∅	∅	∅
lung	∅	∅	∅	∅	∅
heart	∅	∅	∅	∅	∅
liver	∅	∅	∅	∅	∅
spleen	∅	∅	∅	∅	∅
kidney	∅	∅	∅	∅	∅
intestine	∅	∅	∅	∅	∅
Ln. mandibularis	∅	∅	∅	∅	∅
Ln. axillaris	∅	∅	∅	∅	∅
intestinal lymphnode	∅	∅	∅	∅	∅

Ø, negative by PCR; 5 additional pigs housed together with the 5 i.m. infected pigs were investigated at the same intervals and did not carry signs of virus infection; *buffy coat was investigated 0 dpi, 1 dpi, 2 dpi, 3 dpi, 4 dpi, 7 dpi, 11 dpi and 18 dpi and was negative all the time

SUPPLEMENT CHAPTER 5 – COMPARISON OF INFLUENZA INFECTION MODELS

1	*2*	*3*	*4*	*5*
 a) HI antibodies aerosol	 HI antibodies intratracheal	 HI antibodies intranasal	 HI antibodies direct contact	 HI antibodies indirect contact
 b) NT antibodies aerosol	 NT antibodies intratracheal	 NT antibodies intranasal	 NT antibodies direct contact	 NT antibodies indirect contact
 c) NI antibodies aerosol	 NI antibodies intratracheal	 NI antibodies intranasal	 NI antibodies direct contact	 NI antibodies indirect contact

Supplement 6: Comparison of different infection models (1 aerosol infection, 2 intratracheal infection, 3 intranasal infection, 4 direct contact infection, 5 indirect contact infection, each infection group comprised 5 pigs); a, HI antibodies (HI titre reciprocal); b, Neutralising antibodies (ND_{50}); c, Neuraminidase inhibiting antibodies (>50% NI titre); d, Virus excretion (MDCK $TCID_{50}$ /0.1 ml nasal swab solution); e, Rectal temperatures (°C); f, Respiration frequency (min^{-1}); g, Dyspnoea score (Score) after infection with strain A/Jena/VI2688/2010 ($H1_{pdm}N1$) using the same virus suspension ($10^{5.75}$ MDBK $TCID_{50}$/ml (continued next page)

				
d) Virus excretion aerosol	Virus excretion intratracheal	Virus excretion intranasal	Virus excretion direct contact	Virus excretion indirect contact
				
e) Rectal temperatures aerosol	Rectal temperatures intratracheal	Rectal temperatures intranasal	Rectal temperatures direct contact	Rectal temperatures indirect contact
				
f) Respiration frequency aerosol	Respiration frequency intratracheal	Respiration frequeny intranasal	Respiration frequency direct contact	Respiration frequency indirect contact
				
g) Dyspnoea aerosol	Dyspnoea intratracheal	Dyspnoea intranasal	Dyspnoea direct contact	Dyspnoea indirect contact

Supplement 6 continued. d, Virus excretion (MDCK $TCID_{50}$ /0.1 ml nasal swab solution); e, Rectal temperatures (°C); f, Respiration frequency (min^{-1}); g, Dyspnoea score (Score) after infection with strain A/Jena/VI2688/2010 (panH1N1) using the same virus suspension ($10^{5.75}$ MDBK $TCID_{50}$/ml)

SUPPLEMENT CHAPTER 6 – INDIRECT CONTACT INFECTION

Background. The evaluation of data of high-dose aerosol infection revealed remarkable differences in virulence of influenza A viruses. In some of the trials contact infection groups had been carried along. Here, a comparative analysis is provided of the effects of FLUAVs that differ on virulence at high-dose aerosol infection on pigs infected naturally via contact.

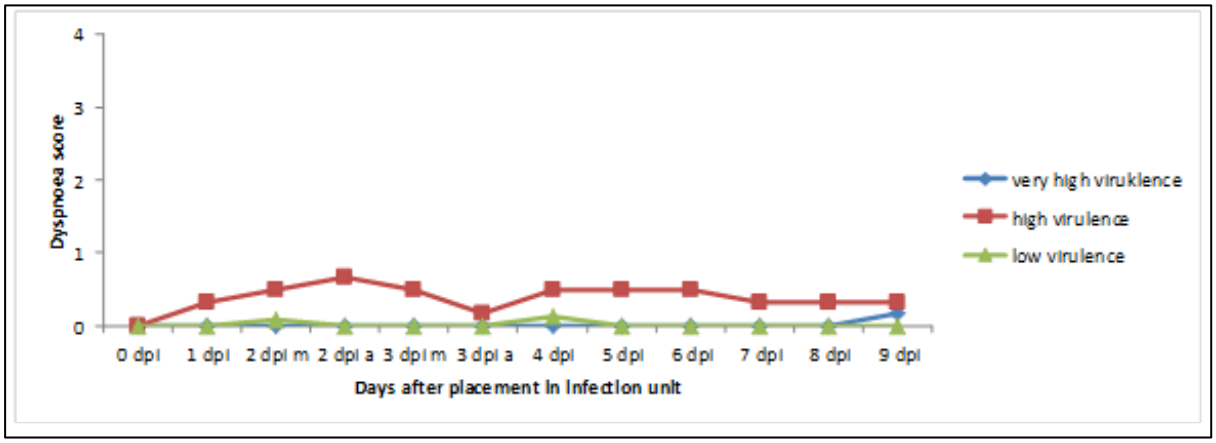
Study design. In some trials pigs were brought into the infection units after aerosol infection had been carried out on the other pigs in order to investigate the effects of transmission via air without direct contact to the other pigs. The data of these investigations are comparatively analysed here. The 12-weeks-old pigs were brought into the infection unit 1 day after aerosol infection had been carried out on the other pigs. They were placed in a separate compartment without contact to the other pigs. Due to the low number of pigs lungs could only be investigated at the end of the trial 8 days after the pigs had been brought into the inferction unit (Supplementary Table 6).

Supplementary Table 6: Overview of the groups included in evaluation of the effects of infection by indirect contact

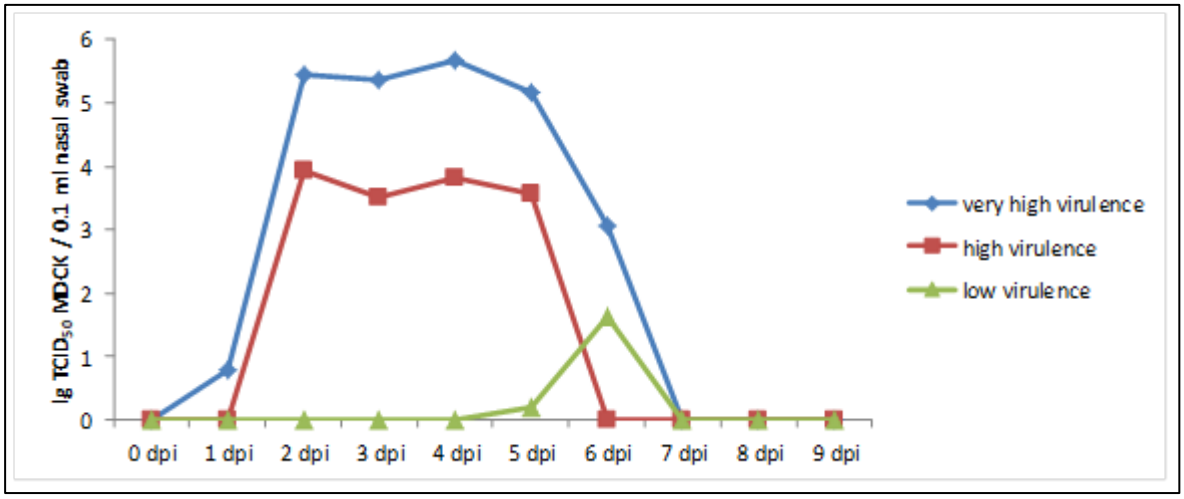
Virulence of virus	Virus used in trial	Number of pigs included*
Very high	FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$)	5
High	FLUAV/sw/Papenburg/12953/2010 ($H1_{pdm}N2$)	3
Low	FLUAV/sw/England/117316/1986 ($H1_{cl}N1$)	3

Results. Indirect contact infection led to no or only slight respiratory disease in the pigs. The $H1_{cl}N1$ 1986 virus induced almost no symptoms whereas the $H1_{pdm}N2$ virus induced slight symptoms. The strain $H1_{pdm}N1$ 2009 (1580) did not cause any disease at all (Supplement 7). The $H1_{pdm}N1$ and $H1_{pdm}N2$ virus replicated well as reflected by virus shedding data whereas the $H1_{cl}N1$ virus provoked only a short period of low virus shedding (Supplement 8). Despite its very high virulence in high-dose aerosol infection the $H1_{pdm}N1$ 2009 strain 1580 did not cause disease in indirect contact infection of healthy pigs without co-infections. Supplement 9 shows the lung lesions on 8 days after indirect contact.

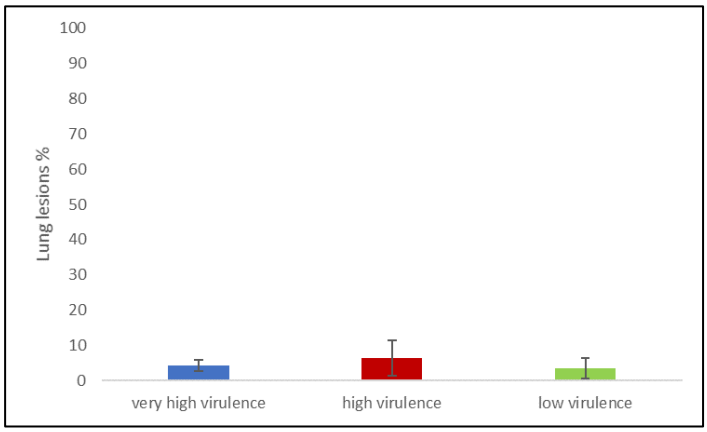
Short interpretation of results. The data show that natural influenza needs co-factors in order to induce disease. The $H1_{pdm}N1$ 2009 virus was the most virulent virus in high-dose aerosol infection but did not induce any disease after indirect contact. The $H1_{pdm}N1$ virus used in the trial had been isolated from a human patient. Despite this, it showed the highest transmissibility and replication in upper respiratory tract. The quick infection by indirect contact indicate the forming of aerosol with high contents of virus by the other pigs which had been infected previously. The other 2 viruses had been isolated from pigs. Among the pig isolates the strain with the higher virulence ($H1_{pdm}N2$) induced slight symptoms after indirect contact infection despite successful infection as reflected by virus shedding. The disease pattern induced by contact infection corresponds to low-dose aerosol, low-dose intratracheal or intranasal infection.



Supplement 7: Symptoms in pigs infected via indirect contact with $H1_{pdm}N1$ virus (dyspnoea scoe)



Supplement 8: Virus excretion in nasal swabs after indirect contact infection with $H1_{pdm}N1$ virus



Supplement 9: Lungs lesion on 8 days after indirect contact

SUPPLEMENT CHAPTER 7 – AEROSOL VERSUS INTRATRACHEAL

COMPARATIVE ANALYSIS OF HIGH-DOSE RESPIRATORY INFECTION MODELS

Background. The outcome of intratracheal infections varies according to the dose injected into the trachea. If the dose is low, the course of infection and disease is similar to intranasal infection. High-dose intratracheal infection can also be used to induce disease in pigs. This involves injecting high doses of virus into the trachea. This can be achieved by concentrating the virus harvest. Higher volumes of virus suspension can also be injected into the trachea to avoid concentration of virus suspension.

Study design. Three independent studies of high-dose aerosol infection and high-dose intratracheal infection were conducted in parallel. The same infectious material was used for infection. A dose equivalent to that contained in 1 m^3 of air in the aerosol challenge was injected into the trachea of each pig in the intratracheal challenge (Supplementary Table 7). To achieve such a high dose, 10 ml of virus suspension had to be introduced into the trachea of each pig. The trials were conducted in parallel in different infection units. Pigs were used at 12 weeks of age. Pigs were taken from the same farrowing event and from the same farm.

Supplementary Table 7: Overview of design of the comparative trials high-dose aserosol versus high-dose intratracheal infection

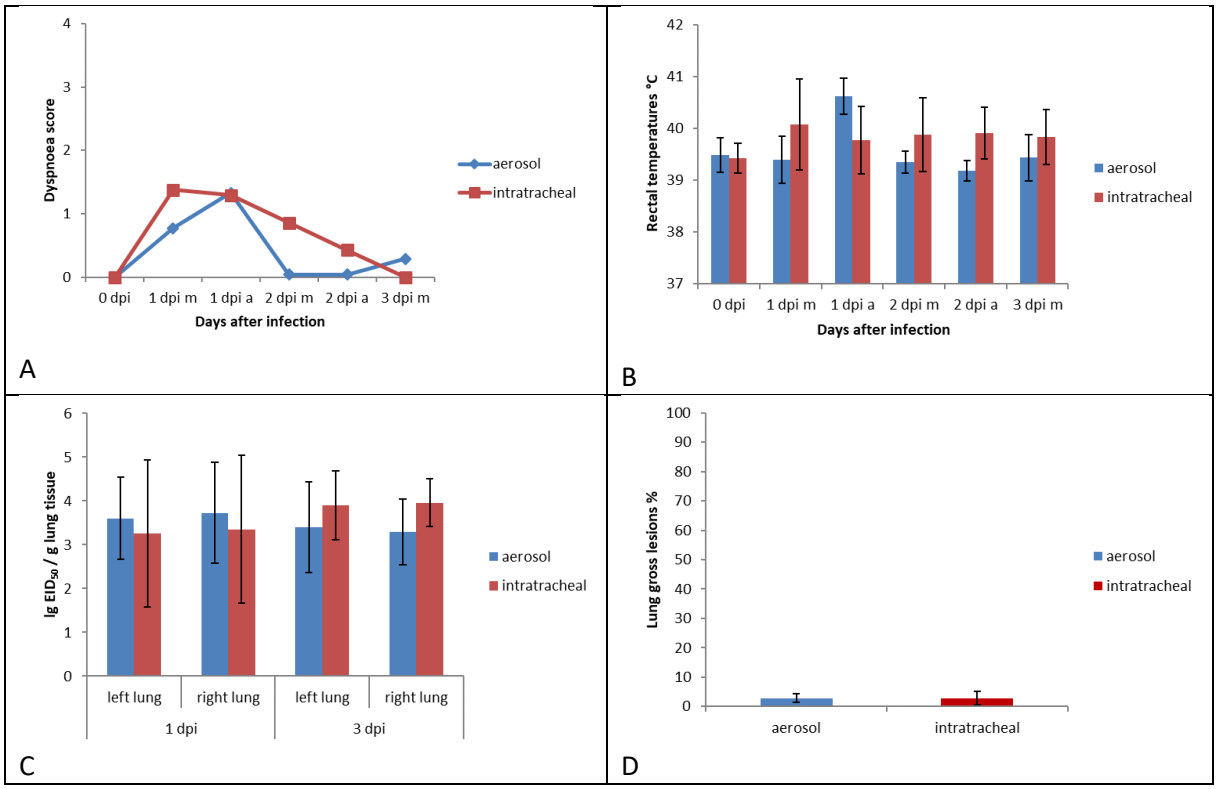
Strain FLUAV/sw/	Infection route	Infection dose $TCID_{50}$	Number of pigs*
Bad Griesbach/5604/2006 ($H1_{av}N1$)	aerosol	8.51/m^3	12
	intratracheal	8.6/pig	13
Kitzen/6142/2007 ($H1_{hu}N2$)	aerosol	8.75/m^3	15
	intratracheal	8.55/pig	15
Damme/5673/2006 (H3N2)	aerosol	9.75/m^3	15
	intratracheal	9.5/pig	15

* age: 12 weeks of life at infection

Results. Both models of infection led to symptoms (Supplement 10, Supplement 11, Supplement 12). Aerosol infection induced significantly higher rectal temperatures at 1 dpi in two studies ($H1_{hu}N2$, H3N2) and significantly higher symptoms in one study (H3N2). There was a trend towards higher lung viral loads in aerosol-infected pigs at 1 dpi; lung viral loads in intratracheally-infected pigs increased from 1 to 3 dpi (Supplement 10, Supplement 11, Supplement 12). Both infection models were suitable for demonstrating vaccine efficacy (data not shown).

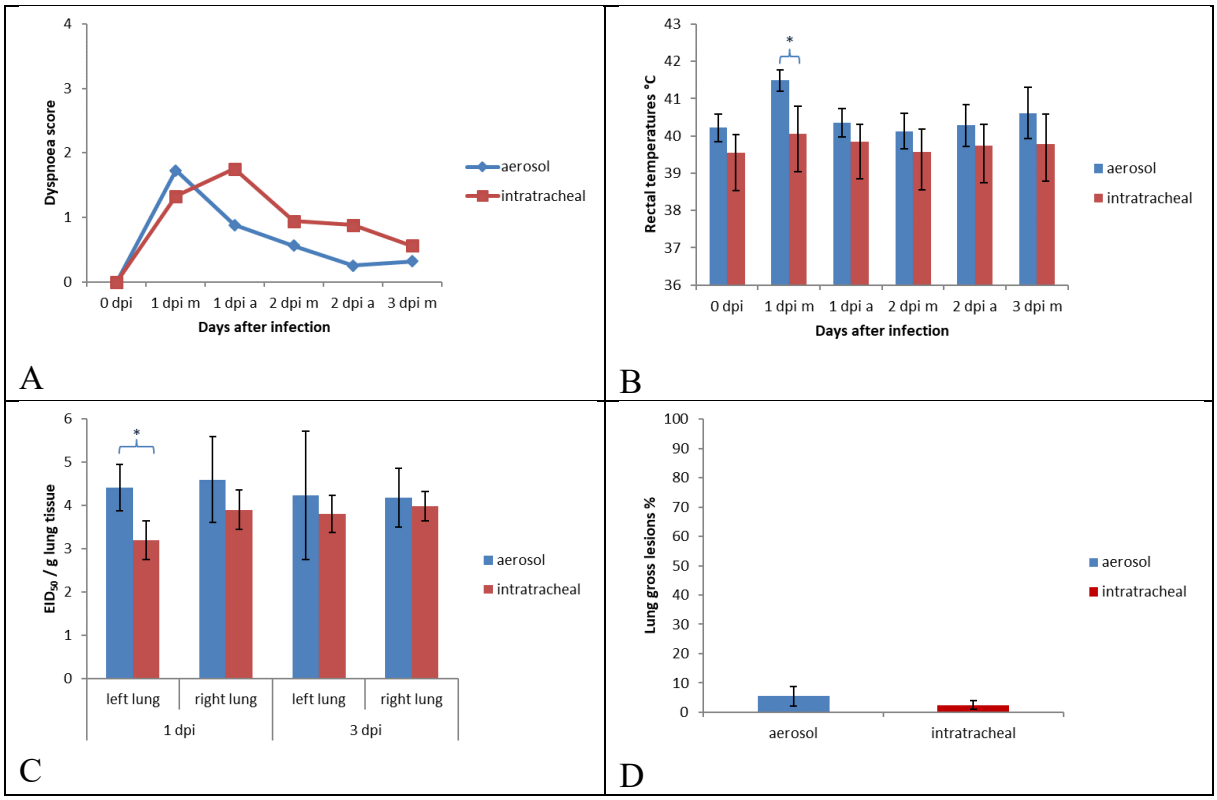
The intratracheal route of infection had several disadvantages: i) injection failure or large differences in lung virus distribution occurred in a few pigs (Supplementary Table 8), (ii) artificial lung lesions induced by injection, iii) more time and staff were needed

to carry out the infection procedure, (iv) some pigs showed signs of respiratory distress after injection.

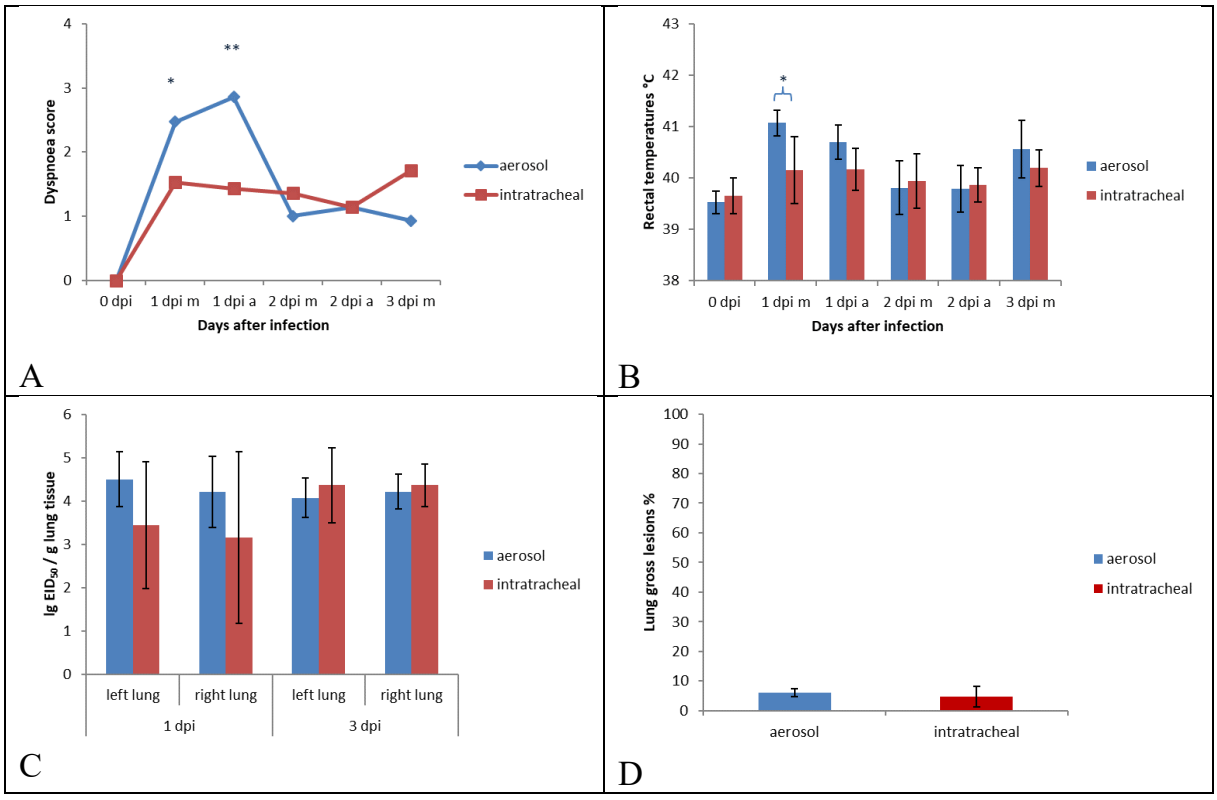


Supplement 10: Comparison of high-dose aerosol (blue) and intratracheal (red) infection with FLUAV/sw/Bad Griesbach/5604/2006 ($H1_{av}N1$); A, Dyspnoea score (arithmetic mean); B, Rectal temperatures (°C); C, Viral lung load (EID_{50}/g lung tissue); D, Lung gross lesions (%), arithmetic means with standard deviation

Short interpretation of results. Some shortcomings of the intratracheal injection procedure must be considered when interpreting the data, such as the possibility of injection failure and uneven distribution of virus in the lungs, as well as the possibility of artificial lung injury. In addition, the handling of the pigs during intratracheal injection may influence the clinical outcome. Due to the longer time required to infect a larger number of pigs by the intratracheal route, the comparability in terms of time after infection is less balanced. There was a trend towards higher lung viral titres at 3 dpi after intratracheal injection compared to 1 dpi, indicating that the highest viral load had not yet been reached at 1 dpi. This is in contrast to aerosol infection, where a high lung viral load is achieved at 1 dpi, suggesting that the pattern achieved by intratracheal infection is intermediate between high-dose aerosol infection and intranasal infection.



Supplement 11: Comparison of high-dose aerosol (blue) and intratracheal (red) infection with FLUAV/sw/Kitzen/6142/2007 ($H1_{hu}N2$); A, Dyspnoea score (arithmetic mean); B, Rectal temperatures (°C); C, Viral lung load (EID_{50}/g lung tissue); D, Lung gross lesions (%), arithmetic means with standard deviation



Supplement 12: Comparison of high-dose aerosol (blue) and intratracheal (red) infection with FLUAV/sw/Damme/5673/2006 (H3N2); A, Dyspnoea score (arithmetic mean); B, Rectal temperatures (°C); C, Viral lung load (EID_{50}/g lung tissue); D, Lung gross lesions (%), arithmetic means with standard deviation

Supplementary Table 8: Individual lung viral loads (EID_{50}/g lung tissue) in pigs infected by high-dose aerosol or intratracheal infection at 1 dpi; cases of injection failure or severe imbalance in virus distribution are shown in bold; no differences were seen at 3 dpi because pigs with injection failure could not be identified as they had since been infected by contact

Aerosol		Intratracheal	
Left lung	Right lung	Left lung	Right lung
$H1_{av}N1$			
3.1	4.5	4.5	4.5
4.9	4.5	4.5	4.5
2.5	3.7	**0**	**0**
4.5	4.5	3.1	3.9
3.7	3.7	3.5	3.7
2.9	3.5	3.9	3.5
$H1_{hu}N2$			
4.3	4.5	**0**	**2.7**
5.3	6.7	4.5	3.5
3.7	4.3	3.1	3.5
4.5	4.5	3.7	4.5
3.9	3.9	3.9	4.5
4.7	4.5	3.3	4.3
H3N2			
4.3	3.1	3.9	3.9
5.9	4.5	4.7	4.,3
4.5	4.5	3.5	4.5
4.5	5.5	3.5	3.5
4.5	3.5	4.3	4.5
4.1	4.5	**0**	**0**
4.5	3.3	**3.3**	**0**
4.5	4.5	4.3	4.5
3.7	4.7	-	-

SUPPLEMENT CHAPTER 8 – DOSE TITRATION OF INFLUENZA VIRUSES

DOSE TITRATION OF PANDEMIC H1N1 2009 VIRUS BY AEROSOL

Background. The dose of infection may be critical for disease induction in experimental influenza A virus infection. Nebulisation of different doses of influenza A virus may provide further insight into this issue.

Study design. Infection dose titration was performed using aerosol infection with strain FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$). The study included six groups of pigs (Supplementary Table 9). Each group contained 6-7 pigs. Five groups were challenged by aerosol. The following doses were nebulised: (1) 2 000 000 $TCID_{50}/m^3$, (2) 20 000 $TCID_{50}/m^3$, (3) 200 $TCID_{50}/m^3$, (4) 2 $TCID_{50}/m^3$, (5) 0.02 $TCID_{50}/m^3$ ($TCID_{50}$ determined using MDBK cells) (Supplementary Table 9). Infection was carried out in successive runs in a separate room close to the infection unit, starting with the lowest dose. The pigs were exposed to the aerosol for 1 h and then returned to the infection unit. In addition, an indirect contact infection group was established by placing pigs in the same infection unit as the aerosol-infected pigs after infection, but without direct contact with the infected groups. Staff performed all procedures on this group first before moving to the other groups. Some pigs were removed from the experiment at 1 and 3 dpi for lung examination. Thus, there were 6-7 pigs in each group up to 1 dpi, 4 pigs up to 3 dpi, and 3 pigs thereafter.

Results. Data were obtained for HI antibodies, neutralising antibodies, neuraminidase inhibiting antibodies, virus shedding, rectal temperatures, dyspnoea score. All groups had similar profiles for antibody kinetics and virus shedding, indicating the same pattern of infection in all groups with a very short viral replication period of 5-6 days and clearance of virus after antibody induction (Supplementary Table 9, Supplement 13).

Supplementary Table 9: Overview of the experimental design of the trial

Infection route	Infection dose*		Number of pigs
aerosol	2000000 $TCID_{50}/m^3$	(lg 6.3)	6
aerosol	20000 $TCID_{50}/m^3$	(lg 4.3)	6
aerosol	200 $TCID_{50}/m^3$	(lg 2.3)	6
aerosol	2 $TCID_{50}/m^3$	(lg 0.3)	6
aerosol	0.02 $TCID_{50}/m^3$	(lg -1.7)	6
Indirect contact	unknown	-	7

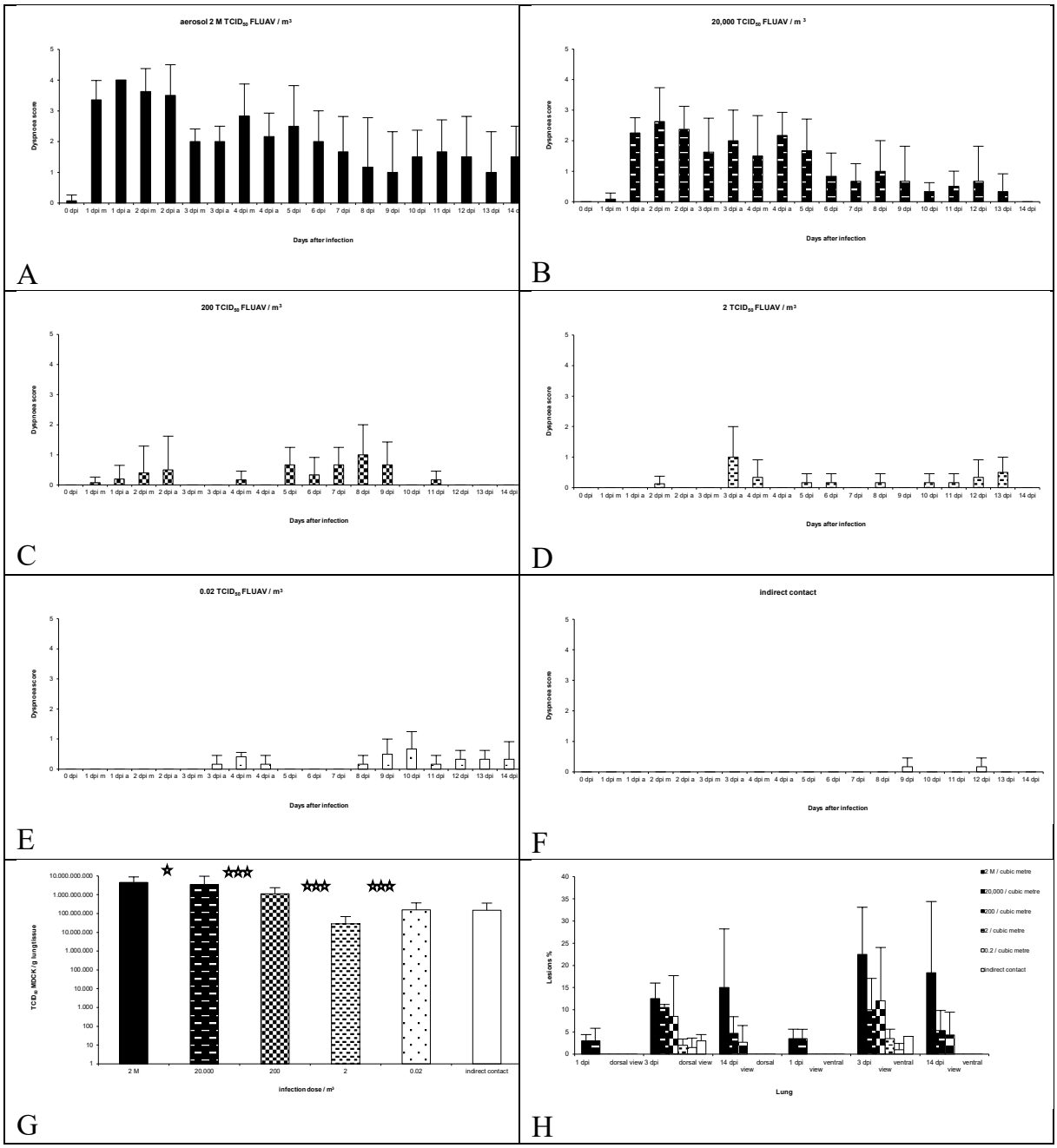
* determined in MDBK cells (for MDCK higher: + lg 2)

In the group that received the highest dose, one pig died on the second day of infection. The notable difference between high and low dose infection was the induction of severe

clinical signs with high dose infection, whereas low dose infection showed almost no signs (Supplement 13, Supplement 18).

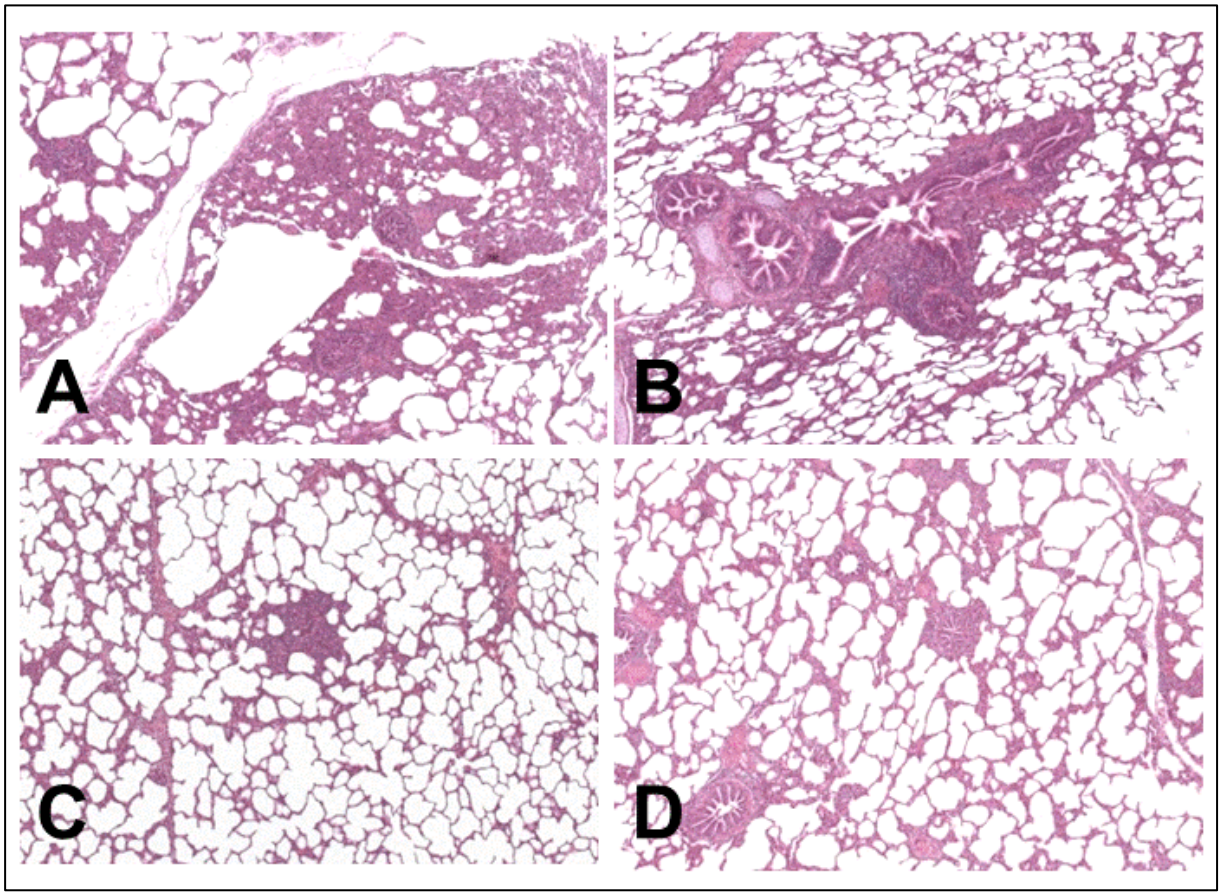
Short interpretation of results. The discrepancy between high-dose and low-dose infection is explained by the lung viral load on day 1 post-infection, which induced disease at $\geq 10^{9.55}$ MDCK $TCID_{50}$/g lung tissue (groups 1, 2) and did not at $\leq 10^{9.05}$ MDCK $TCID_{50}$/g lung tissue (all other groups).

This suggests that quantitative mechanisms are important in influenza pathogenesis. Despite the high lung viral load of $10^{9.05}$ MDCK $TCID_{50}$/g on average, the lung tissue of the pigs in group 3 did not develop pronounced symptoms. This suggests that the lung can cope with such high lung viral loads without cytokine storm induction and disease expression. This discrepancy between high and low doses may also be helpful in explaining human deaths after H5N1 infection. It underlines the importance of avoiding primary and secondary high-dose conditions of influenza virus infection (primary high-dose conditions: exposure to high doses of virus, leading to high viral lung loads in the vulnerable period within the first days after infection, before immune system responses act as shown here; secondary high-dose conditions: immunosuppression and co-infections, which can also lead to high viral lung loads in the later stages of infection). Detailed comparison of the effects of different infection doses within the first hours after aerosol infection with FLUAV/Hamburg/NY1580/2009 revealed more severe disease and faster disease induction at higher doses (Supplement 19).
The pigs that were infected with the higher dose reflected a higher level of inflammation in their lungs (Supplement 14). Photographs of the macroscopic and microscopic changes in the respiratory tract are shown in Supplement 15, Supplement 16, Supplement 17.

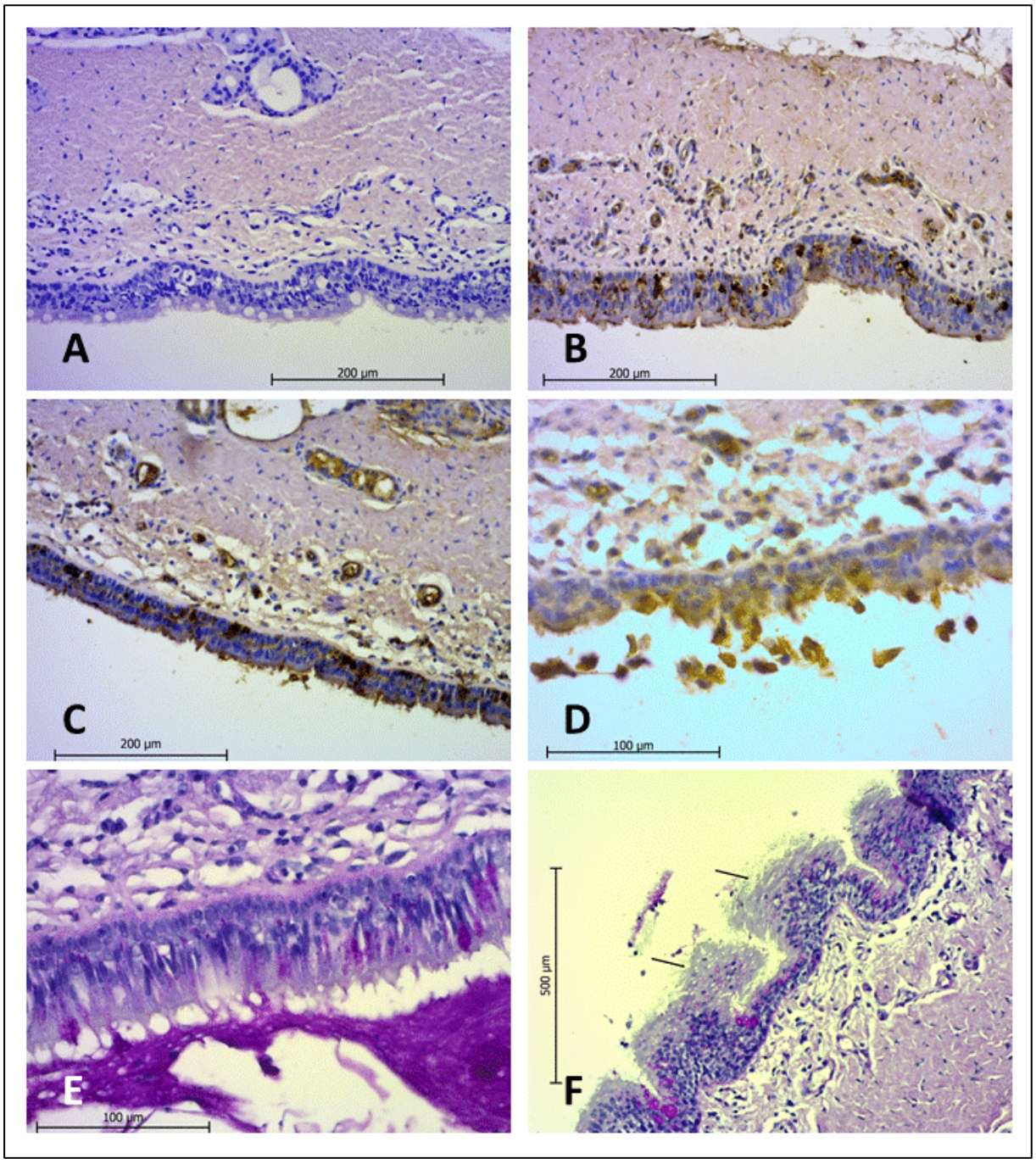


Supplement 13: Titration of infection dose (aerosol infection with FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$)

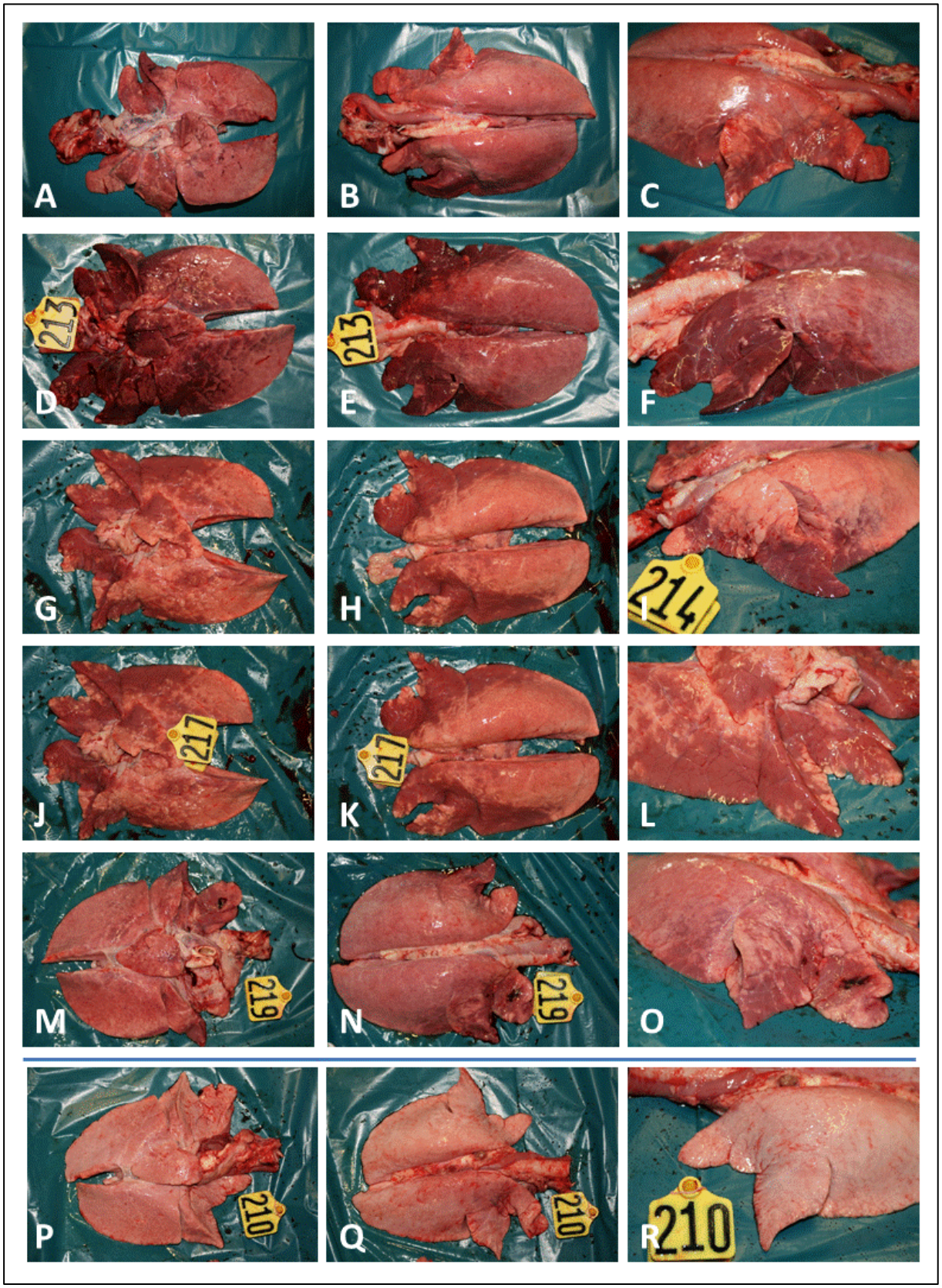
A-F, Clinical outcome (dyspnoea sore), for additional data (rectal temperatures, shedding, antibodies) see Supplement 18; 2000000 $TCID_{50}$ /m^3 (A); 20000 $TCID_{50}$/m^3 (B); 200 $TCID_{50}$/m^3 (C); 2 $TCID_{50}$/m^3 (D); 0,02 $TCID_{50}$/m^3 (E) indirect contact infection (F). G, Viral lung load ($TCID_{50}$ MDCK/g lung); arithmetic mean values based on lung samples from 2 pigs each group taken from 16 different locations within the lung of each pig; statistical differences: 2000000:20000 $TCID_{50}$/m^3 $p=0.010$, 20000:200 $TCID_{50}$/m^3 $p=0.003$, 200:2 $TCID_{50}$/m^3 $p<0.001$. 2:0.02 $TCID_{50}$/m^3 $p<0.001$, 0.002: indirect contact $p= 0.775$, Mann-Whitney U test, asymptotic, 2-sided. H, Lung lesions (%)



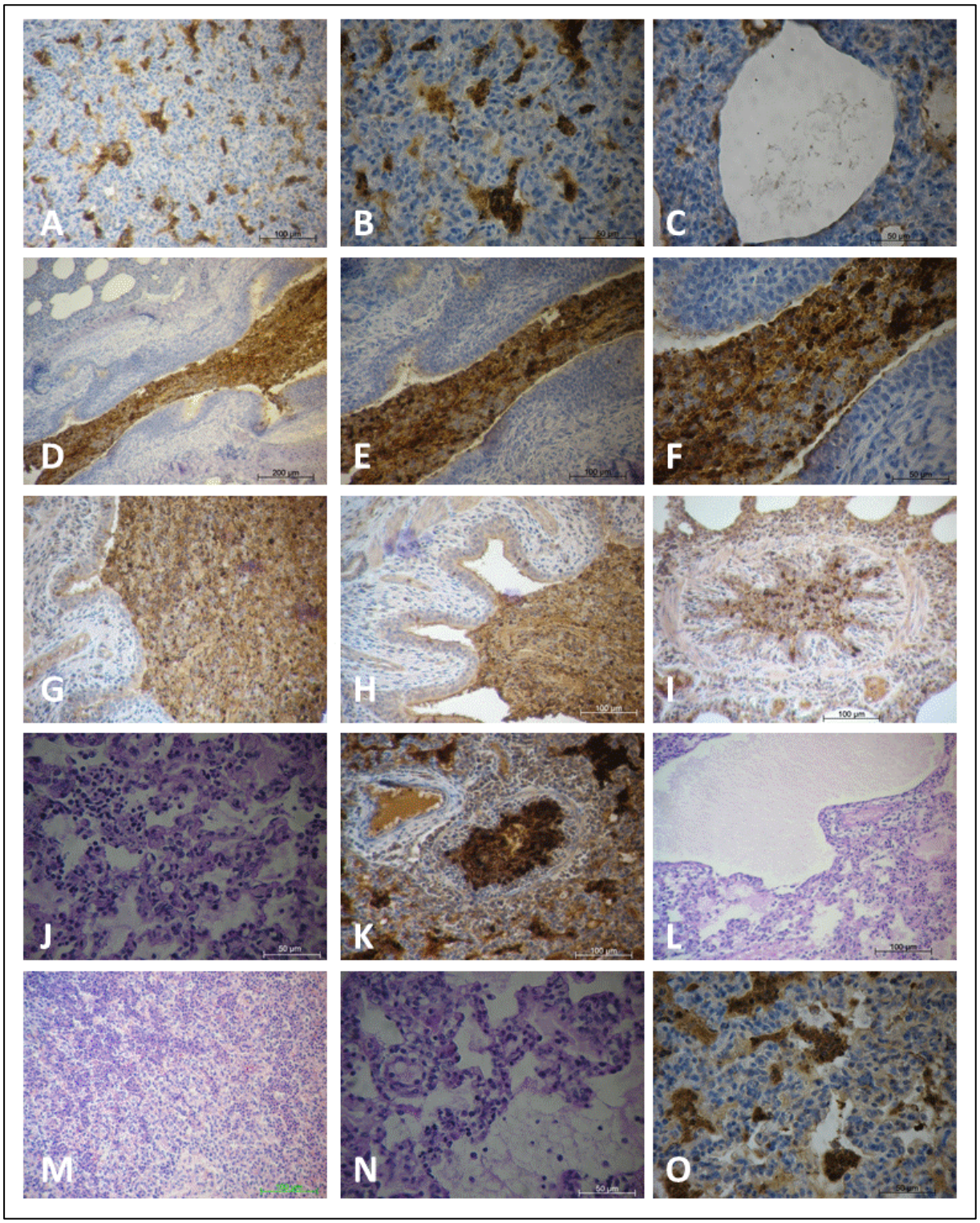
Supplement 14: H/E stainings of lung tissue sections (1 day after infection) revealed a dose dependent effect regarding the evolvement of inflammation with large areas of infiltrations in pigs infected with high doses (A, 2,000,000 $TCID_{50}/m^3$) and reduced pathology in pigs infected with lower virus doses (B, 20,000 $TCID_{50}/m^3$, C, 2 $TCID_{50}/m^3$, D, 0.02 $TCID_{50}/m^3$); H/E stainings were done by Dr. M. Sauter, Prof. Dr. K. Klingel, Tübingen



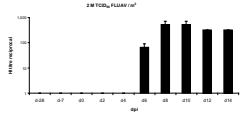
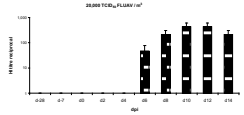
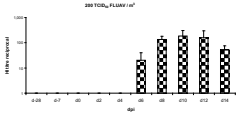
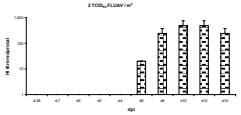
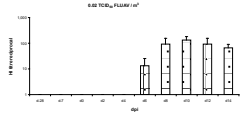
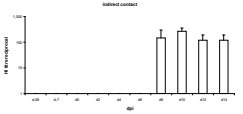
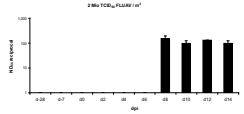
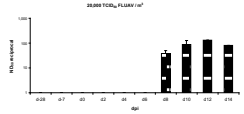
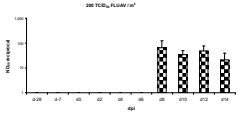
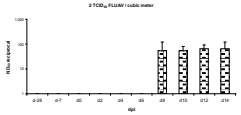
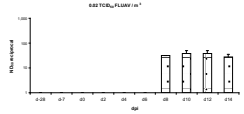
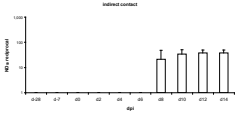
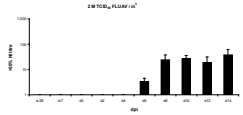
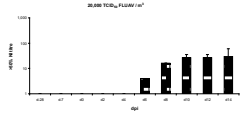
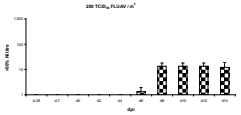
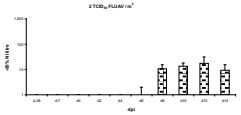
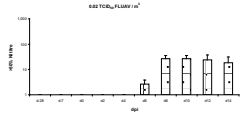
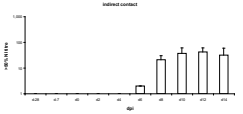
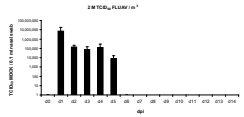
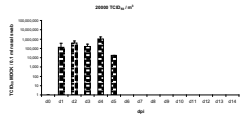
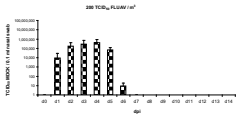
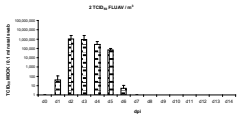
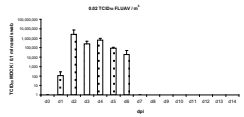
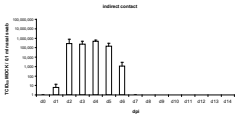
Supplement 15: Trachea of pigs after infection with A/Hamburg/NY1580/2009 $H1_{pdm}N1$ virus, A, $H1_{pdm}N1$-vaccinated pig 1 dpi: neutrophil exocytosis, intracytoplasmatic vacuolae, microvilli intact, some luminal macrophages (HE); B, the same pig (SABC staining): virus-specific staining of intraepithelial and intravacuolar macrophages and eptithelial cells of microvilli; C, not vaccinated pig 1 dpi (SABC staining): intact epithel and luminal macrophages, submucosal mononuclear infiltrates, virus-specific staining of macrophages and epithelial cells; D, not vaccinated pig 3 dpi (SABC staining) staining of epithelial cells and submucosal and epithelial macrophages, desquamation of macrophages, partial destruction of microvilli, neutropihli exocytosis; E, not vaccinated pig 1 dpi (PAS): fibrin on microvilli, submucosal mononuclear infiltration, intact epithel; F, $H1_{pdm}N1$-vaccinated pig 3 dpi (PAS): the arrows indicate fibrin on epithelial cells; some macrophages are visible and a moderate neutrophil exocytosis into the tracheal epithel



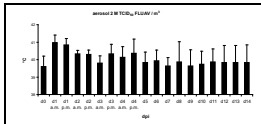
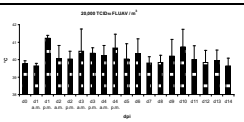
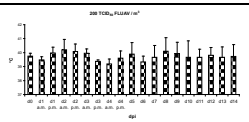
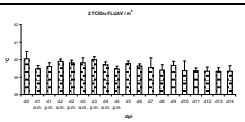
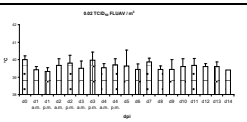
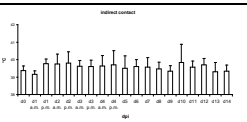
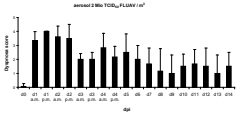
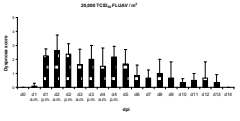
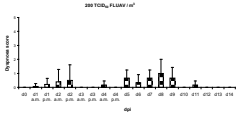
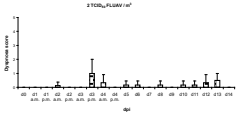
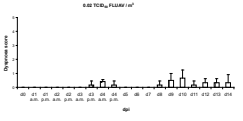
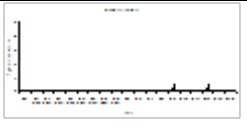
Supplement 16: Lungs of pigs after experimental aerosol infection with $H1_{pdm}N1$ April 2009 virus (A/Hamburg/NY1580/2009); A, B, C, ventral, dorsal and lateral view of lung of a pig (235) which died 2 dpi; D, E, F, ventral, dorsal and lateral view of lung of a another pig which died 2 dpi; G, H, I, ventral, dorsal and lateral view of lung of a pig which was slaughtered on 3 dpi; J, K, L, ventral, dorsal and lateral view of lung of another pig which was investigated on 3 dpi; M, N, O, ventral, dorsal and lateral view of lung of another pig which was investigateded on 3 dpi; P, Q, R, ventral, dorsal and lateral view of lung of a $H1_{pdm}N1$-vaccinated pig which was investigated on 3 dpi; all pigs were from the same farrowing event, 3 months old at infection and all pigs were investigated in the same trial



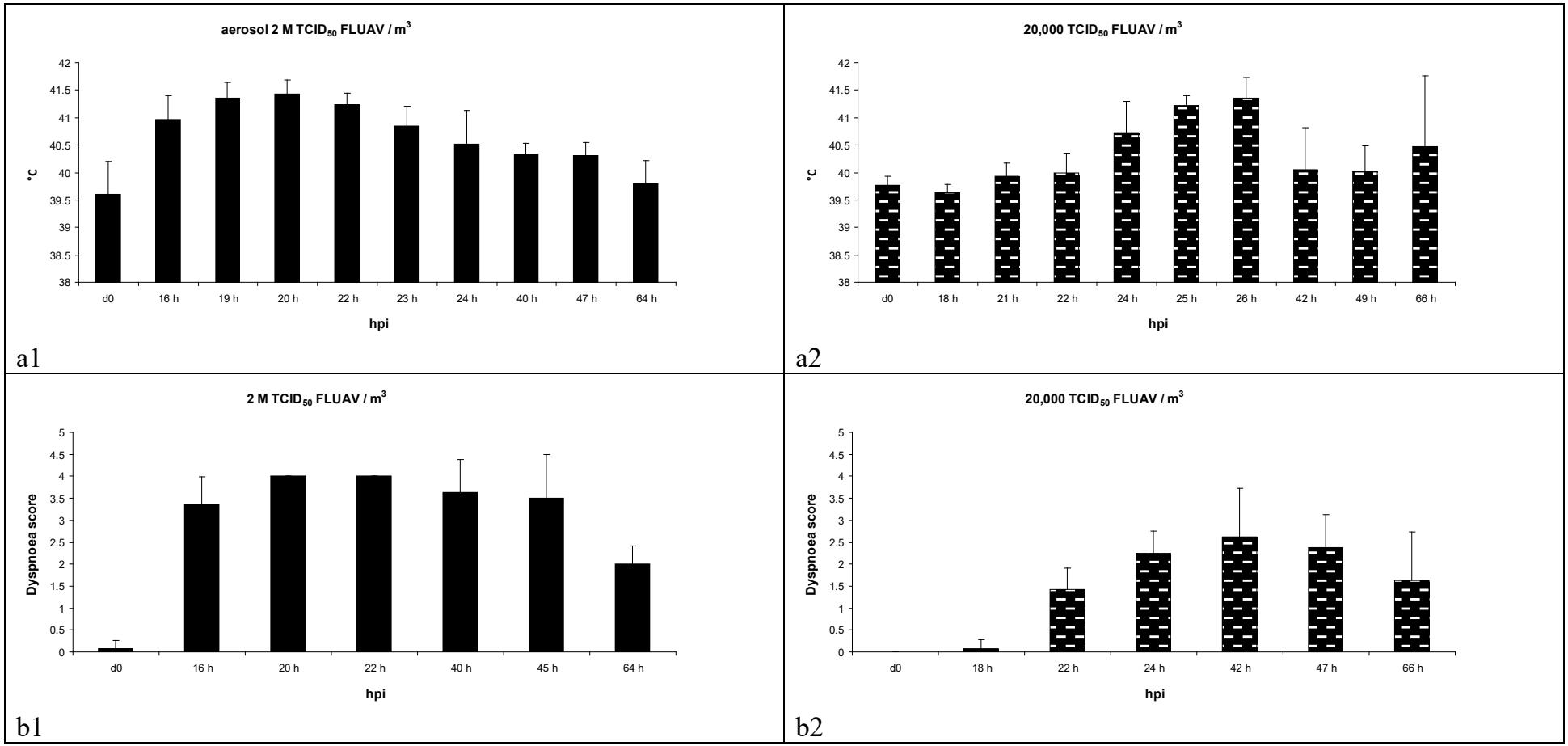
Supplement 17: Histological investigation of lungs from pigs shown in the supplement before (A-F); A-I, SABC; A-F, part from lung of pig 235 with lesion; A, massive interstitial mononuclear infiltration and atelectasis; B, same as in A at higher magnification; C, oedema; D-F, interstitial mononuclear infiltrates, partial atelectasis, massive broncholuminal alveolar macrophages and adhesion of macrophages to epithelial cells of bronchi (different magnifications; G-I, investigation of a part of the lung without macroscopic visible lesion: infiltrations occupy smaller areals as in A-F, virus-specific staining of macrophages and epithelial cells; J-O, lung of pig 213 from areals with strong lesions; J, interstitial neutrophil granulocytes (PAS); K, virus-specific staining of macrophages and epithelial cells (SABC); L, alveolar and parenchymal edema (PAS); M, massive mononuclear infiltration affecting large areals of the lung tissue (HE); N, alveolar and parenchymal edema (PAS); O, virus-specific staining of macrophages, massive infiltration of mononuclear cells (neutrophil garnulocytes) and partial atelectasis

1	2	3	4	5	6
 a) HI antibodies 2 M $TCID_{50}/m^3$	 HI antibodies 20,000 $TCID_{50}/m^3$	 HI antibodies 200 $TCID_{50}/m^3$	 HI antibodies 2 $TCID_{50}/m^3$	 HI antibodies 0.02 $TCID_{50}/m^3$	 HI antibodies Indirect contact
 b) Neutralising antibodies 2 M $TCID_{50}/m^3$	 Neutralising antibodies 20,000 $TCID_{50}/m^3$	 Neutralising antibodies 200 $TCID_{50}/m^3$	 Neutralising antibodies 2 $TCID_{50}/m^3$	 Neutralising antibodies 0.02 $TCID_{50}/m^3$	 Neutralising antibodies Indirect contact
 c) NI antibodies 2 M $TCID_{50}/m^3$	 NI antibodies 20,000 $TCID_{50}/m^3$	 NI antibodies 200 $TCID_{50}/m^3$	 NI antibodies 2 $TCID_{50}/m^3$	 NI antibodies 2 $TCID_{50}/m^3$	 NI antibodies 2 $TCID_{50}/m^3$
 d) Virus shedding 2 M $TCID_{50}/m^3$	 Virus shedding 20,000 $TCID_{50}/m^3$	 Virus shedding 200 $TCID_{50}/m^3$	 Virus shedding 200 $TCID_{50}/m^3$	 Virus shedding 200 $TCID_{50}/m^3$	 Virus shedding 200 $TCID_{50}/m^3$

Supplement 18: Titration of infection dose (aerosol infection with FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$) using: (1) 2,000,000 $TCID_{50}$ /m^3, (2) 20,000 $TCID_{50}/m^3$, (3) 200 $TCID_{50}/m^3$, (4) 2 $TCID_{50}/m^3$, (5) 0.02 $TCID_{50}/m^3$ ($TCID_{50}$ determined using MDBK cells), (6) Indirect contact infection by the same strain; a), HI antibodies (HI titre reciprocal); b), Neutralising antibodies (ND_{50} reciprocal); c), Neuraminidase inhibiting antibodies (>50% Neuraminidase inhibition titre); d), Virus excretion ($TCID_{50}$ MDCK/0.1 ml nasal swab solution); e), Rectal temperatures (°C); f), Dyspnoea score (Score); 6-7 pigs in each group until 1 dpi, 4 pigs in each group till 3 dpi, 3 pigs each group thereafter (continued next page); arithmetic means with standard deviation, for antibodies geometric means

 e) Rectal temperatures 2 M $TCID_{50}/m^3$	 Rectal temperatures 20,000 $TCID_{50}/m^3$	 Rectal temperatures 200 $TCID_{50}/m^3$	 Rectal temperatures 200 $TCID_{50}/m^3$	 Rectal temperatures 200 $TCID_{50}/m^3$	 Rectal temperatures 200 $TCID_{50}/m^3$
 f) Dyspnoea 2 M $TCID_{50}/m^3$	 Dyspnoea 20,000 $TCID_{50}/m^3$	 Dyspnoea 200 $TCID_{50}/m^3$	 Dyspnoea 200 $TCID_{50}/m^3$	 Dyspnoea 200 $TCID_{50}/m^3$	 Dyspnoea 200 $TCID_{50}/m^3$

Supplement 18 continued: e), Rectal temperatures (°C); f), Dyspnoea score (Score); 6-7 pigs in each group until 1 dpi, 4 pigs in each group till 3 dpi, 3 pigs each group thereafter; *arithmetic means with standard deviation, for antibodies geometric means*



Supplement 19: Comparison of the effects of different infection doses within the first hours after aerosol infection with FLUAV/Hamburg/NY1580/2009 (panH1N1) *using: 1. 2 M = 2,000,000 $TCID_{50}$ /m^3; 2. 20,000 $TCID_{50}$/m^3) reflects more severe disease and faster disease induction by higher doses; a), Rectal temperatures (°C); b), Dyspnoea score (Score). One pig of the group receiving the infection dose of 2,000,000 $TCID_{50}$/m^3 suffered from extremely severe dyspnoea and died 1.5 days pi resulting in an experimental case fatality rate of 14% (each group comprised 6 pigs); hpi, hours post infectionem; arithmetic means with standard deviation*

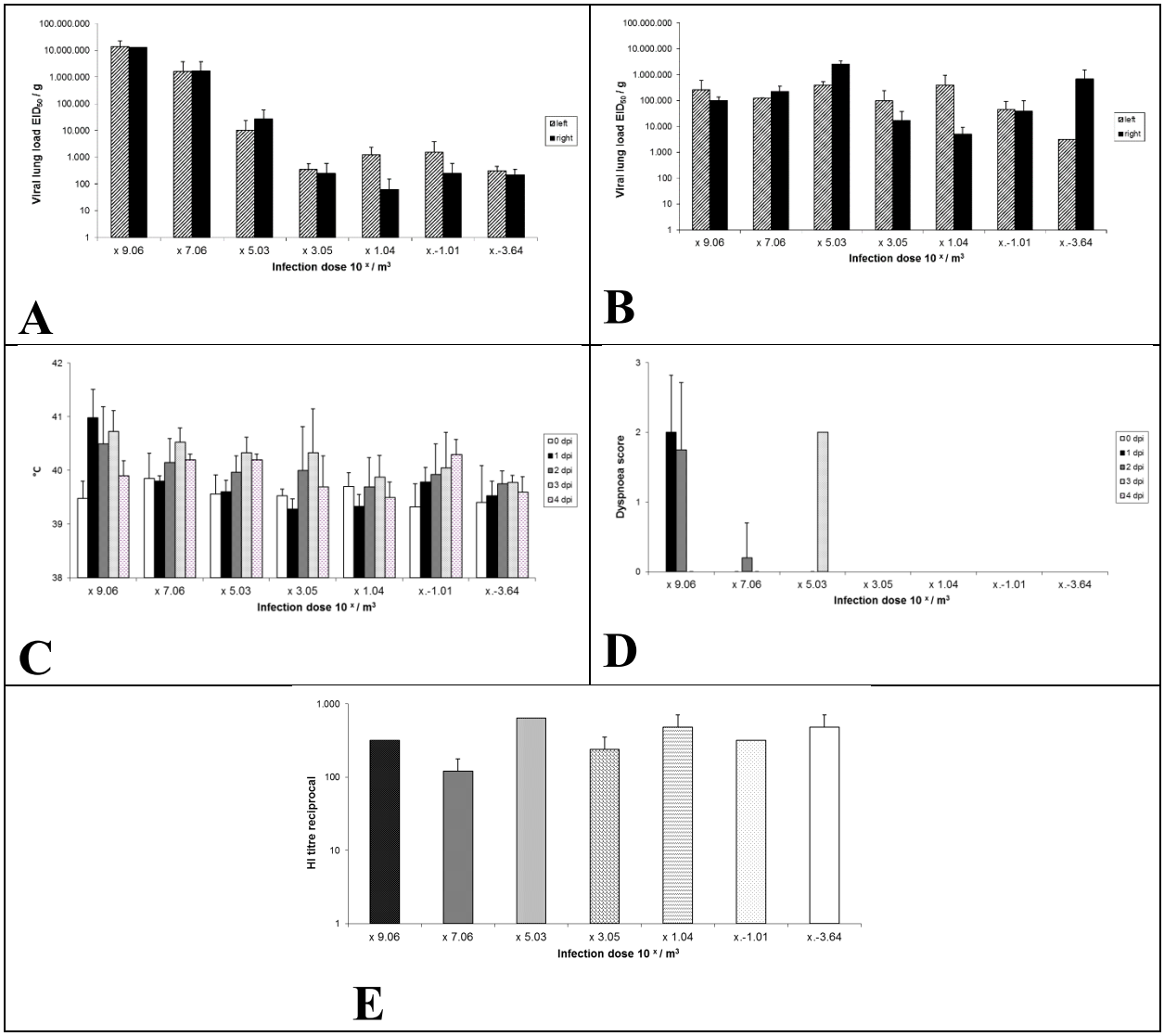
DOSE TITRATION OF SWINE HUMAN-LIKE H3N2 VIRUS BY AEROSOL

Background. Dose titration data for H3N2 were still lacking.

Study design. Titration of aerosol infection dose of H3N2 virus was performed using strain A/sw/Bakum/1769/2003 (H3N2).

Results. Viral lung load on 1 dpi and 3 dpi, rectal temperatures, dyspnoea score, antibody response against the homologous H3N2 strain (HI) were established (Supplement 20). No antibodies had been induced against $H1_{av}N1$ and $H1_{hu}N2$ virus (data not shown). 6 pigs were included in each group, 2 were taken out of the study on 1 dpi, 2 on 3 dpi, 2 pigs remained in the study until 14 dpi.

Short interpretation of results. The data reflect a correlation between infection dose and viral lung load on day 1 after infection (dpi). A high viral lung load on 1 dpi is crucial for dyspnoea and fever induction. Although viral lung titres increased until 3 dpi in the groups which received lower infection doses the viral lung load did not reach the level which the highest infection dose had on 1 dpi.



Supplement 20: Titration of aerosol infection dose of H3N2 virus (strain A/sw/Bakum/1769/2003); A, Viral lung load 1 dpi; B, Viral lung load 3 dpi; C, Rectal temperatures; D, Dyspnoea score; E, Antibody response against the homologous H3N2 strain (HI), arithmetic means with standard deviation, geometric means for antibodies

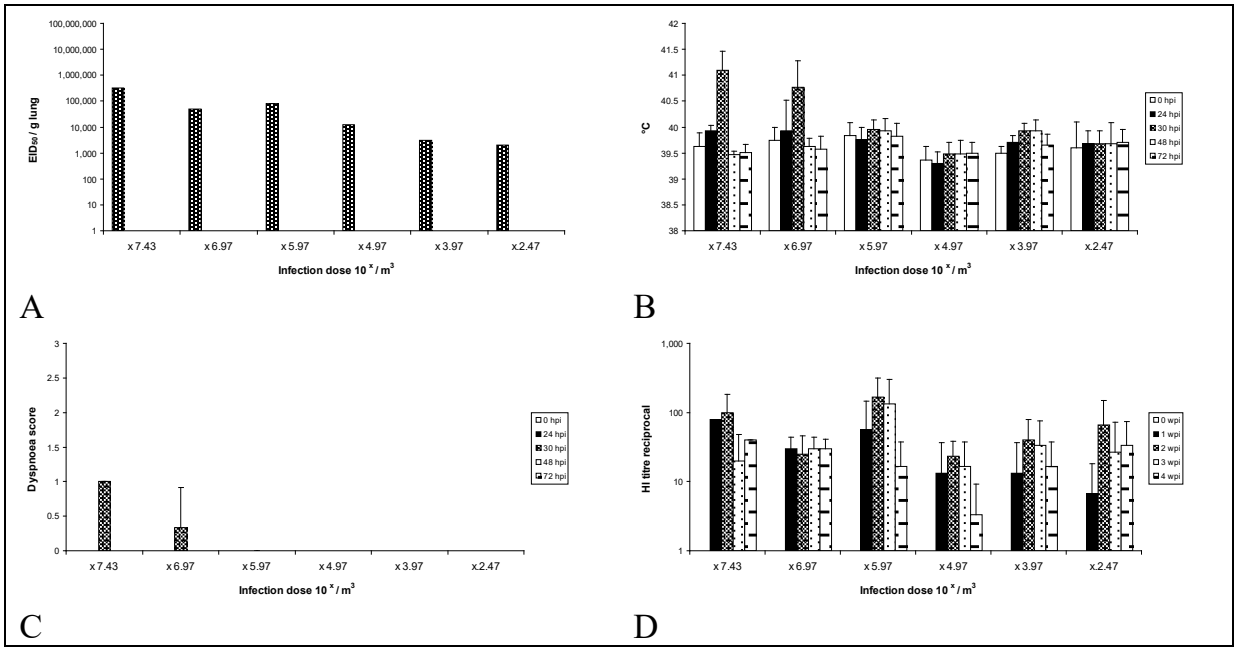
DOSE TITRATION OF SWINE AVIAN-LIKE H1N1 VIRUS BY AEROSOL

Background. Dose titration data for $H1_{av}N1$ had not yet been established.

Study design. Titration of aerosol infection dose of $H1_{av}N1$ virus was done by using strain A/sw/Vechta/2623/2003 $H1_{av}N1$).

Results. Data of viral lung load 1 dpi, rectal temperatures (note that there is only a limited period of rise in body temperature around 30 hpi), dyspnoea score, antibody response against the homologous $H1_{av}N1$ strain (HI) were established (Supplement 21). No antibodies had been induced against H3N2 and $H1_{hu}N2$ virus (data not shown). 5 pigs were included in each group, 1 slaughtered on 1 dpi, 4 pigs remained in the study until 14 dpi.

Short interpretation. The virulence of this virus is very low. There were effects of a high initial infection dose on the clinical outcome.



Supplement 21: Titration of aerosol infection dose of $H1_{av}N1$ virus (strain A/sw/Vechta/2623/2003); A, Viral lung load 1 dpi; B, Rectal temperatures (note that there is only a limited period of rise in body temperature around 30 hpi); C, Dyspnoea score; D, Antibody response against the homologous $H1_{av}N1$ strain (HI). No antibodies had been induced against H3N2 and $H1_{hu}N2$ virus. 5 pigs were included in each group, 1 slaughtered on 1 dpi, 4 pigs remained in the study until 14 dpi; arithmetic means with standard deviation, geometric means for antibodies

hpi, hours post infectionem, wpi, weeks post infectionem.

DOSE TITRATION OF CLASSICAL SWINE H1N1 VIRUS BY AEROSOL

Background. $H1_{pdm}N1$ reflected a very high virulence. Therefore it was of interest to investigate wheter a closely related virus like $H1_{cl}N1$ is highly virulent or not.

Study design. In order to investigate the virulence of classical swine influenza A H1N1 virus ($H1_{cl}N1$) a virus isolate from pigs in UK of 1986 was used. FLUAV/sw/England/117316/1986 ($H1_{cl}N1$) was cultivated on MDBK cells. The virus replicated to titres of 5.5 lg $TCID_{50}$/ml in roller bottles. In order to increase the amount of viruses the harvest was concentrated 10:1. The harvest was diluted 1:100 and 1:10000. Five concentrations were available for infection.
Pigs of the same farrowing event were divided randomly into several groups and infected on their 96th day of life (Supplementary Table 10). One group served as indirect contact control and was housed together with the other pigs after infection in the same infection room in order to mimic natural infection. A strict control group consisted of 4 pigs and was kept in the farm outside of the infection unit. On dpi 1 and 3 lung samples were taken (from 5 pigs of group 1 and 2 pigs of groups 2-6 and 1 pig of group 7). On 9 dpi lung samples were taken from the remaining pigs, and the trial was finished. Regarding all other parameters the trial procedure followed the general outline as it was done with the other trials (observation and record of clinical symptoms, taking nasal swabs and blood samples daily; weighing).

Supplementary Table 10: Overview of experimental design of infection trial $H1_{cl}N1$

Group	Infection dose lg $TCID_{50}$ / m^3	Abbreviation	Number of pigs included
1	7.71	7.71 $TCID_{50}$ / m^3	15
2	6.71	6.71 $TCID_{50}$ / m^3	8
3	4.71	4.71 $TCID_{50}$ / m^3	8
4	2.71	2.71 $TCID_{50}$ / m^3	8
5	0.71	0.71 $TCID_{50}$ / m^3	8
6	Indirect contact	Indirect contact	8
7	None (strict control)	-	4

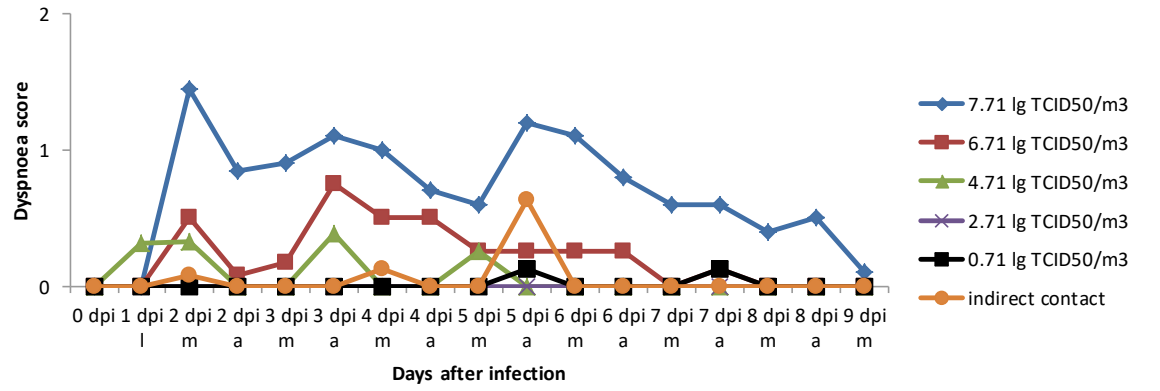
Results. The data are summarized in Supplement 22. The virus induced moderate symptoms. A clear expression of respiratory disease was only detectable in the group that received the highest infection dose. Pigs of the strict control showed no symptoms, no changes in the lungs and no antibodies against influenza during the entire trial (data not shown).
Virus excretion kinetics had its peak on 3-5 dpi in groups which had been exposed to the highest infection doses whereas pigs of the other groups shed the highest amount of virus on 6 dpi.
There was a slight increase in rectal temperatures in some individual pigs of the groups 1 and 2 which were exposed to the highest doses but no induction of fever. This again reflects the low virulence of this virus after decades of circulation in the pig population.

Pigs responded to infection by antibodies on 7-9 dpi. Pigs exposed to the highest infection doses responded already on 7 dpi.

The viral lung load on 1 dpi was highest in the groups that had been exposed to the highest doses and followed a linear trend. On 3 dpi the viral lung load had been decreased and equilibrated in these groups. The groups which had been received the lowest doses reflected only low viral lung load. The pigs of the indirect contact control group had no virus in their lungs on 1 and 3 dpi. The pigs of the strict control group had no virus in their lungs, no lesions and no histological alterations (data not shown).

Lung lesions were highest in the pigs of groups 1-3 on 3 dpi and decreased thereafter whereas in the groups which had been exposed to lower doses and in the indirect contact control group lung lesions were at their peak on day 9 dpi.

The degree of inflammation varied according to the day after infection. Pigs exposed to the lower doses responded later to the virus indicating that infection of the lung was postponed which is in agreement with the data obtained for viral lung load. Differences in body weights were observed on 8 dpi. This may due to the differences in starting body weights.



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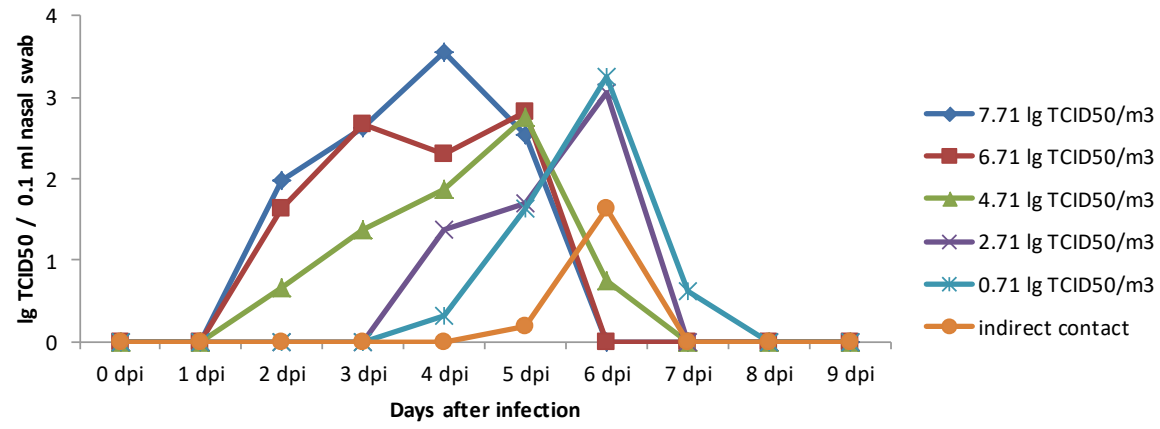

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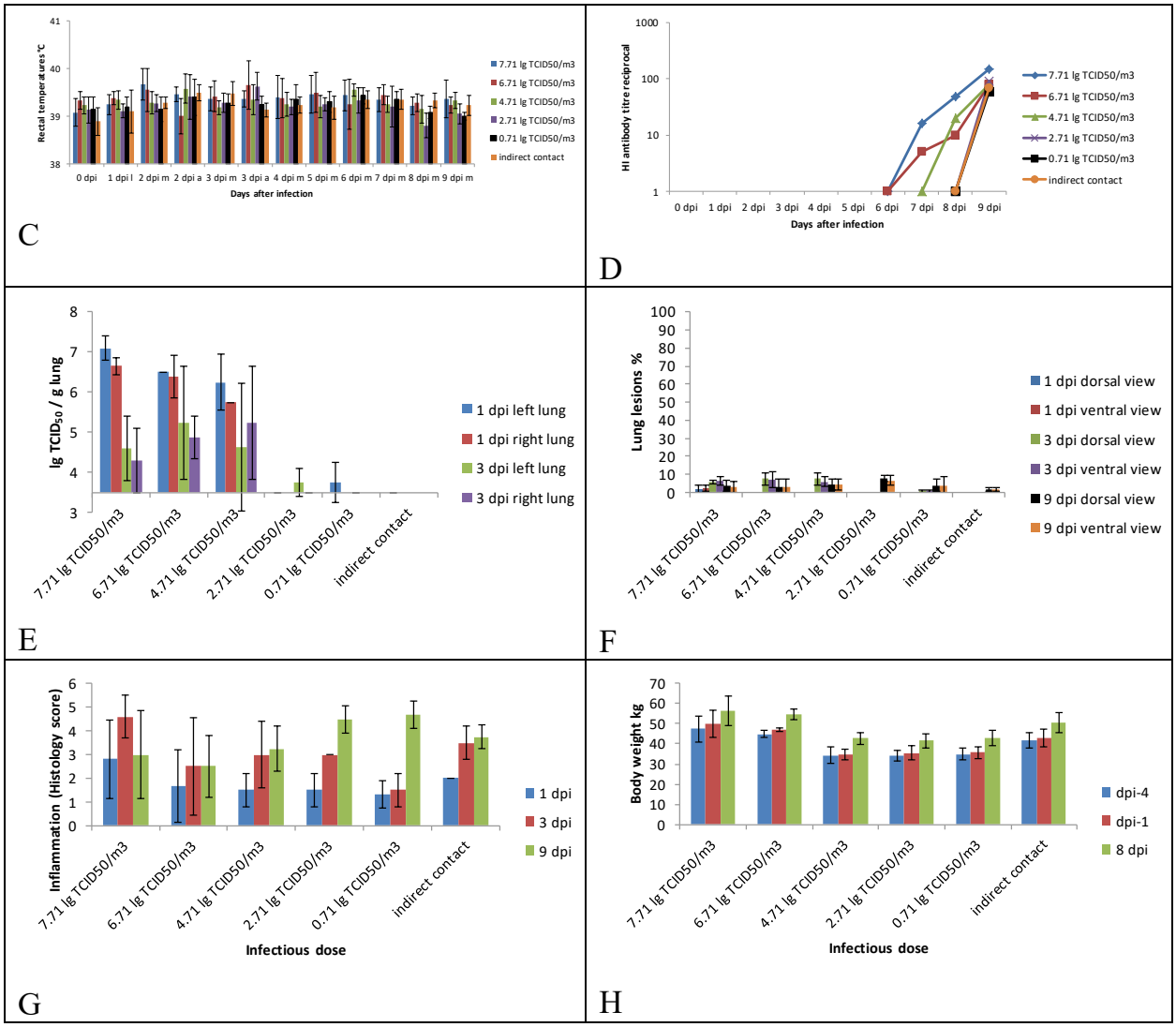


Supplement 22: Parameters after infection of pigs with different infectious doses of FLUAV/sw/England/117316/1986 ($H1_{cl}N1$); A, Symptoms (dyspnoea score; arithmetic mean values without standard deviation), there were significant differences between group 7.71 lg $TCID_{50}/m^3$ and the other groups from 2-8 dpi (, $p<0.05$) but not in between the other groups; B, Virus shedding kinetics (lg $TCID_{50}$/0.1 ml nasal swab solution), there were no significant differences between group 7.71 + 6.71 lg $TCID_{50}/m^3$ and the other groups but significant differences (*, $p<0.05$) between both groups and group 4.71 lg $TCID_{50}/m^3$ from 1-4 dpi and with all the other groups; groups 2.71 + 0.71 lg $TCID_{50}/m^3$ reflected a delay in shedding similar to the indirect contact group but shed significantly more virus than the latter (*, $p<0.05$); C, Rectal temperatures (°C); there were no significant differences between the groups but groups 7.71 + 6.71 lg $TCID_{50}/m^3$ showed a higher standard deviation on 2 and 3 dpi indicating higher temperatures in a few pigs; D, Antibody induction (HI titre); groups receiving higher doses responed earlier and with higher antibody titres; E, Viral lung load (lg $TCID_{50}$/g); viral lung load was higher in the groups which got higher infectious doses (group 7.71 versus 6.71 lg $TCID_{50}/m^3$ left lung 1 dpi *, $p<0.05$; group 6.71 versus 5.71 lg $TCID_{50}/m^3$; right lung *, $p<0.05$; no significant differences within the other parts and days of groups 7.71, 6.71, and 4.71 lg $TCID_{50}/m^3$, in the other groups which received lower doses viral lung load was very low; F, Lung lesions (%); lung gross lesions were moderate but stronger in the groups which got the higher infectious doses; G, Inflammation (histological score) differed significantly between groups 7.71, 6.71, and 4.71 lg $TCID_{50}/m^3$ and 2.71, 0.71, $TCID_{50}/m^3$ and indirect contact on 3 and 9 dpi; higher on 3 dpi in groups receiving higher doses and higher on 9 dpi in groups receiving lower doses; H, Body weights (kg); there was no influence of infection on body weight development: all groups gained weight; arithmetic means with standard deviation, for antibodies geometric means are shown*

Dyspnoea followed a linear trend. The steep of the trend straight line was low indicating a very low virulence of this virus.

Short interpretation of results. The data reflect that the strain displays a very low virulence which is in contrast to $H1_{pdm}N1$ 2009 virus which contains also the hemagglutinin of the classical swine H1N1 viruses.

DOSE TITRATION OF SWINE PANDEMIC H1N2 2010 VIRUS BY AEROSOL

Background. In 2010 a new swine influenza A virus lineage emerged as a result of reassortment between $H1_{pdm}N1$ and H3N2 virus. This $H1_{pdm}N2$ virus contained 7 segments of $H1_{pdm}N1$ and the segment coding for the neuraminidase of H3N2 virus.

Study design. The first virus of this lineage which was isolated in Germany was used for initial investigation of its virulence: strain FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$). Seventy five days old commercial cross bred pigs were challenged by aerosol (Supplementary Table 11). Pigs were treated in their 3rd and 7th week of life with tulathromycin (Draxxin™) in order to prevent bacterial co-infections. In their 11th week of life challenge infection was performed in a small chamber of a large infection unit starting with the lowest dose. Exposure time was 1 hour for each group. After the infection procedure had been finished for all groups, the indirect contact control was brought into the large infection unit but kept in a separated pen without direct contact to the other pigs. The pigs of the strict control were kept outside the building. On days 1 and 3 after infection lung samples were taken from 5 pigs of each group (groups 1-4). On day 9 after infection the lungs were taken from the remaining pigs of each group (1-4) and the pigs of the indirect contact group and strict control. Parameters were recorded as outlined in materials and methods.

Supplementary Table 11: Overview of the experimental design of dose titration of infection dose $H1_{pdm}N2$ virus

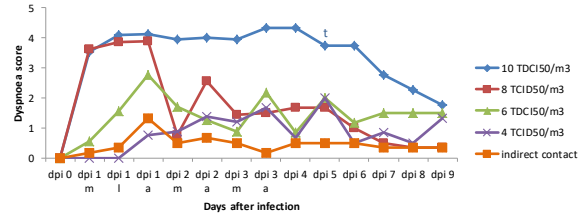
Group	Infection dose lg $TCID_{50}$ / m^3	Abbreviation	Number of pigs included
1	10.21	10 $TCID_{50}$ / m^3	13
2	8.46	8 $TCID_{50}$ / m^3	13
3	6.21	6 $TCID_{50}$ / m^3	13
4	4.71	4 $TCID_{50}$ / m^3	13
5	Indirect contact control	Indirect contact	3
6	None (strict control)	-	4

Results. In relation to the infection dose pigs developed different degrees of symptoms (Supplement 23):

- The group exposed to the highest infection dose displayed high and long lasting symptoms with severe dyspnoea and strong lung lesions and inflammation; one pig of this group died on 5 dpi,

- Pigs exposed to the second highest dose developed severe dyspnoea on 1 dpi but recovered soon,
- Pigs which got 6.21 lg $TCID_{50}/m^3$ developed disease reflecting that this strain belongs to the more virulent strains in comparison to $H1_{av}N1$, $H1_{hu}N2$, and H3N2 viruses,
- Pigs exposed to 4.71 lg $TCID_{50}/m^3$ reflected symptoms similar to the indirect contact control group.

Virus shedding reflected a bell-shaped excretion curve. Virus excretion followed the pattern which is typical for influenza virus infection reflecting peaks in shedding between 2-4 dpi; there were no significant differences between the groups. All pigs responded to the infection with an increase in rectal temperatures on 1 and 3 dpi. Fever was induced in groups exposed to higher amounts of nebulised virus. All pigs raised specific antibodies against the infection strain between 5-9 dpi. The viral lung load was high in all groups and did not differ significantly between the groups although groups which received higher doses displayed higher mean values in trend. Lung lesions were significantly higher in groups exposed to the 2 highest infectious doses. The average degree of inflammation increased from 1 dpi till 3/9 dpi; it was highest in the group which had been exposed to the highest dose but significant differences to the other groups could not be demonstrated due to the low number of animals remaining in the group until 9 dpi (n=3). There were differences in body weight gains: pigs exposed to doses 10 and 8 lg $TCID_{50}/m^3$ had significantly lower body weight gains than groups 6 and 4 lg $TCID_{50}/m^3$. The indirect control group moved in between groups 10+8 and 6+4 lg $TCID_{50}/m^3$ in body weight development but the number of pigs in this group was too low to conduct statistical analysis. Strong inflammation was shown by immunohistology.



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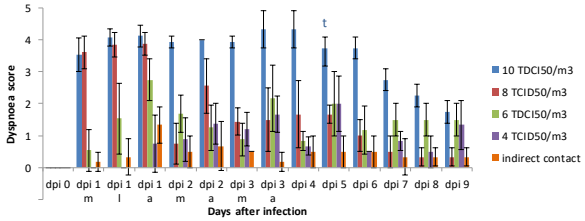

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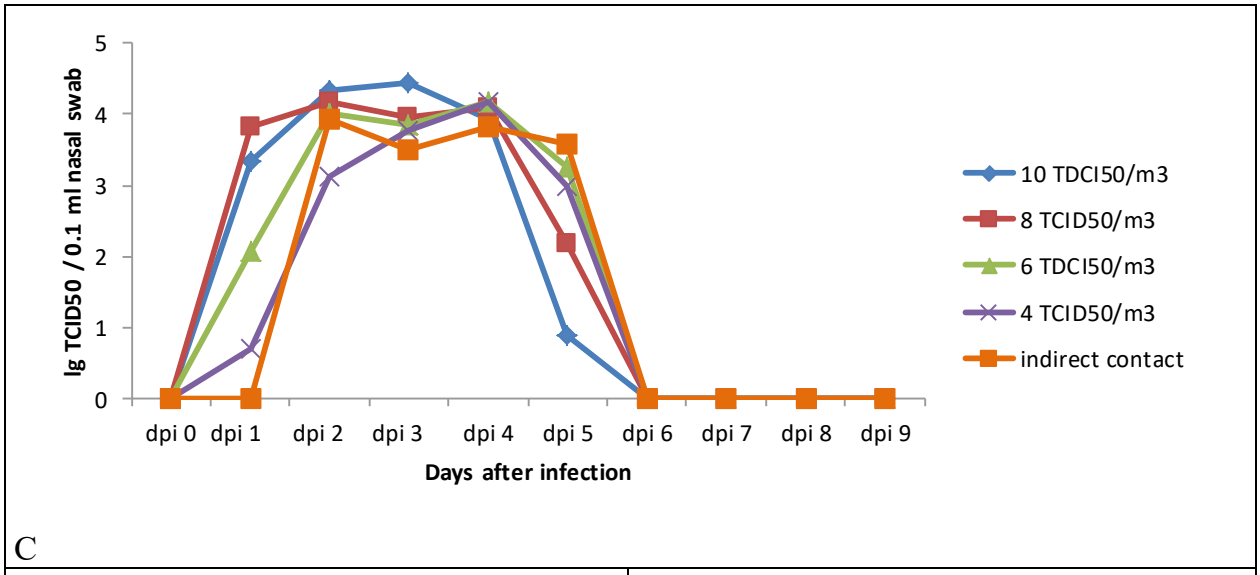

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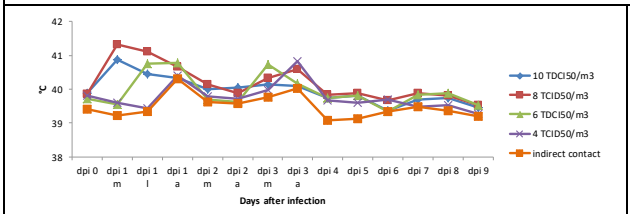

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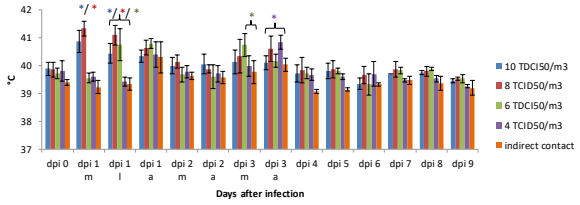

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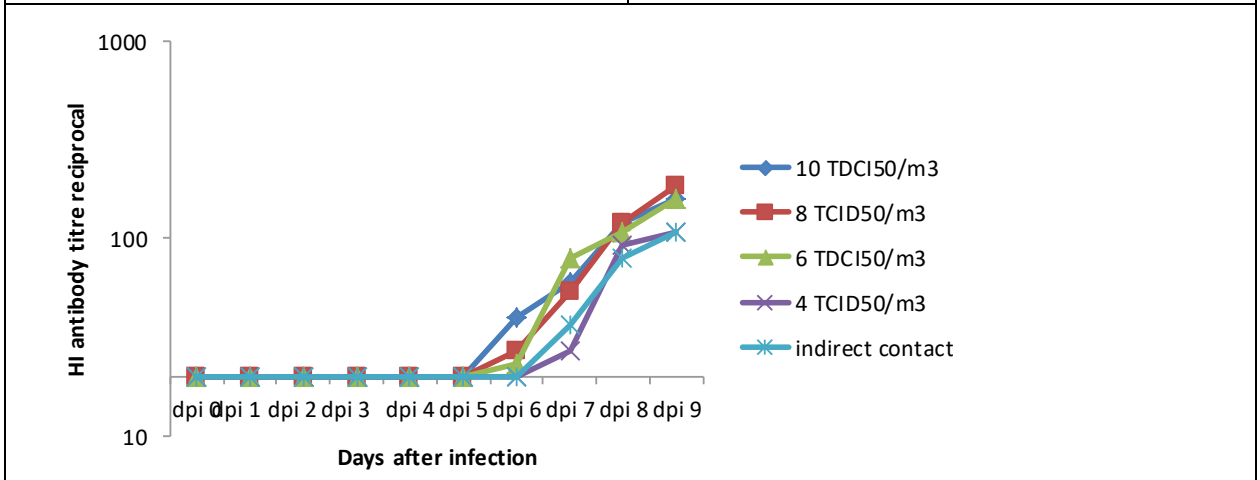

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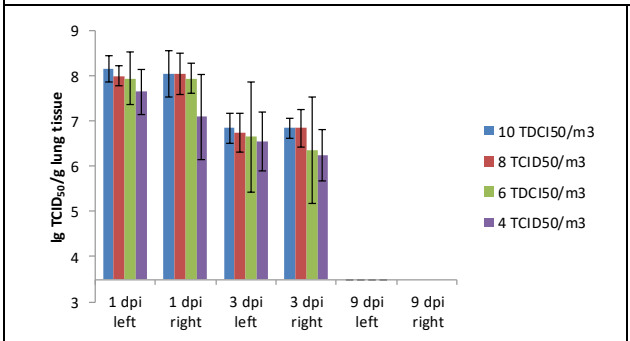

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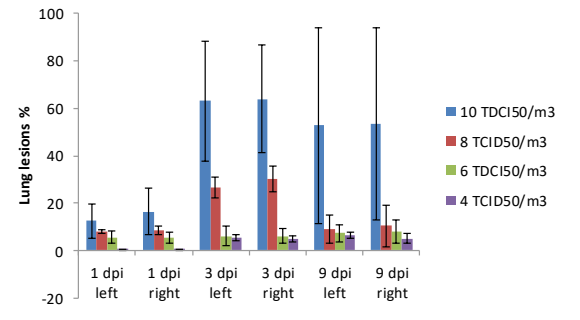

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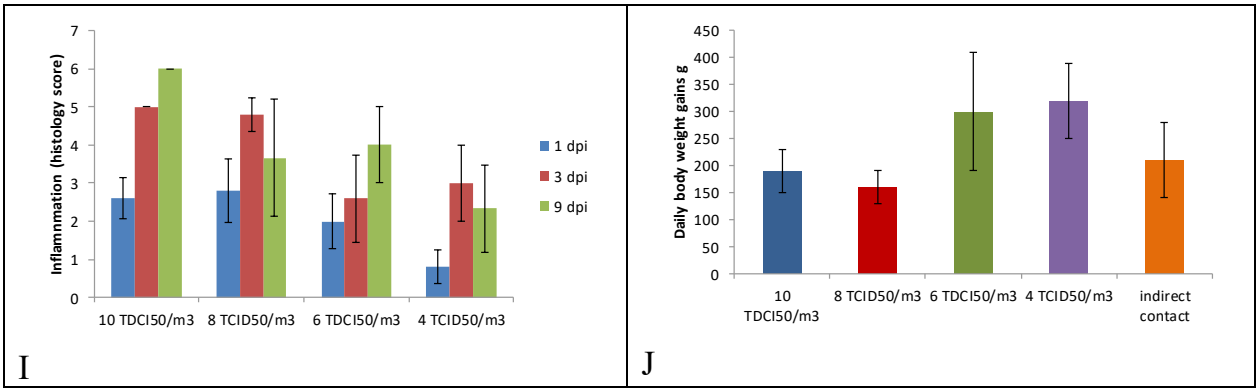


Supplement 23: Results of dose titration of FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$); A, Symptoms dyspnoea score, arithmetic mean); B, Symptoms (mean dyspnoea score with standard deviation); C, Virus excretion (lg $TCID_{50}$/0.1 ml nasal swab), there were no significant differences between the groups; D, Rectal temperatures (°C, arithmetic mean); E, Rectal temperatures (°C, arithmetic mean with standard deviation and significance levels); F, Antibody kinetics (HI titre, tested against the challenge strain): pigs exposed to higher doses responed earlier and stronger; G, Viral lung load (lg $TCID_{50}$/g, arithmetic mean), despite decreasing viral load with decreasing infectious dose no significant differences were calculated; H, Lung pathology: lung lesions (%) were significantly larger in group 10 lg $TCID_{50}/m^3$ in comparison to all other groups on 3 and 9 dpi (, $p<0.05$) and in group 8 lg $TCID_{50}/m^3$ in comparison to groups 6 + 4 lg $TCID_{50}/m^3$ on 3 dpi (*, $p<0.05$); I, Lung histology (inflammation score, arithmetic mean), inflammation was significantly stronger in groups 10+8+6 lg $TCID_{50}/m^3$ in comparison to group 4 lg $TCID_{50}/m^3$ on 1 dpi (*, $p<0.05$), in groups 10+8 lg $TCID_{50}/m^3$ on 3 dpi (*, $p<0.05$), in group 10 lg $TCID_{50}/m^3$ on 9 dpi, (*, $p<0.05$); J, Daily body weight gains from -1 dpi until 9 dpi reflect significantly lower body weight gains in groups 1+2 (10+8 $TCID_{50}/m^3$) in comparison to groups 3+4 (6+4 $TCID_{50}/m^3$, *, $p<0.05$); m, morning; l, lunch time; a, afternoon; arithmetic means with standard deviation, for antibodies geometric means are shown*

Short interpretation of results. The $H1_{pdm}N2$ virus proved to be more virulent than other HxN2 viruses, but less virulent than the $H1_{pdm}N1$ virus of April 2009. This may be important as this virus has established a stable lineage in pigs in Germany and Denmark. As there has been no decrease in virulence of HxN2 viruses so far and the N2 neuraminidase is associated with higher virulence, it may be possible that this virus will maintain its virulence in the future.

DOSE TITRATION OF SWINE PANDEMIC H1N1 2014 VIRUS BY AEROSOL

Background. From 2009 onwards $H1_{pdm}N1$ viruses were detected in the German swine population. There were frequent transmissions of $H1_{pdm}N1$ viruses from humans to pigs. The frequent detection of $H1_{pdm}N1$ viruses all around the year in pigs which does not follow the seasonality of influenza A viruses circulating in the human population indicates the establishment of lineages of $H1_{pdm}N1$ virus in the swine population. After 5 years of circulation of $H1_{pdm}N1$ 2009 virus it was of interest to investigate the virulence of such a swine $H1_{pdm}N1$ virus.

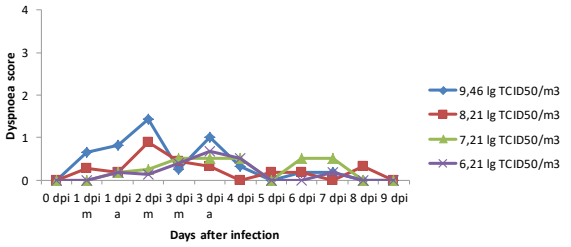
Study design. A virus isolated from pigs in 2014 was used in this study: strain FLUAV/sw/Schallern/19989/2014 ($H1_{pdm}N1$). Fifty two 3 months old commercial cross

bred pigs were infected with different doses of virus by aerosol Supplementary Table 12. Pigs were treated in their 3rd and 7th week of life with tulathromycin (Draxxin™) in order to prevent bacterial co-infections. In their 12th week of life challenge infection was performed in a small chamber of a large infection unit starting with the lowest dose. Exposure time was 1 hour for each group. On days 1 and 3 after infection lungs samples were taken from 5 pigs of each group (groups 1-4). On day 9 after infection lungs were collected from the remaining pigs of each group (1-4). Parameters were recorded as outlined in materials and methods.

Supplementary Table 12: Overview of the experimental design of dose titration of infection dose of swine $H1_{pdm}N1$ 2014 virus

Group	Infection dose lg $TCID_{50}$ / m^3	Abbreviation	Number of pigs included
1	9.46	9.46 $TCID_{50}$ / m^3	13
2	8.21	8.21 $TCID_{50}$ / m^3	13
3	7.21	7.21 $TCID_{50}$ / m^3	13
4	6.21	6.21 $TCID_{50}$ / m^3	13

Results. Respiratory symptoms were mild in comparison to those caused by $H1_{pdm}N1$ viruses of 2009. More pronounced symptoms were only induced in the groups which received the higher doses (groups 9.46 and 8.21 $TCID_{50}$ / m^3; Supplement 24 A, B). Here, the pigs infected with the highest dose reflected also the highest symptoms (Supplement 24 A, B). The virus shedding curves reaches the earliest peak in the group receiving the highest dose (Supplement 24 C, D). Viral lung load was significantly higher in groups 9.46 and 8.21 $TCID_{50}$ / m^3 in comparison to the other two groups (Supplement 24 E, F). This was accompanied by more pronounced lung gross lesions in the groups receiving the higher doses (Supplement 24 G, H). Increases in rectal temperatures were only seen in the groups 9.46 and 8.21 $TCID_{50}$ / m^3 and were moderate (Supplement 24 I, J). Body weights were influenced strongest in the group which was infected with the highest dose; the group receiving the second highest dose showed a stagnation in body weight gains 2 dpi and recovered afterwards; the best performance showed the group receiving the third highest dose; the group which received the lowest dose had the lowest body weights at start of infection (Supplement 24 K, L). Pigs of all groups responded with antibodies to infection around 8-9 dpi (Supplement 24 M, N).



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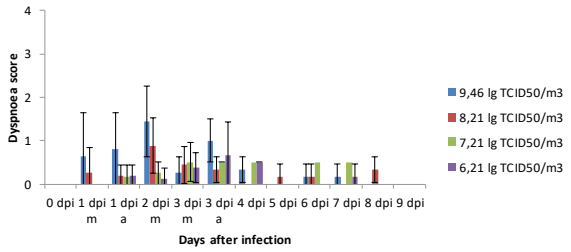

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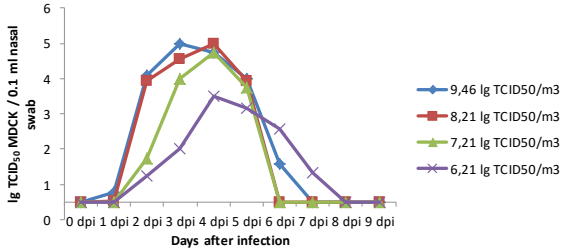

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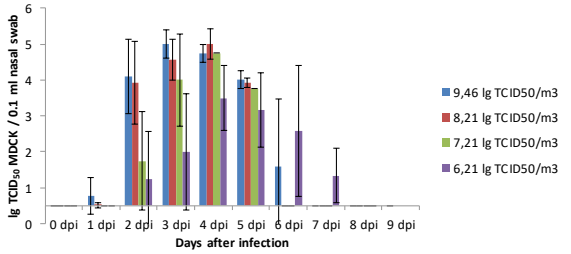

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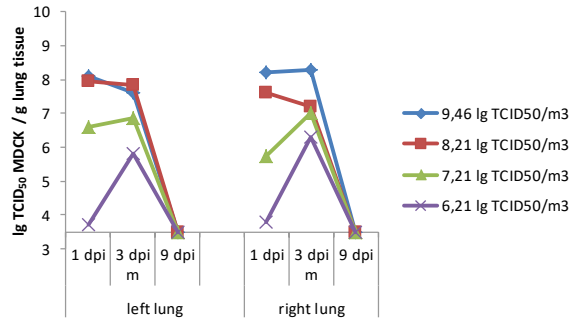

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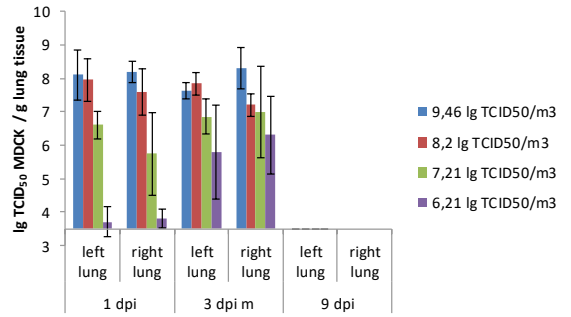

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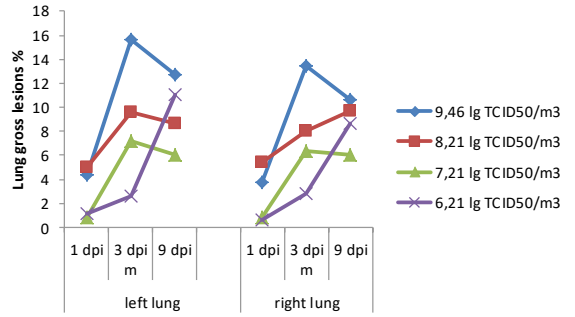

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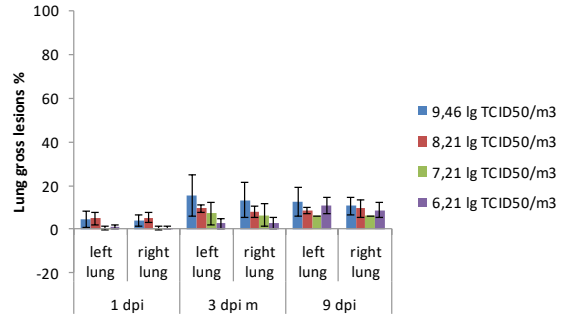

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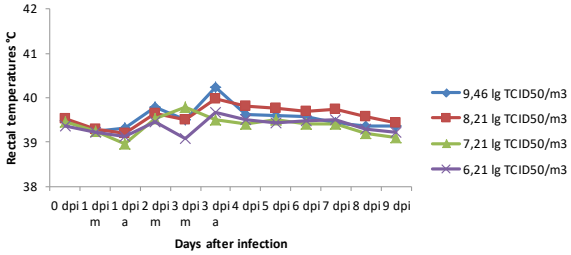

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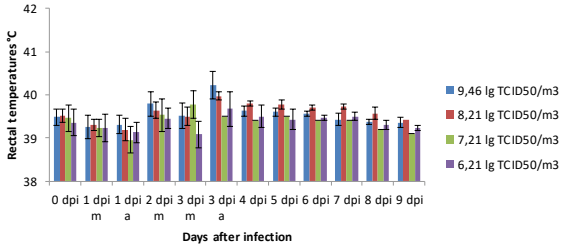

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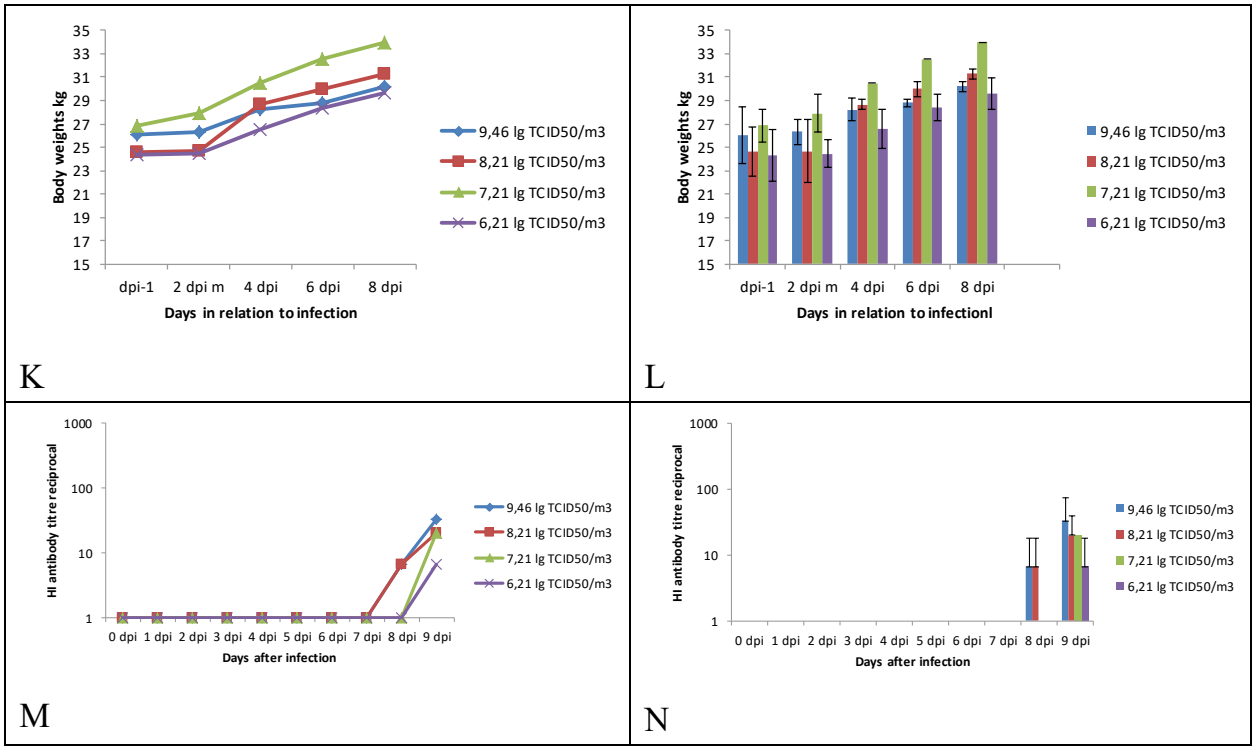


Supplement 24: Results of the infectious dose titration experiment using FLUAV/sw/Schallern/19989/2014 ($H1_{pdm}N1$) virus; A, Symptoms (dyspnoea score) trends reflected by arithmetic mean values; B, Symptoms (dyspnoea score) arithmetic mean values with standard deviation; C, Virus excretion (lg $TCID_{50}$ MDCK/0.1 ml nasal swab) trends reflected by arithmetic mean values; D, Virus excretion (lg $TCID_{50}$ MDCK/0.1 ml nasal swab) arithmetic mean values with standard deviation; E, Viral lung load (lg $TCID_{50}$ MDCK/g lung tissue) trends reflected by arithmetic mean values; F, Viral lung load (lg $TCID_{50}$ MDCK/g lung tissue) arithmetic mean values with standard deviation; G, Lung lesions (%) trends reflected by arithmetic mean values; H, Lung lesions (%) arithmetic mean values with standard deviation; I, Rectal temperatures (°C) trends reflected by arithmetic mean values; J, Rectal temperatures (°C) arithmetic mean values with standard deviation; K, Body weights (kg) trends reflected by arithmetic mean values; L, Body weights (kg) arithmetic mean values with standard deviation; M, Antibody kinetics (HI titre), trends reflected by geometric mean values; N, Antibody kinetics (HI titre), geometric mean with standard deviation; Signifances: 9.46 and 8.21 lg $TCID_{50}$ groups had significantly higher (, $p<0.05$) dyspnoea (1 dpi), viral lung loads (1 dpi), virus shedding (1+2 dpi), lung lesions (3 dpi) in comparison to groups 7.21 and 6.21 lg $TCID_{50}$; group 7.21 lg $TCID_{50}$ performed significantly better in body weight development than group 9.46 lg $TCID_{50}$ (the other two groups were not included into statistical analysis due to significantly lower body weights at start)*

SUPPLEMENT CHAPTER 9 – STABILITY OF NEBULIZED VIRUSES

Supplementary Table 13: Investigation of virus content in suspensions before and after nebulisation by generator SAG-1; the virus strains were grown in MDBK and MDCK cells, respectively ($H1_{pdm}N1$ viruses were grown only in MDCK cells), nebulised and tested on MDCK and MDBK cells thereafter

	Before nebulisation	**After nebulisation sample 1**	**sample 2**
A/Jena/**VI2688**/2010 ($H1_{pdm}N1$) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{7.83}$	$10^{7.67}$	$10^{8.0}$
$TCID_{50}$/ml MDBK	$10^{5.67}$	$10^{4.67}$	$10^{4.83}$
HA titre	64	64	64
pH	7.3	7.86	7.84
A/Hamburg/**NY1580**/2019 ($H1_{pdm}N1$) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{7.75}$	$10^{7.5}$	$10^{7.5}$
$TCID_{50}$/ml MDBK	$10^{4.5}$	$10^{3.75}$	$10^{3.5}$
HAtitre	2	< 2	< 2
pH	7.71	7.45	8.28
A/sw/Bakum/**3543**/98 ($H1_{av}N1$) **MDBK**			
$TCID_{50}$/ml MDCK	$10^{6.25}$	$10^{6.75}$	$10^{6.25}$
$TCID_{50}$/ml MDBK	$10^{5.5}$	$10^{6.0}$	$10^{5.5}$
HA titre	128	128	128
pH	6.82	8.19	8.12
A/sw/Bakum/**3543**/98 ($H1_{av}N1$) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{3.0}$	$10^{4.5}$	$10^{4.25}$
$TCID_{50}$/ml MDBK	$10^{2.5}$	$10^{2.5}$	$10^{3.5}$
HA titre	<2	2	2
pH	6.47	7.89	7.86
A/sw/Ennigerloh/**5803**/06 ($H1_{av}N1$) **MDBK**			
$TCID_{50}$/ml MDCK	$10^{8.75}$	$10^{8.25}$	$10^{8.5}$
$TCID_{50}$/ml MDBK	$10^{7.75}$	$10^{8.0}$	$10^{7.75}$
HA titre	256	512	512
pH	7.21	8.44	8.36
A/sw/Ennigerloh/**5803**/06 ($H1_{av}N1$) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{7.75}$	$10^{8.0}$	$10^{8.0}$
$TCID_{50}$/ml MDBK	$10^{7.0}$	$10^{6.5}$	$10^{6.5}$
HA titre	128	128	128
pH	7.19	8.08	8.09

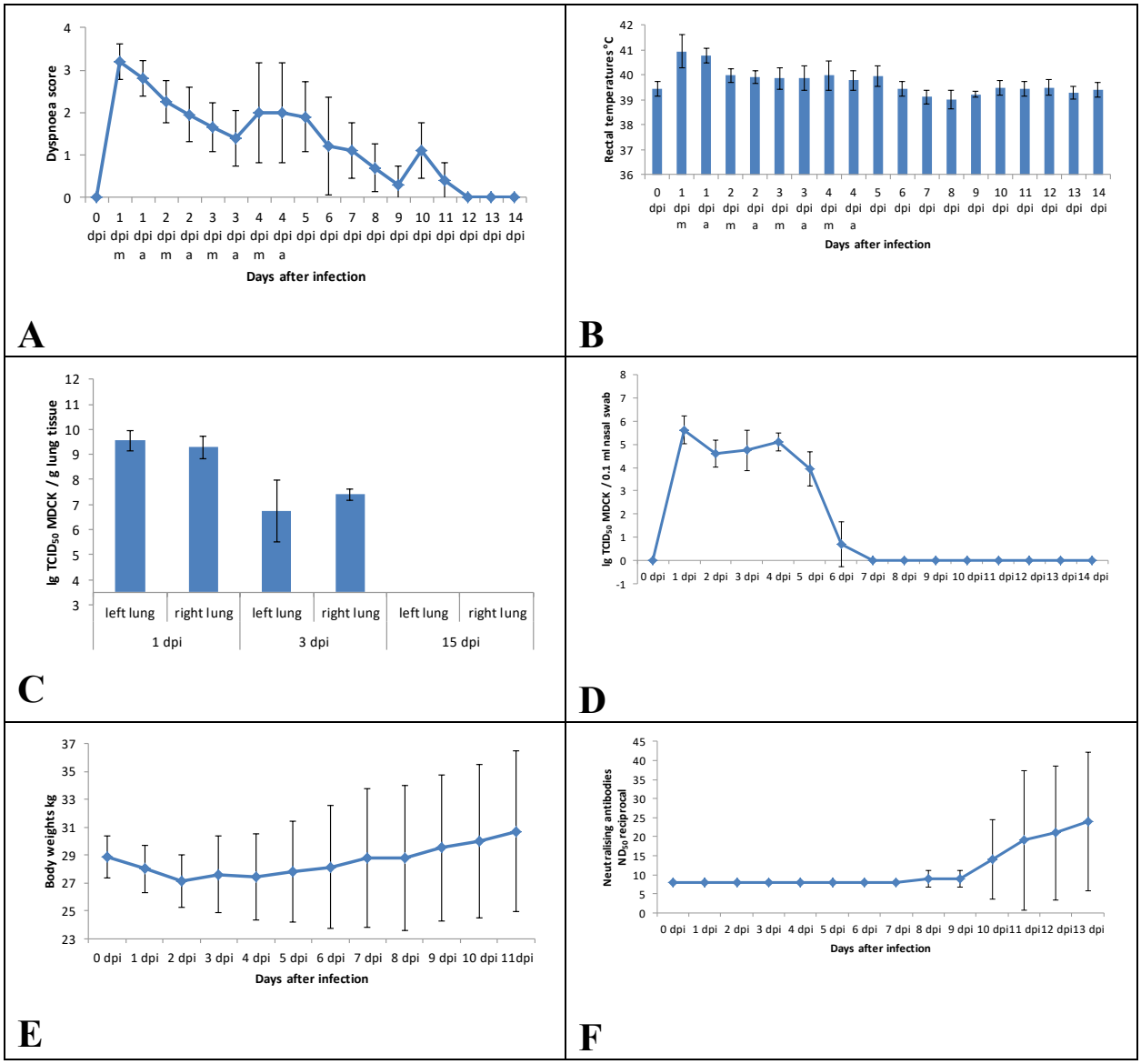
Supplementary Table continued on next page

	Before nebulistion	After nebulisation sample 1	sample 2
A/sw/Bakum/**1832**/00 ($H1_{hu}N2$) **MDBK**			
$TCID_{50}$/ml MDCK	$10^{8.0}$	$10^{8.0}$	$10^{7.75}$
$TCID_{50}$/ml MDBK	$10^{6.75}$	$10^{6.5}$	$10^{7.0}$
HA titre	128	256	256
pH	7.03	8.19	8.18
A/sw/Bakum/**1832**/00 ($H1_{hu}N2$) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{8.5}$	$10^{8.75}$	$10^{8.0}$
$TCID_{50}$/ml MDBK	$10^{6.25}$	$10^{7.0}$	$10^{6.75}$
HA titre	32	64	64
pH	6.84	8.22	8.09
A/sw/Kitzen/**6142**/07 ($H1_{hu}N2$) **MDBK**			
$TCID_{50}$/ml MDCK	$10^{8.25}$	$10^{8.0}$	$10^{8.5}$
$TCID_{50}$/ml MDBK	$10^{7.0}$	$10^{7.25}$	$10^{7.25}$
HA titre	128	256	256
pH	6.98	8.24	8.12
A/sw/Kitzen/**6142**/07 ($H1_{hu}N2$) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{8.0}$	$10^{7.75}$	$10^{8.0}$
$TCID_{50}$/ml MDBK	$10^{6.25}$	$10^{6.5}$	$10^{6.75}$
HA titre	32	64	32
pH	7.53	8.12	8.08
A/sw/Bakum/**909**/09 (H3N2) **MDBK**			
$TCID_{50}$/ml MDCK	$10^{8.0}$	$10^{8.5}$	$10^{8.0}$
$TCID_{50}$/ml MDBK	$10^{7.75}$	$10^{7.5}$	$10^{7.5}$
HA titre	256	512	512
pH	6.68	8.34	8.34
A/sw/Bakum/**909**/09 (H3N2) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{8.25}$	$10^{7.75}$	$10^{8.75}$
$TCID_{50}$/ml MDBK	$10^{7.0}$	$10^{7.5}$	$10^{7.5}$
HA titre	128	128	128
pH	6.62	8.14	8.24
A/sw/Bondelum/**5959**/07 (H3N2) **MDBK**			
$TCID_{50}$/ml MDCK	$10^{8.0}$	$10^{8.5}$	$10^{8.0}$
$TCID_{50}$/ml MDBK	$10^{7.5}$	$10^{8.0}$	$10^{7.75}$
HA titre	256	1024	1024
pH	7.35	8.42	8.33
A/sw/Bondelum/**5959**/07 (H3N2) **MDCK**			
$TCID_{50}$/ml MDCK	$10^{7.25}$	$10^{7.75}$	$10^{7.0}$
$TCID_{50}$/ml MDBK	$10^{6.5}$	$10^{6.5}$	$10^{6.75}$
HA titre	256	256	256
pH	7.37	8.20	8.17

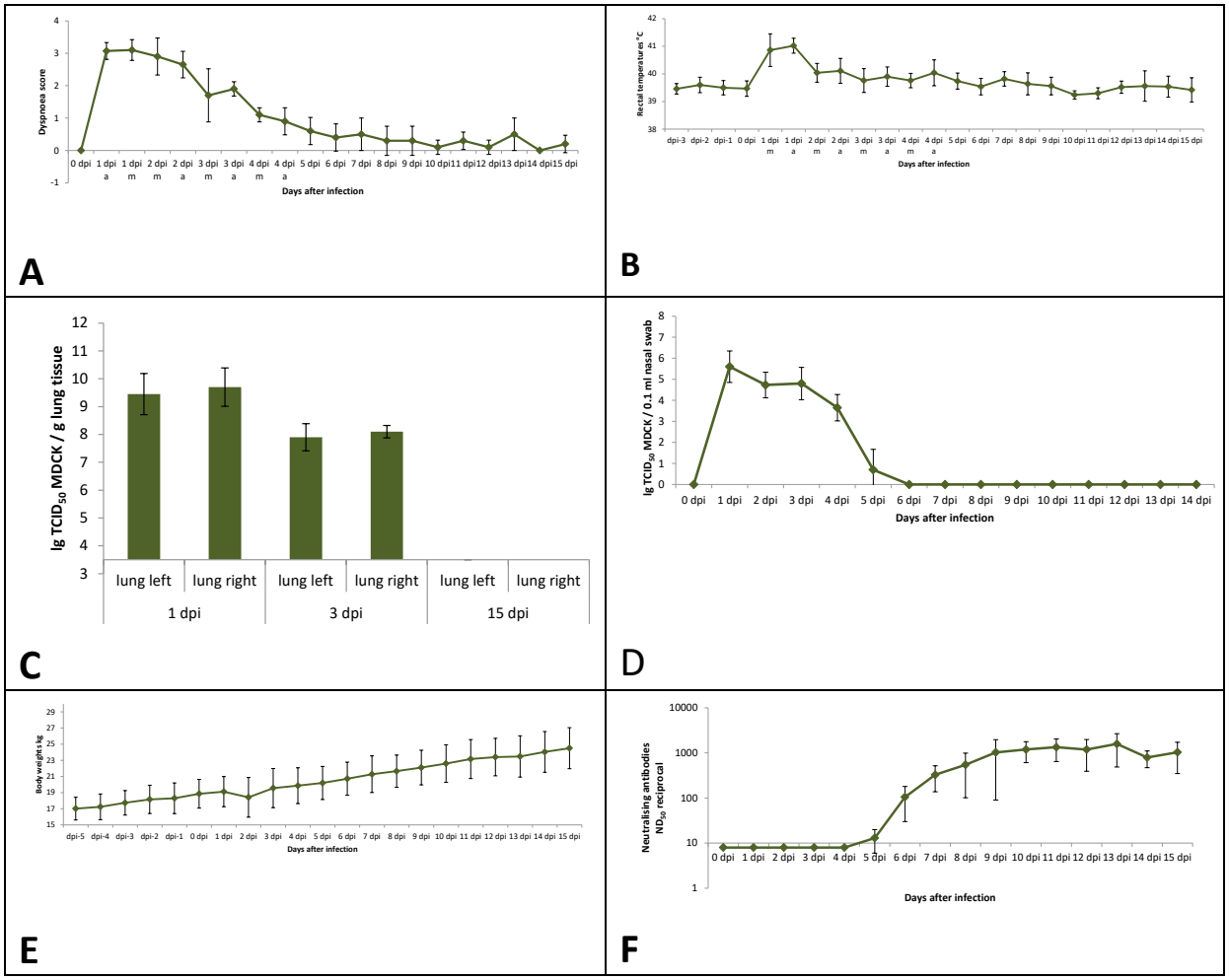
SUPPLEMENT CHAPTER 10 – ANALYSIS OF $H1_{PDM}N1$ VIRUSES DIFFERING IN VIRULENCE

CLINICAL DATA

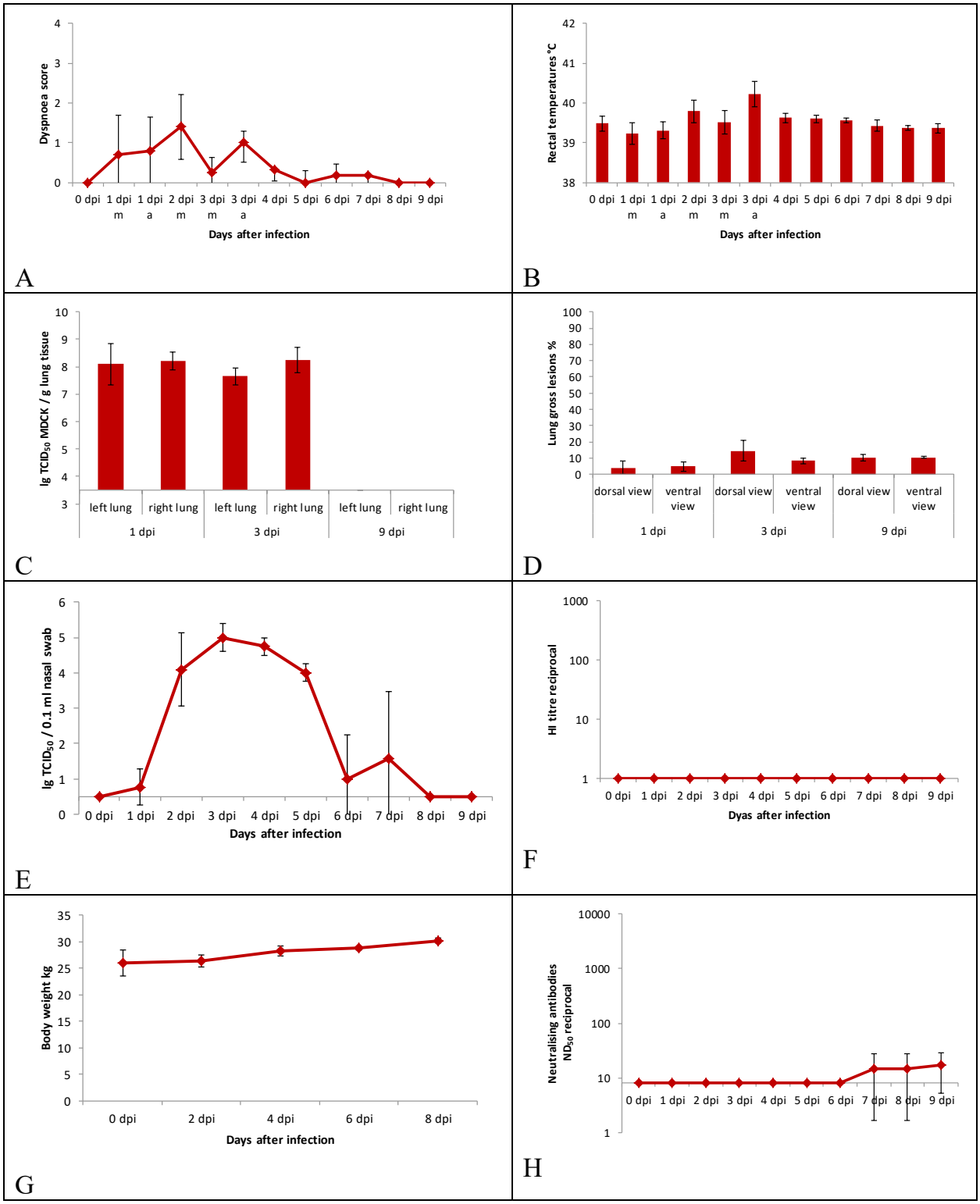
In Supplement chapter 8 clinical and virological data for the highly virulent $H1_{pdm}N1$ April 2009 virus were shown. The following figures provide details for the less virulent $H1_{pdm}N1$ viruses (Supplement 25, Supplement 26, Supplement 27, Supplement 28, Supplement 29)



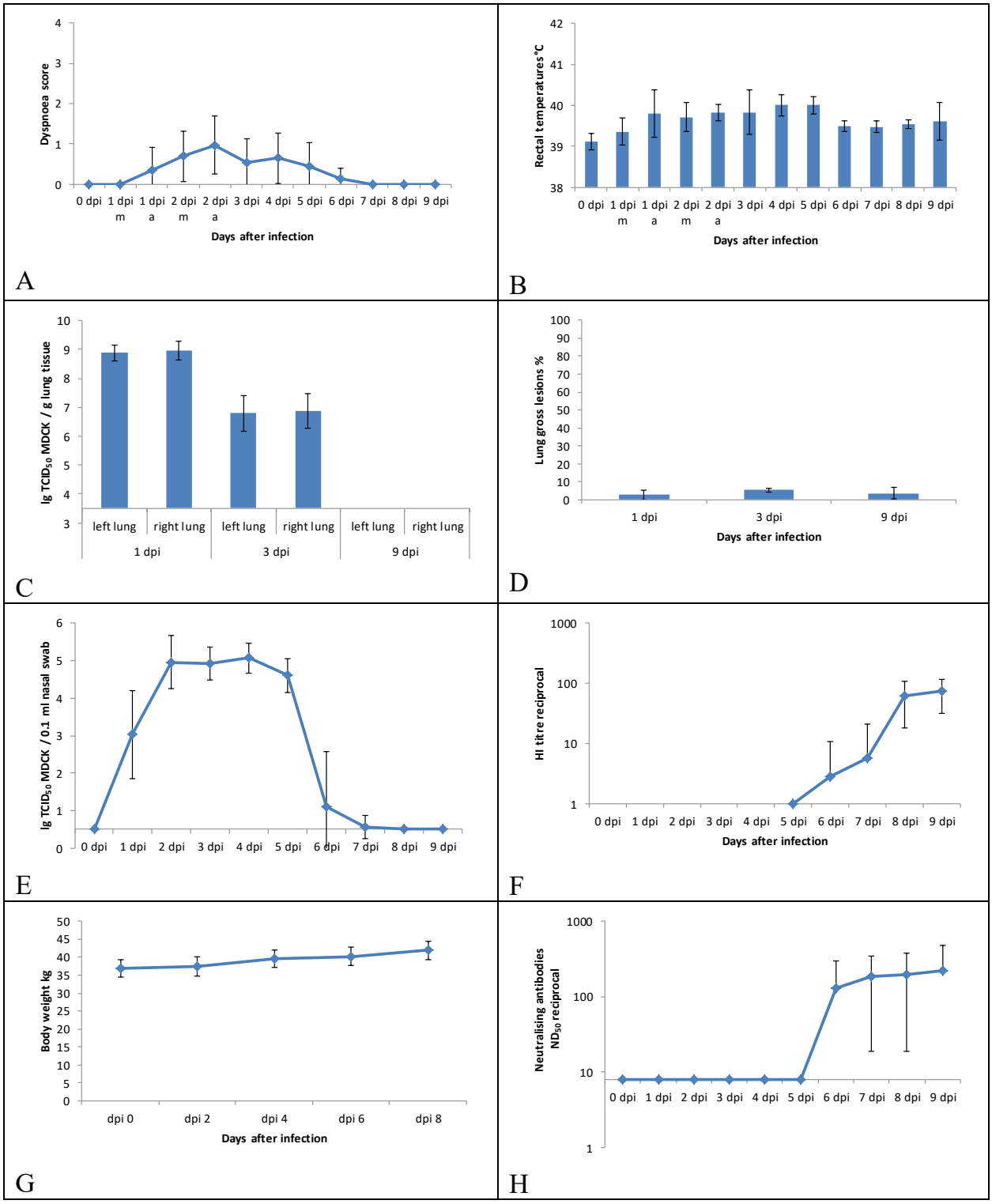
Supplement 25: Parameters after infection with FLUAV/Jena/VI5258/2009 ($H1_{pdm}N1$); A, Dyspnoea (score), B, Rectal temperatures (°C); C, Viral lung load (lg $TCID_{50}$ MDCK/ g lung tissue), D, Virus excretion (lg $TCID_{50}$ MDCK/0.1 ml nasal swab); body weights (kg); F, Neutralising antibodies (ND_{50} reciprocal); arithmetic means are shown, for antibodies geometric mean



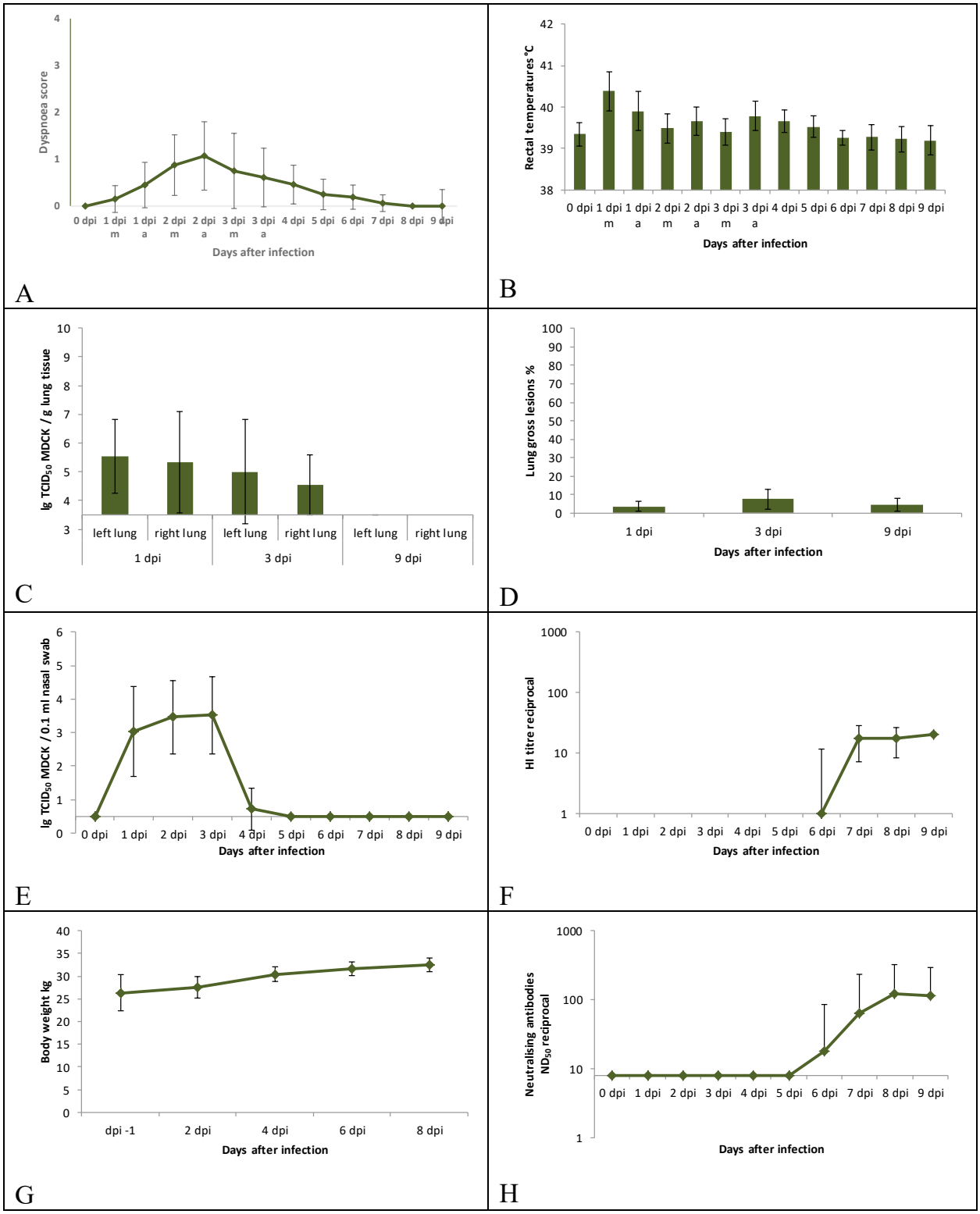
Supplement 26: Parameters after infection with FLUAV/Jena/VI2688/2010 ($H1_{pdm}N1$); A, Dyspnoea (score), B, Rectal temperatures (°C); C, Viral lung load (lg $TCID_{50}$ MDCK/ g lung tissue), D, Virus excretion (lg $TCID_{50}$ MDCK/0.1 ml nasal swab); body weights (kg); F, Neutralising antibodies (ND_{50} reciprocal); arithmetic means are shown, for antibodies geometric mean



Supplement 27: Parameters after infection with FLUAV/Schallern/19989/2014 ($H1_{pdm}N1$); A, Symptoms (dyspnoea score); B, Rectal temperatures (°C); C, Viral lung load (lg $TCID_{50}$ MDCK/g lung tissue); D, Lung gross lesions (%); E, Virus excretion (lg $TCID_{50}$ MDCK/0.1 ml nasal swab); F, HI antibodies (HI titre reciprocal); G, Body weights (kg); H, Neutralising antibodies (ND_{50} reciprocal); antibody assays measured against infection strain (note that there is no measurable HI titre until 9 dpi); arithmetic means are shown, for antibodies geometric mean



Supplement 28: Parameters after infection with FLUAV/Kiel/18909686/2015 ($H1_{pdm}N1$); A, Symptoms (dyspnoea score); B, Rectal temperatures (°C); C, Viral lung load (lg $TCID_{50}$ MDCK/g lung tissue); D, Lung gross lesions (%); E, Virus excretion (lg $TCID_{50}$ MDCK/0.1 ml nasal swab); F HI antibodies (HI titre reciprocal); G, Body weights (kg); H, Neutralising antibodies (ND_{50} reciprocal); antibody assay done with infection strain; arithmetic means are shown, for antibodies geometric mean



Supplement 29: Parameter after infection with FLUAV/Tesp/2110/2015 ($H1_{pdm}N1$); A, Symptoms (dyspnoea score); B, Rectal temperatures (°C); C, Viral lung load (lg $TCID_{50}$ MDCK/g lung tissue); D, Lung gross lesions (%); E, Virus excretion (lg $TCID_{50}$ MDCK/0.1 ml nasal swab); F, HI antibodies (HI titre reciprocal); G, Body weights (kg); H, Neutralising antibodies (ND_{50} reciprocal); antibody assay done with infection strain; arithmetic means are shown, for antibodies geometric mean

REPLICATION KINETICS OF $H1_{PDM}N1$ VIRUSES IN CELL CULTURE

REPLICATION KINETICS OF $H1_{PDM}N1$ VIRUSES IN MBCK CELLS

Background. As all infectious doses refer to titrations in cell culture, some differences in virus replication in cell culture should be mentioned. For all viruses used, titration of the infectious dose was performed in MDBK cells to obtain comparable results. Due to the characteristic uniform monolayer obtained with MDBK cells, the reading of the results is clear and follows a strong linearity when viruses are titrated in MDBK cells. Influenza A viruses replicate faster in MDCK cells which sometimes results in less antigen being produced compared to MDBK cells (low haemagglutination titres). When the first titrations were performed with the newly emerged $H1_{pdm}N1$ 2009 virus, the viruses were more difficult to grow in MDBK cells. Therefore, MDCK cells were used for cultivation. This revealed differences in the virus titres after cultivation in both cell lines.

Study design. The viruses were cultured in MDBK and MDCK cells and the virus titres were compared. For the non-cytopathogenic viruses, cells were lysed by freezing after each passage and the thawed suspensions were used to infect the next passage. Due to the lower replication characteristics of $H1_{pdm}N1$ viruses in MDBK cells, the viruses were adapted to these cells by passaging.

Results. $H1_{pdm}N1$ April 2009 virus did not induce cytopathic effects in the first passages in MDBK cells, but entered the cells and produced antigen detectable by immunofluorescence staining (Supplementary Table 14). In MDCK cells, $H1_{pdm}N1$ April 2009 virus induced cytopathic effects and grew to higher infectious titres (Supplementary Table 14). In contrast to the 2009 $H1_{pdm}N1$ viruses, $H1_{pdm}N1$ viruses isolated in July 2009 and later were able to induce cytopathic effects in MDBK cells from the first passage in these cells (Supplementary Table 15). July 2009 viruses showed less pronounced cytopathic effect. However, replication increased during passaging in MDBK cells (Supplementary Table 14).

Short interpretation of results. Early $H1_{pdm}N1$ 2009 viruses differed from other viruses in their replication characteristics, indicating that they are not yet as well adapted. They did not induce cytopathic effects in their first passages in MDBK cells and grew to lower titres in this cell culture system. In contrast, $H1_{pdm}N1$ 2014/2015 viruses induced cytopathic effects in MDBK cells from the first passage in these cells and did not reflect such large differences in cell culture titration between MDBK and MDBK cells as did early $H1_{pdm}N1$ 2009 viruses, thus being identical in their replication pattern to other swine influenza A viruses. This could be due to adaptations in the polymerase complex but also due to differences in neuraminidase activity. It has been shown that there was a switch at the position N340K during the first months of circulation of this virus in 2009 (see below HA09 from April 2009 contains PB2 640N - JE09 from July 2009 and all other $H1_{pdm}N1$ viruses

contain 640K). It is possible that this mutation facilitated adaptation to a broader spectrum of mammalian cells, which could explain why the first $H1_{pdm}N1$ viruses were not able to cause cytopathic effects in MDBK cells, but the later viruses were able to do so. Positon 340 is known to be a residue in the cap-binding domain of PB2[498]. It has been demonstrated that the K at position 340 is associated with mammalian adaptation[499]. PB2 340K is found in almost all swine influenza viruses[500]. The PB2 of $H1_{pdm}N1$ viruses is of avian origin and contains amino acids 627E and 631M, which are not adapted to the human ANP32A protein, which supports the formation of a dimer between viral RNA polymerases[501]. Nevertheless, the viruses can circulate in humans, suggesting that the interactions within the $H1_{pdm}N1$ PB2 and the human ANP32A protein are more complex and may involve additional amino acids within PB2. After the 1918 pandemic, a switch was observed in later viruses, here PB2 M631L[502] which did not occur in $H1_{pdm}N1$ 2009 viruses. However, it could also be that the lack of cytopathic effect in MDBK cells is a sign of non-optimised neuraminidase activity and that the neuraminidase was not able to detach virions from the cell membrane.

Supplementary Table 14: Passaging of $H1_{pdm}N1$ April 2009 virus (1580) in MDBK and MDCK cells

	ps1	ps2	ps3	ps4	ps5	ps6	ps7	ps8	ps9	ps10
$H1_{pdm}N1$ April 2009 (1580)										
MDBK	4.75	4.5	5	4.5	4.75	5	4.75	4.5	5	5.5
CPE	no	no	no	no	no	no	no	no	no	no
HU	16	16	32	16	16	32	32	32	32	32
MDCK	8.5	8.25	8.75	8.5	8.5	8.75	8.5	8.75	8.5	8.75
CPE	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
HU	32	32	32	64	32	64	32	64	64	64
$H1_{pdm}N1$ July 2009 (5555)										
MDBK	5	4.5	5	5.5	5.75	6	6.5	7.5	7.75	8.5
CPE	yes*	yes*	yes*	yes*	yes*	yes*	yes*	yes*	yes*	yes
HU	32	32	32	64	64	64	128	128	256	512
MDCK	8.5	8.75	8.25	8.25	8	8.75	8.5	8.75	8.5	9
CPE	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
HU	32	64	32	32	32	64	32	64	64	128

ps, passage in MDBK cells; CPE, cytopathic effect; MDBK, titration in MDBK cells (lg $TCID_{50}$ is shown); MDCK, titration in MDCK cells (lg $TCID_{50}$ is shown), HU, hemagglutinating units; strain 1580/2009 $H1_{pdm}N1$ was used in this experiment which had been before 3x passaged in MDCK cells in which it had been originally isolated; * CPE was less pronounced compared to ps10

Supplementary Table 15: Effects of $H1_{pdm}N1$ viruses on MDBK cells after primary infection

Virus		$H1_{pdm}N1$ 1580 April 2009	$H1_{pdm}N1$ 5258 July 2009	$H1_{pdm}N1$ 5555 July 2009	$H1_{pdm}N1$ 19989 April 2014	$H1_{pdm}N1$ 18909686 January 2015
CPE ps1	MDBK	no	yes*	yes*	yes	yes

CPE, cytopathic effect; ps1, passage 1; moi = 0.005 (multiplicity of infection); * CPE was less pronounced compared to 2014 and 2015 viruses

REPLICATION KINETICS OF H1$_{PDM}$N1 VIRUSES IN MDCK CELLS

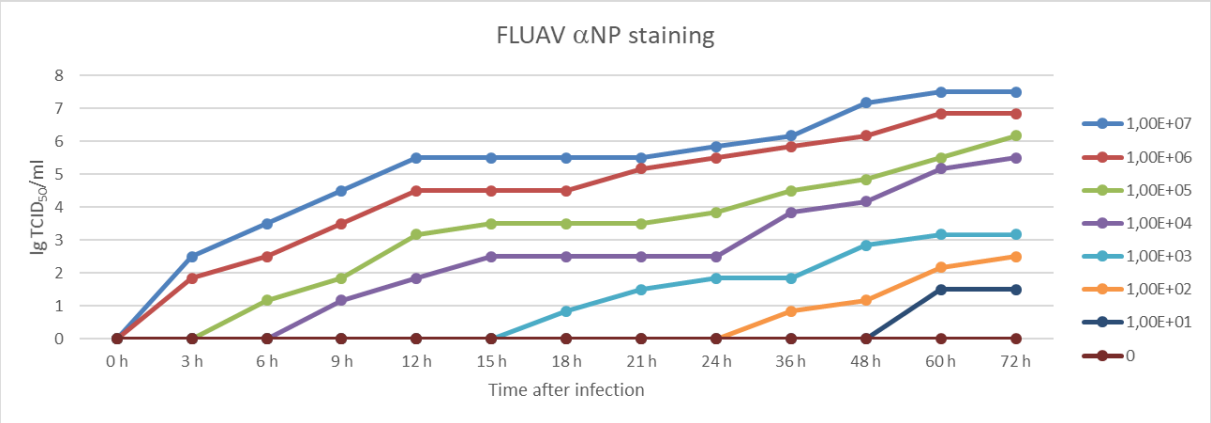
Background. To investigate the biological properties of H1$_{pdm}$N1 viruses, which induce different patterns of virulence in pigs, replication kinetics in the MDCK cell line (MDCK-SIAT cells^{503}) were established.

Study design. In a first study, virus A/Hamburg/NY1580/2009 was titrated and the effect of the different dilutions on MDCK cells was investigated.

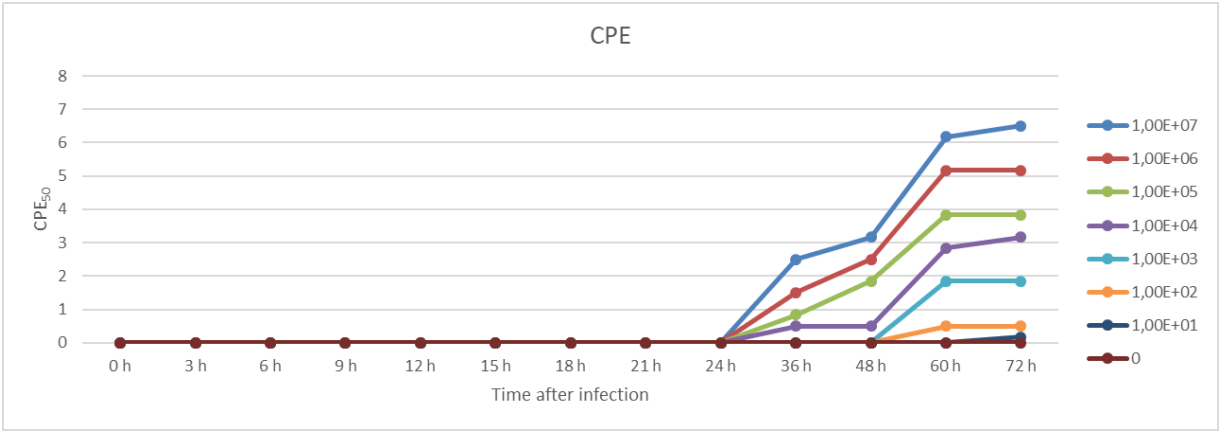
In a second study, several viruses were adjusted to 10^4 TCID$_{50}$ and tested in MDCK-Siat cells. These viruses included one of the first H1$_{pdm}$N1 viruses to be isolated (A/California/07/2009) together with the highly virulent virus A/Hamburg/NY1580/2009 from April 2009 (about 20% mortality in pigs), the virulent virus A/Jena/VI5258/2009 from July 2009 (about 20% severe disease in pigs but no mortality), the medium virulent virus A/Jena/VI2688/2010 of March 2010 (moderate influenza in pigs), the low virulent viruses A/sw/Schallern/19989/2014 of April 2014 and A/Kiel/18909686/2015 (almost no signs of disease in pigs) of January 2015 and other H1$_{pdm}$N1 viruses from the collection of the German National Influenza Centre. Confluent MDCK cells were infected with 10000 TCID$_{50}$ of each strain and the kinetics of cytopathic effect and virus titres were measured. Titres were determined by fixing the cells and immunostaining with a monoclonal antibody directed against the nucleoprotein (see Materials and methods).

Results. Virus growth in cell culture was clearly influenced by the initial infection dose. Compared to lower doses, higher doses led to earlier expression of viral antigen and earlier induction of cytopathic effects (Supplement 30, Supplement 31).

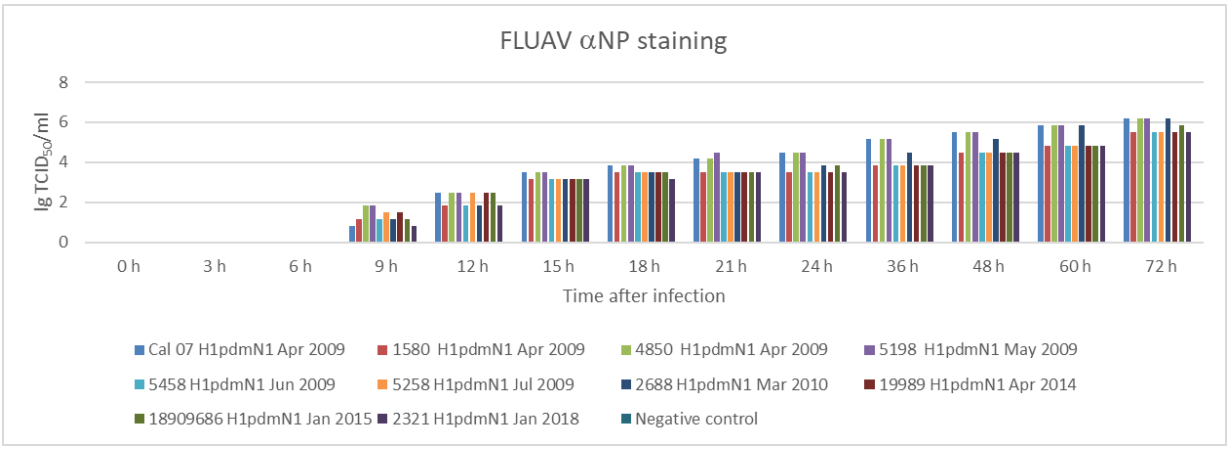
There were no differences in replication kinetics between the different viruses when adjusted to the same initial infection dose. The A/California/07/2009 virus, as well as the highly virulent 2009 H1$_{pdm}$N1 influenza viruses, did not differ significantly in the kinetics of antigen expression and induction of cytopathic effects compared to low virulent viruses isolated in 2010 and later (Supplement 32, Supplement 33).



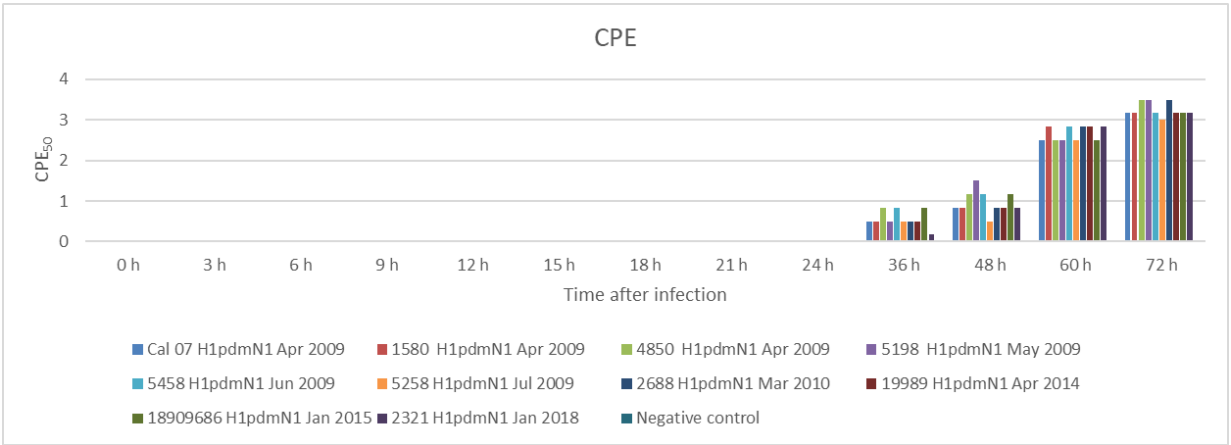
Supplement 30: Kinetics of expression of nucleoprotein in MDCK-SIAT cells after infection with different doses of virus A/Hamburg/NY1580/2009 (H1$_{pdm}$N1)



Supplement 31: Kinetics of cytopathic effects in MDCK-SIAT cells after infection with different doses of virus A/Hamburg/NY1580/2009 ($H1_{pdm}N1$)



Supplement 32: Kinetics of antigen expression (against NP) after infection of MDCK-SIAT cells with different $H1_{pdm}N1$ viruses



Supplement 33: CPE kinetics after infection of MDCK-SIAT cells with different $H1_{pdm}N1$ viruses

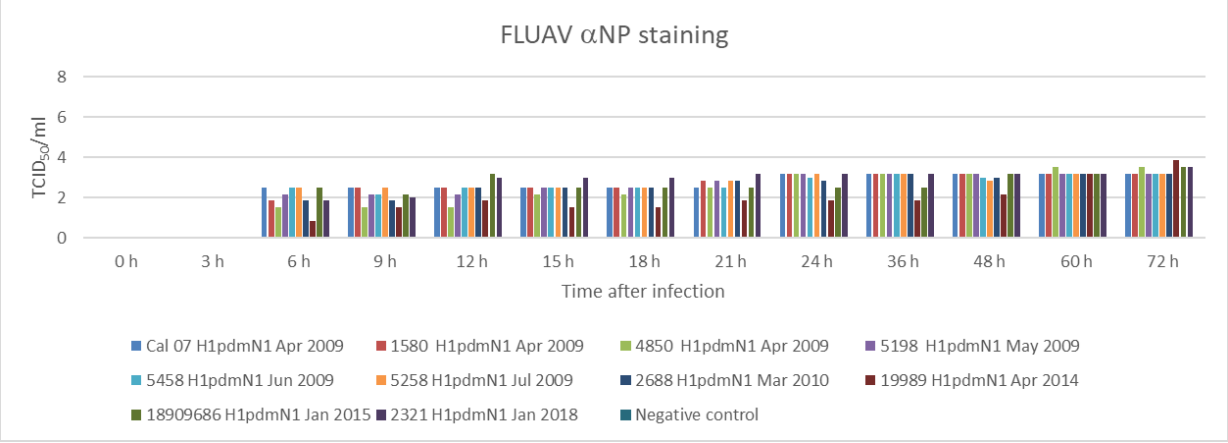
Short interpretation of results. There were no differences in replication between virulent and low virulent viruses in MDCK-SIAT cells. Therefore, features that determine replication patterns cannot explain differences between high and low virulence viruses. However, there was a clear effect of initial infection dose on MDCK-SIAT cells, resulting in earlier antigen expression and induction of cytopathic effects.

REPLICATION KINETICS OF $H1_{PDM}N1$ VIRUSES IN A549 CELLS

Background. To investigate the replication pattern in lung cells, the A549 cell line was used for further studies. The A 549 cell line is a continuously cultured line derived from a human lung adenocarcinoma that has morphological and biochemical characteristics of pulmonary alveolar type II pneumocytes[504,505].

Study design. Confluent A549 cells were infected with 10000 $TCID_{50}$ of each strain and the kinetics of virus titres were measured. Titres were determined by fixing the cells and immunostaining with a monoclonal antibody directed against the nucleoprotein (see Materials and Methods). A 549 cells require serum supplementation in the medium. Therefore, after infection and incubation with trypsin-supplemented medium for 2 hours, serum-containing medium without trypsin was again added to the wells.

Results. There were no significant differences in antigen expression between the different virulent viruses (Supplement 34). Antigen expression in A 549 cells occurred 3 hours earlier than in MDCK-SIAT cells, but remained at lower levels compared to MDCK-SIAT cells. In A549 cells, there was no induction of cytopathic effects.

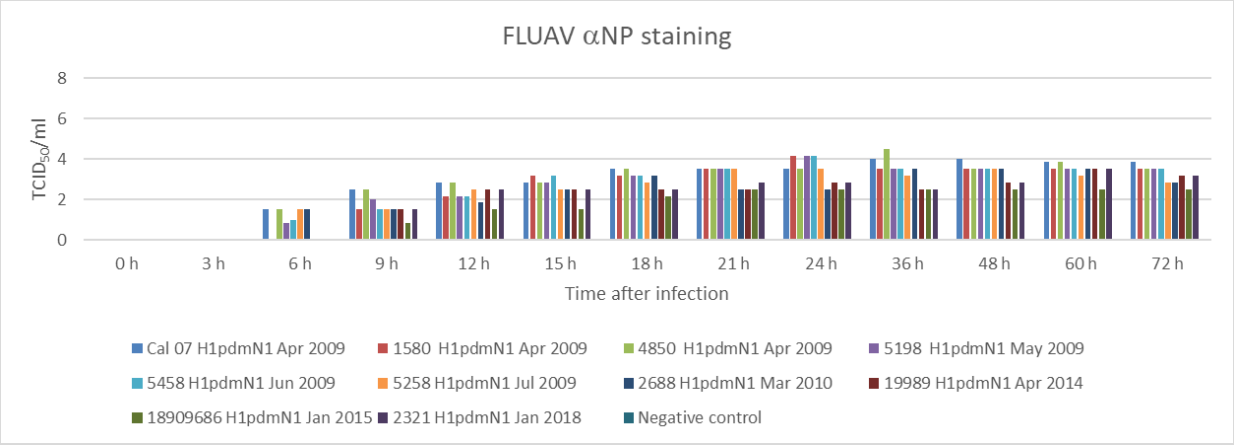


Supplement 34: Kinetics of antigen expression (against NP) after infection of A 549 cells with different $H1_{pdm}N1$ viruses

Short interpretation of results. In type II pneumocytes, there were also no differences in replication between virulent and low virulent viruses. Therefore, features that determine replication patterns cannot explain differences between high and low virulence viruses.

REPLICATION IN OF H1$_{PDM}$N1 VIRUSES IN CALU CELLS

Background. To investigate the replication pattern in bronchial cells, the Calu-3 cell line was used for further studies. Calu-3 cells (ATCC HTB-55) are a cell line derived from human bronchial submucosal glands[506,507].
Study design. Confluent Calu-3 cells were infected with 10000 TCID$_{50}$ of each strain and the kinetics of virus titres were measured. Titres were determined by fixing cells and immunostaining with anti-nucleoprotein monoclonal antibody (see Materials and Methods). Calu-3 cells require serum supplementation in the medium. For this reason, after infection and incubation with trypsin-supplemented medium for 2 hours, medium containing serum, but without trypsin, was again added to the wells.
Results. There were no significant differences in antigen expression between the different virulent viruses (Supplement 35). Antigen expression in Calu-3 cells occurred 3 hours earlier than in MDCK-SIAT cells, but remained at lower levels compared to MDCK-SIAT cells. In Calu-3 cells, there was no induction of cytopathic effects.
Short interpretation of results. In bronchial submucosal gland cells, there were also no differences in replication between virulent and low virulent viruses. Therefore, features that determine replication patterns cannot explain differences between high and low virulence viruses



Supplement 35: Kinetics of antigen expression (against NP) after infection of Calu-3 cells with different H1$_{pdm}$N1 viruses

REPLICATION KINETICS OF H1$_{PDM}$N1 VIRUSES IN AIRWAY EPITHELIAL CELLS

This was investigated by Fu et al. (2019)[401] at the Tierärztliche Hochschule Hannover using the same H1$_{pdm}$N1 viruses representative of the different groups in virulence as used in this study. In these *in vitro* investigations the virulent viruses from 2009/2010 differed from the less virulent 2014/2015 viruses by (i) increased release of infectious virus 48 – 72 hous

after infection, (ii) a more pronounced loss of ciliated cells, (iii) a reduced thickness of the epithelial cell layer.

GENETIC CHARACTERISATION OF INFLUENZA A VIRUSES DIFFERING IN VIRULENCE

Background. To determine the genetic characteristics of $H1_{pdm}N1$ viruses that induce different patterns of virulence in pigs, the genetic composition of viruses belonging to different virulence groups was investigated.
Study design. Five A(H1N1)pdm09 viruses were selected from the collection of viruses available at the time: A/Hamburg/NY1580/2009 (HA09), A/Jena/VI5258/2009 (JE09), A/Jena/VI2688/2010 (JE10), A/Kiel/18909686/2015 (KI15) and A/sw/Schallern/19989/2014 (SC14). The viruses belong to A(H1N1)pdm09 influenza virus clade 1 (JE10), 2 (HA09, JE09) and 6 (SC14, KI15). HA09 was isolated in April 2009, JE09 in July 2009, JE10 in April 2010, SC14 in April 2014 and KI15 in January 2015. These viruses differed in their virulence in pigs in the order HA09 (very highly virulent virus causing about 20% mortality in 3-months-old pigs) > JE09 (highly virulent virus causing severe disease with no mortality) > JE10 (virulent virus causing moderate disease) > SC14 and KI15 (low virulent viruses causing mild or almost no respiratory disease).
The GenBank acc. nos. of viral genes of the viruses are: HA09: EPI296157, EPI296174-EPI296179, EPI296981; JE09: KJ549775-KJ549782; SC14: KX013010-KX013017; JE10: MK159113-MK159120, and KI15 MK159105-MK159112[401].
The virulent viruses HA09, JE09 and JE10 were compared to analyse the slight loss of virulence of HA09 ► JE09 ► JE10. In addition, the virulent viruses were compared with the low-virulent viruses to identify amino acid substitutions that might be associated with loss of virulence (Supplementary Table 16).
Phylogenetic analysis of the sequences was done using Mega7 (NJ, K2, partial deletion 5, Supplement 36, page CXIII).
Results. Virus HA09 differs by two amino acid exchanges from all other viruses in polymerase genes (PA: I118V, PB2: K340N). Virus JE09 has distinguishing mutations in HA: K119E, PA: E688K, PB1: D41E, N77D, K430K/R, PB2: T76N, NS1: V65M, and M1: K113Q (Supplementary Table 16). JE10 reflects a lot of amino acid exchanges in comparison to all other viruses (Supplementary Table 16). The virulent viruses (HA09, JE09, JE10) were compared with the low-virulent viruses (SC14, KI15). Among the different influenza virus genes, a total of 20 amino acid changes were found that distinguished these two groups of viruses (Supplementary Table 17, Supplement 37, Supplement 38, Supplement 39).

Short interpretation of results. Few mutations were identified that distinguished the viruses. It was not possible to attribute the slightly reduced virulence of JE09 and JE10 to a specific amino acid. In PB2 there was a K340N and the PA I118V exchange: PB2 340N and PA 118V were found in viruses that caused 20% mortality; PB2 340K and PA 118I were found in viruses of different virulence that did not cause fatal cases. The K340N mutation in PB2 and the I118V substitution in PA of the highly virulent HA09 virus were the only mutations found only in a virus that caused about 20% mortality in pigs, but not in viruses that did not cause fatal cases. These mutations are not known to be markers of virulence. In agreement with the MDBK cell culture data reported above (no CPE in these cells after infection of the 1st MDBK passage) it is most probably an adaptive mutation caused by replication in mammals. It also suggests that either American swine triple reassortant viruses carrying avian PB2 are not yet fully adapted to mammalian cells, or that avian viruses were involved in the generation of the 2009 $H1_{pdm}N1$ virus, since European swine viruses cause cytopathic effects in MDBK cells. JE09 virus has distinctive mutations in HA: K139E and PB1: N77D, but these cannot be a marker of virulence because they are not shared with the highly virulent HA09 virus (Supplementary Table 16). JE10 reflects a large number of amino acid exchanges compared to all other viruses, but most of these are not shared with the low virulent viruses, indicating its separate position (in clade 2) rather than the results of time-dependent continued evolution, with the exception of the PB2 K340N and the PA I118V exchange: PB2 N and PA V were found in viruses that caused 20% mortality; PB2 K and PA I were found in viruses of different virulence that did not cause fatal cases. 20 mutations were identified that distinguish virulent viruses from less virulent ones. All sequences of human A(H1N1)pdm09 viruses of 2014/15 and 2016/17 isolated in different parts of Germany and deposited in the GISAID repository share the 20 amino acid changes mentioned above (Supplementary Table 17). None of these amino acids are known to be virulence markers, with the exception of HA D222G found in virus JE10. This virus was isolated from a pregnant woman who died of influenza, but it did not cause fatal influenza in pigs, unlike virus HA09 which did not have this mutation. Therefore, there is no evidence for genetic markers of virulence. This is supported by the cell culture study reported above, which also showed no differences between virulent and less virulent viruses. Virulence may be determined by other factors such as morphological characteristics of the virus. This is important because most analyses of virulence focus on sequence information, but this is not the whole picture. It is important to note the interaction between H1 haemagglutinin, N1 neuraminidase and the host cell membrane. The N1 neuraminidases in European and American birds have evolved into two distinct clusters that differ in their compatibility with H1 haemagglutinin. After reassortment, $H1_{cl}$ and European N1 or $H1_{av}$ and American N1 must first adapt to each other in order to allow optimised interaction with rapid virion release. The genetic relationships within the H1 haemagglutinins as well as the N1 and N2 neuraminidases are shown in Supplement 40 and Supplement 41.

Supplementary Table 16: Substitutions in HA and NA distinguishing virulent viruses (HA09, JE09, JE10) from each other (with additional data for the low virulent viruses: SC14, KI15)

Virus	HA*									NA					
nt pos.	4 (4)	32 (49)	119 (136)	130 (147)	203 (220)	222 (239)	286 (303)	460 (477)	520 (537)	81	106	248	434	438	443
HA09	I	L	K	K	T	D	I	I	V	V	I	D	N	T	I
JE09	I	L	E	K	T	D	I	I	V	V	I	D	N	T	I
JE10	T	I	K	I	S	G	V	V	A	A	V	N	H	A	T
SC14	I	L	K	K	T	D	I	I	V	V	I	D	N	T	I
KI15	I	L	K	K	T	D	I	I	V	V	V	D	N	T	I

Supplementary Table 6 continued

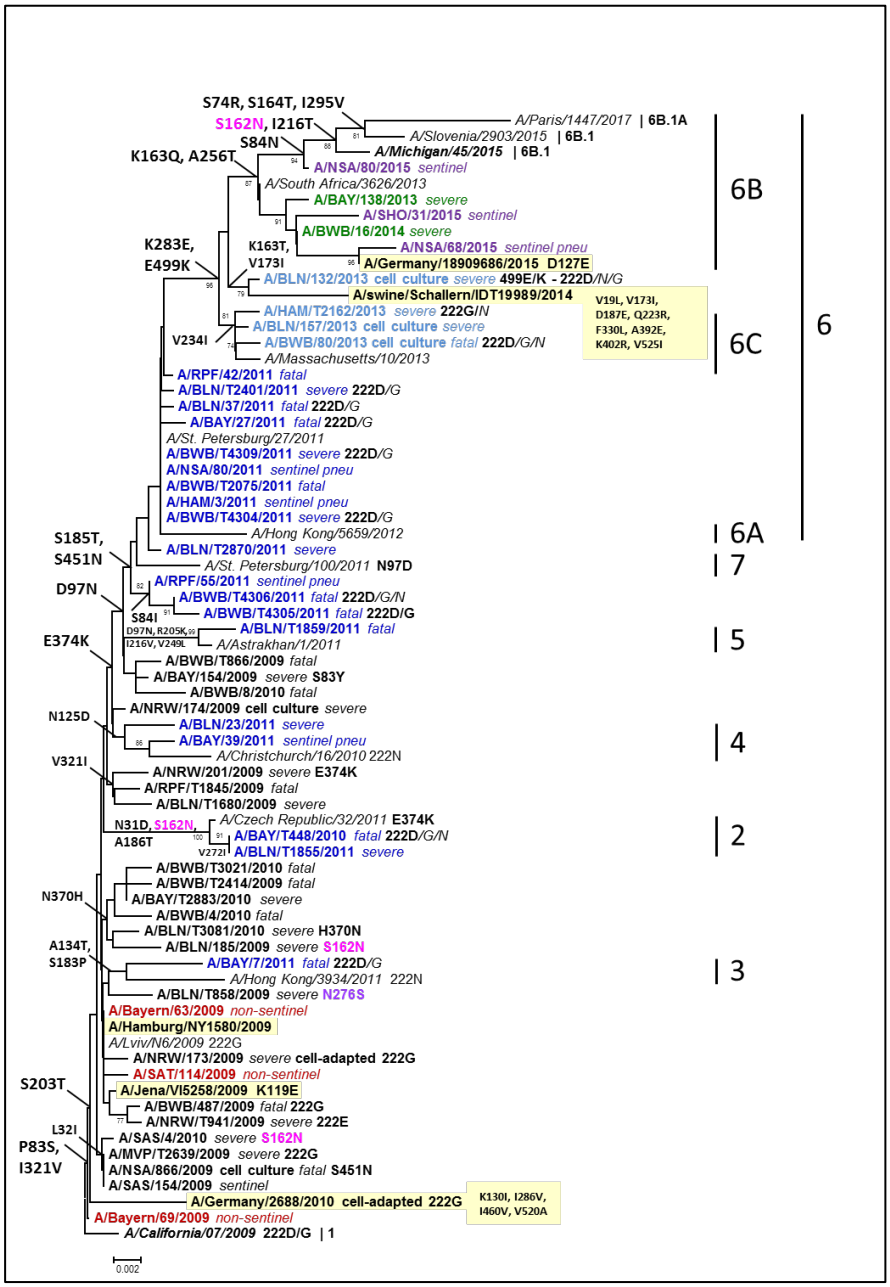
Virus	PA							PB1							PB2			NP	NS1			M1	M2
nt pos.	3	118	369	488	553	562	688	41	77	317	353	398	430	568	76	340	570	65	65	123	180	113	10
HA09	D	V	A	K	A	F	E	D	N	M	K	D	K	I	T	N	M	R	V	V	V	K	P
JE09	D	I	A	K	A	F	K	E	D	M	K	D	K/R	I	N	K	M	R	M	V	V	Q	P
JE10	E	I	V	Q	S	Y	E	D	N	I	R	E	K	T	T	K	I	S	V	I	I	K	H
SC14	D	I	A	K	A	F	E	D	N	M	K	D	K	I	T	K	M	R	V	I	I	K	P
KI15	D	I	A	K	A	F	E	D	N	M	K	D	K	I	T	K	M	R	V	V	V	K	P

* H1 numbering, see Buke & Smith (2014)[508]; in brackets numbering starting from start codon M; red, mutations only found in HA09, blue amino acids only seen in JE09; green, amino acids only found in JE10 few of them shared with SC14 or KI15

Supplementary Table 17: Mutations distinguishing virulent viruses (HA09, JE09, JE10) from low virulent viruses (SC14, KI15)

Virus	HA *							NA		PA			PB2		NP			NS1	NS2	M1
nt pos.	97 (104)	163 (180)	185 (202)	283 (300)	374 (391)	451 (468)	499 (516)	241	369	100	321	330	344	354	425	444	498	205	48	80
HA09	D	K	S	K	E	S	E	V	N	V	N	I	V	I	V	V	S	N	T	V
JE09	D	K	S	K	E	S	E	V	N	V	N	I	V	I	V	V	S	N	T	V
JE10	D	K	S	K	E	S	E	V	N	V	N	I	V	I	V	V	S	N	T	V
SC14	N	T	T	E	K	N	K	I	K	I	K	V	M	L	I	I	N	S	A	I
KI15	N	Q	T	E	K	N	K	I	K	I	K	V	M	L	I	I	N	S	A	I

* H1 numbering (in brackets numbering starting from start codon M of the sequence);
for H3 numbering see Fu et al. (2019)[401]

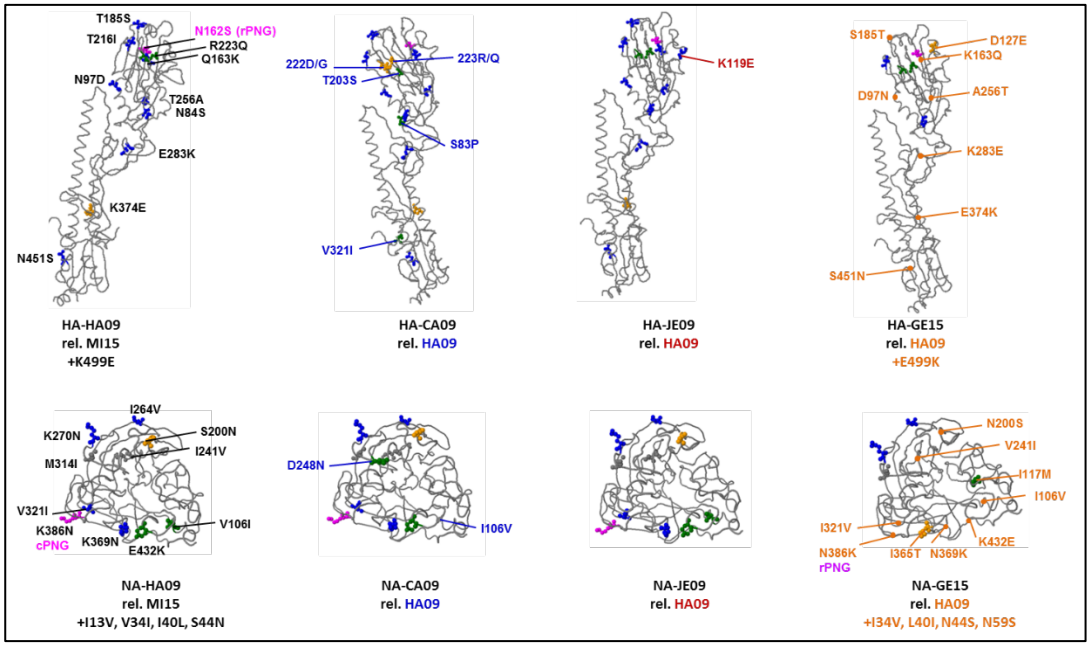


Supplement 36: Phylogenetic analysis of HA genes of A(H1N1)pdm09 viruses circulating between the outbreak in 2009 and the season 2014-2015

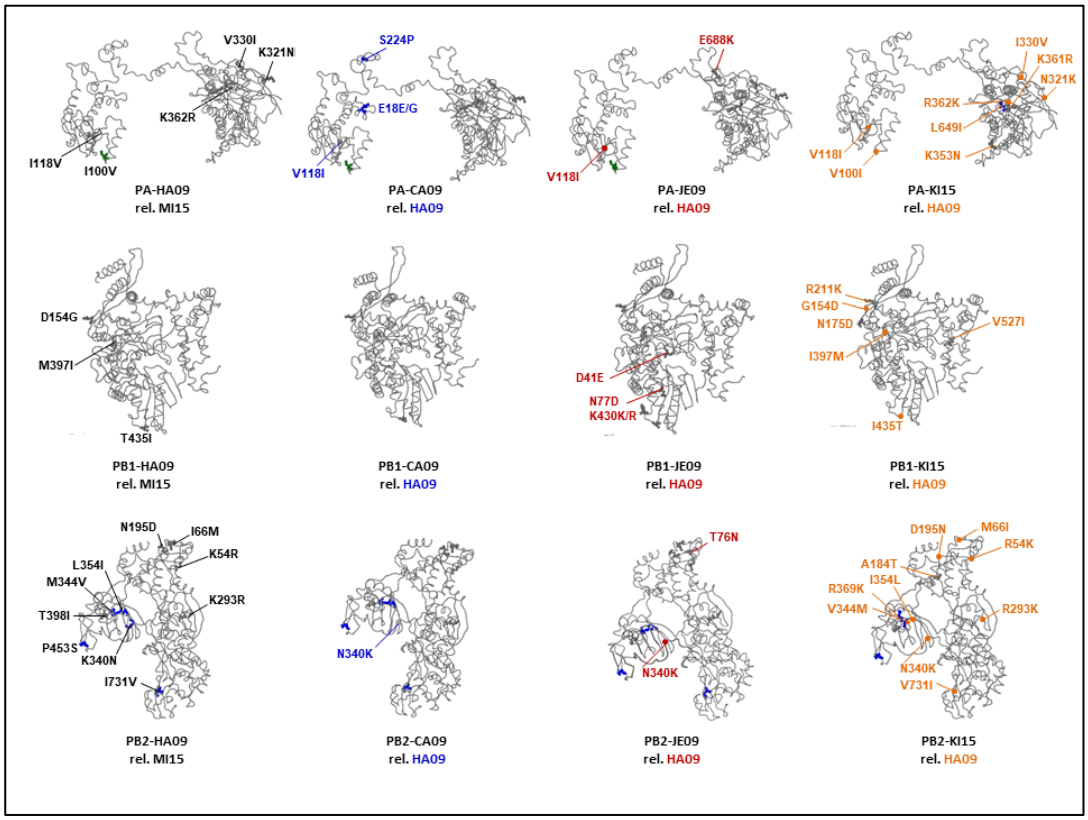
HA genes were sequenced according to Sanger and were phylogenetically evaluated with Mega7 (Neighbor-Joining method, bootstrap test with 1000 replicates, Kimura 2-parameter method). Five cell culture isolates specified according to their virulence (A/Hamburg/NY1580/2009-very high virulent, A/Jena/VI5258/2009-high virulent, A/Germany/2688/2010-virulent, A/swine/Schallern/IDT19989/2014-moderately virulent, A/Germany/18909686/2015-moderately virulent, highlighted in yellow) and 53 viruses (original material=47, cell culture isolates=6) were phylogenetically analyzed: HA nt138 – nt1244 (numbering of nucleotide positions (nt) starting from the first nucleotide of the gene). Almost all viruses were isolated from patients with a severe course of A(H1N1)pdm09 infection (fatal=18, severe=23, community-acquired pneumonia=5 (sentinel pneu), non-sentinel NRZ-orders=3 and AGI-sentinels=3). Substitutions in the deduced HA amino acid sequences were identified by FluSurver database. The identified HA substitutions are displayed according to H1-numbering. H1-numbering starts after the signal peptide (-17). Polymorphisms are indicated as e.g. 222D/G and displayed in italics when they are detected exclusively by pyrosequencing technique (minority variants less than 20%). Clade und sub-clade specific substitutions are indicated and virus specific substitutions are shown for the five virulence specified viruses (see above) relative to A/California/07/2009. According to FluSurver the substitutions are displayed that are associated with creation (pink) or removal (purple) of a potential N-glycosylation site.

The HA sequences are marked as follows: viruses collected during the outbreak in 2009 (red), in the season 2009-2010 (black), 2010-2011 (blue), 2012-2013 (light blue), 2013-2014 (green) and 2014-2015 (purple). The WHO reference sequences are marked in black / italics and the vaccine strains are additional highlighted in bold.

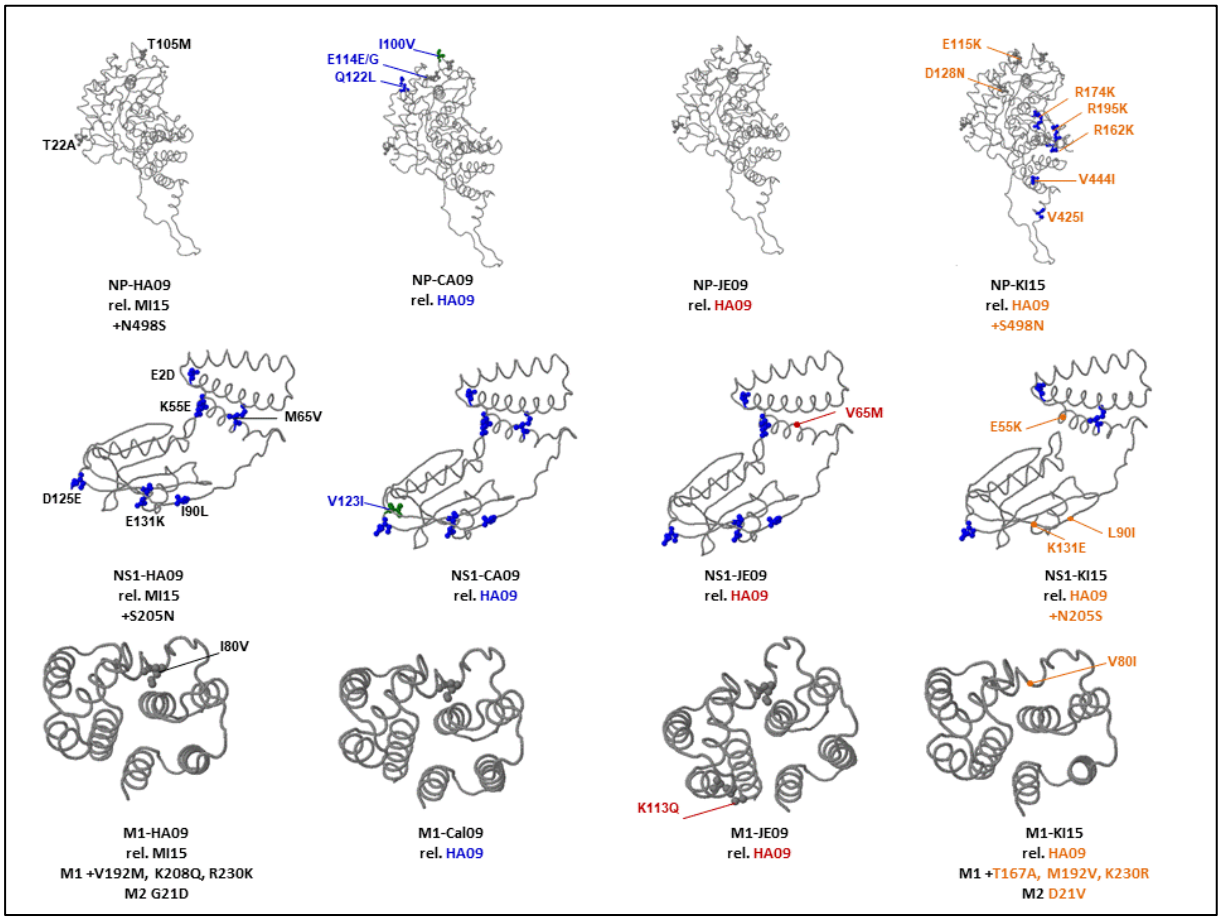
The following abbreviations are used for the names of the virus isolates: BWB: Baden-Württemberg, BAY: Bavaria, BLN: Berlin, BBG: Brandenburg, BRE: Bremen, HAM: Hamburg, HES: Hessen, MVP: Mecklenburg-Vorpommern, NSA: Niedersachsen, NRW: North Rhine-Westphalia, RPF: Rhineland-Palatinate, SAS: Saxony, SAT: Saxony-Anhalt, SAL: Saarland, SHO: Schleswig-Holstein, THR: Thuringia.



Supplement 37: Structural models of (A) HA and NA, (B) PA, PB1 and PB2, and (C) NP, NS1 and M1 of A(H1N1)pdm09 viruses: Substitutions (H1-numbering) detected in the high virulent strain A/Hamburg/NY1580/2009 (HA09) are displayed relative to the reference strain A/Michigan/45/2015 (black). Additionally, the substitutions relative to HA09 are shown for following viruses: A/California/07/2009 (Cal09, blue), A/Jena/VI5258/2009 (JE09, red) and A/Germany/18909686/2015 (GE15, orange). According to FluSurver the substitutions are displayed that are associated with creation (cPNG, pink) or removal (rPNG, purple) of a potential N-glycosylation site; analyses were done via https://flusurver.bii.a-star.edu.sg/



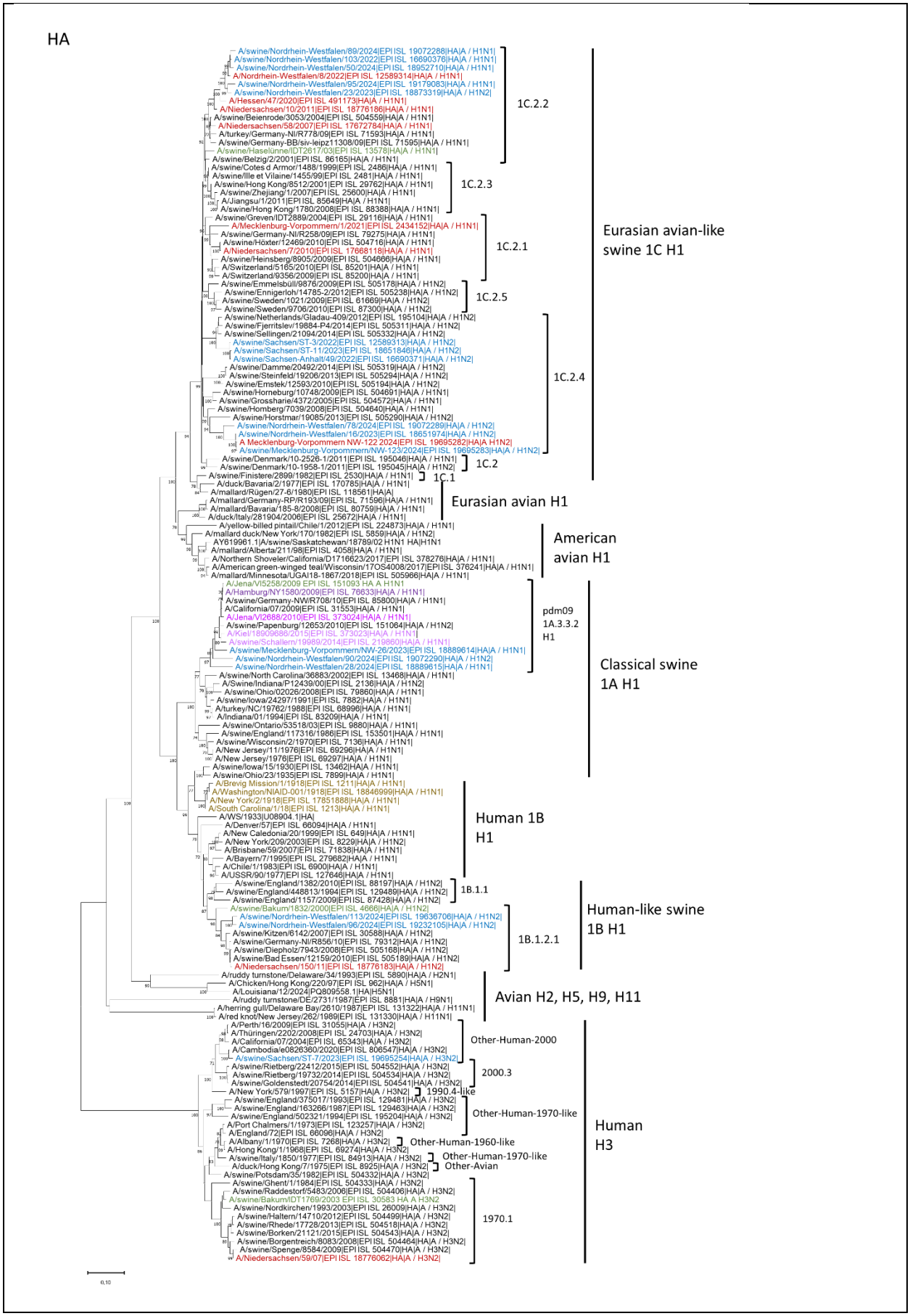
Supplement 38: Structural models of (PA, PB1 and PB2 of A(H1N1)pdm09 viruses: Substitutions (H1-numbering) detected in the high virulent strain A/Hamburg/NY1580/2009 (HA09) are displayed relative to the reference strain A/Michigan/45/2015 (black). Additionally, the substitutions relative to HA09 are shown for following viruses: A/California/07/2009 (Cal09, blue), A/Jena/VI5258/2009 (JE09, red) and A/Germany/18909686/2015 (GE15, orange). According to FluSurver the substitutions are displayed that are associated with creation (cPNG, pink) or removal (rPNG, purple) of a potential N-glycosylation site; analyses were done via https://flusurver.bii.a-star.edu.sg/



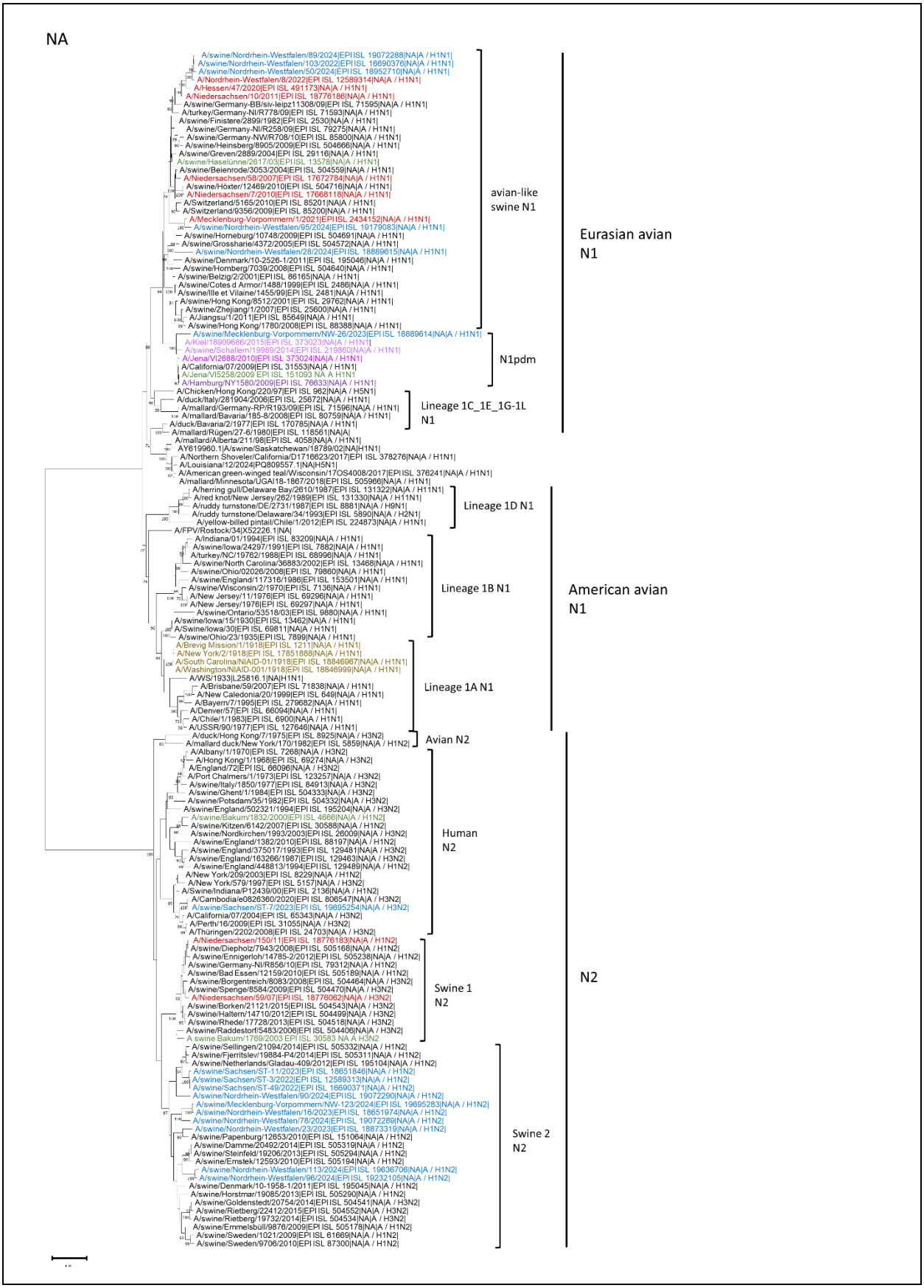
Supplement 39: Structural models of NP, NS1 and M1 of A(H1N1)pdm09 viruses: Substitutions (H1-numbering) detected in the high virulent strain A/Hamburg/NY1580/2009 (HA09) are displayed relative to the reference strain A/Michigan/45/2015 (black). Additionally, the substitutions relative to HA09 are shown for following viruses: A/California/07/2009 (Cal09, blue), A/Jena/VI5258/2009 (JE09, red) and A/Germany/18909686/2015 (GE15, orange). According to FluSurver the substitutions are displayed that are associated with creation (cPNG, pink) or removal (rPNG, purple) of a potential N-glycosylation site; analyses were done via https://flusurver.bii.a-star.edu.sg/

In the following two phylogenetic trees, the relationships between H1 and N1 are shown and supplemented by other groups of haemagglutinins and neuraminidases (Supplement 40, Supplement 41). Blue: current swine influenza viruses, red: influenza viruses from zoonotic swine-human transmissions (for details on zoonotic transmissions see volume 1 of this monograph[1]), green: vaccine strains of swine influenza vaccines, purple: in descending intensity from high virulent to low virulent: $H1_{pdm}N1$ viruses and brown: influenza viruses from 1918.

Influenza reference sequences representing the genome of avian, seasonal, and swine influenza lineages were downloaded from GISAID-database and GenBank. MAFFT-Alignment of nucleotide sequences was created using CLC Genomics Workbench (24.0.1) and evaluated with Mega (11.0.11) using Neighbour-Joining method, bootstrap test with 1000 replicates, Kimura 2-parameter model, partial deletion: site coverage cutoff 5%. Influenza gene nucleotide sequences were deposited in GISAID (http://gisaid.org) and are available under accession numbers in figure (trees).



Supplement 40: The phylogenetic tree reflects the genetic relationships within H1 and H3 hemagglutinins



Supplement 41: The phylogenetic tree reflects the genetic relationships within N1 and N2 neuraminidases; neuraminidases that reassort with haemagglutinin can induce higher virulence if they originate from an NA group that has not yet adapted to haemagglutinin; note that there a two N1 groups: Eurasian and American avian N1; Classical swine H1 ($H1_{cl}$) has been adapted to American N1; reassortment of Classical swine H1 with Eurasian avian N1 in 2009 resulted in a virus of high virulence; therefore, reassortment of European swine H1 with American avian N1 may also result in a virus of high virulence

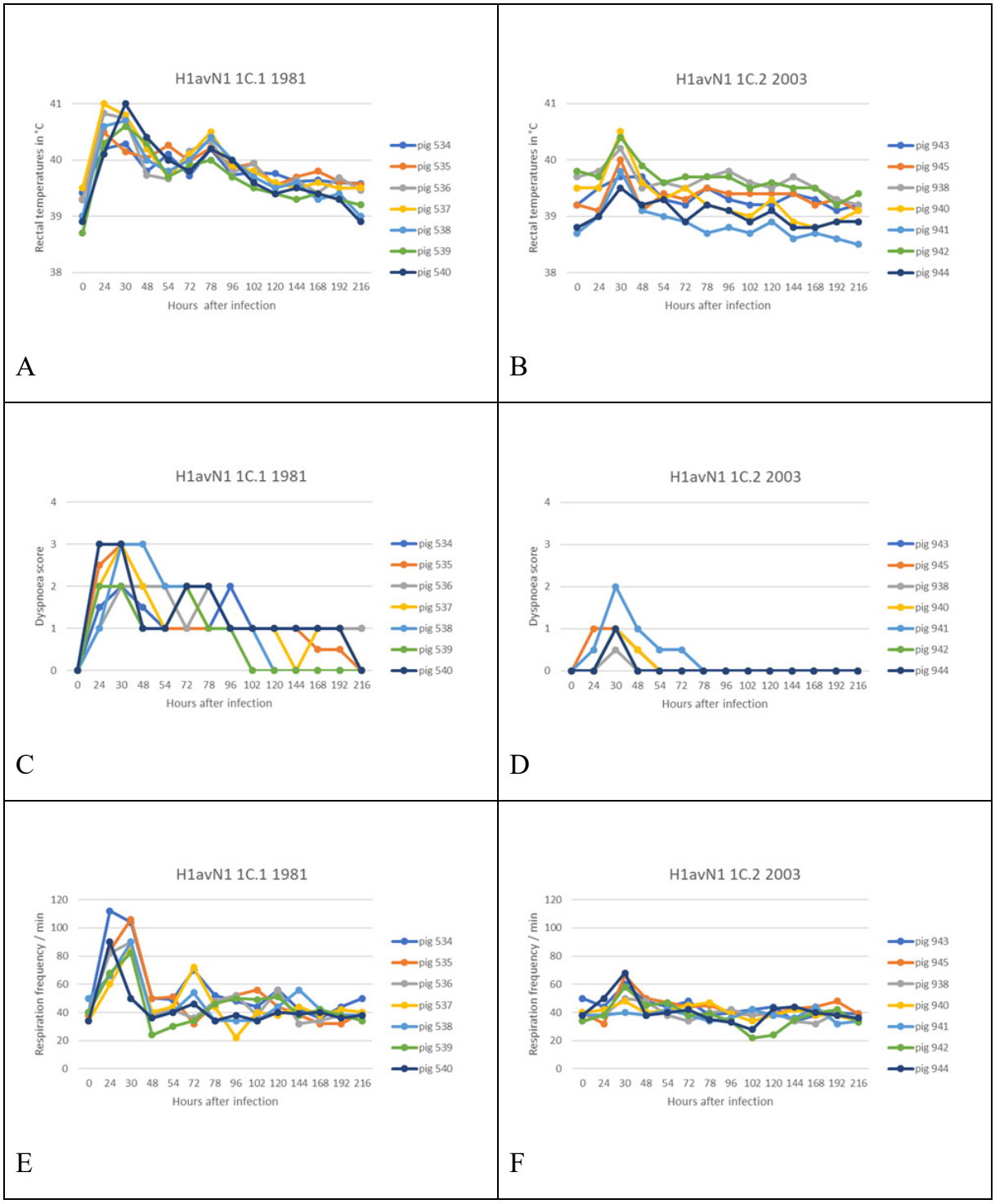
SUPPLEMENT CHAPTER 11 – VIRULENCE OF $H1_{AV}N1$ 1C.1 AND 1C.2 VIRUSES

Background. Following the demonstration of the significant decline in virulence of the $H1_{pdm}N1$ influenza A viruses, it was interesting to take a closer look at the $H1_{av}N1$ influenza A viruses.

Methods: Control pigs from challenge trials with $H1_{av}N1$ influenza A viruses of clusters 1C.1 and 1C.2 were compared with regard to symptoms after infection with an infectious dose of $10^8/m^3$ by aerosol (for 1C.2 a 2003 $H1_{av}N1$ 1C.2.2 virus was used).

Results. The cluster 1C.1 influenza viruses showed clear fever reactions already after 24 hours and a second peak of temperature increases 3 days after infection, while the $H1_{av}N1$ 1C.2.2 influenza viruses only had slight temperature increases 30 hours after infection and the second temperature increase was absent. Similarly, dyspnoea and respiratory rate were more pronounced with infection with $H1_{av}N1$ 1C.1 influenza A viruses (Supplement 42).

Short interpretation of results. With the emergence of 1C.2 influenza viruses, $H1_{av}N1$ influenza viruses are also showing a decline in virulence. The reasons for the longer evolutionary period of the $H1_{av}N1$ influenza viruses compared to the $H1_{pdm}N1$ influenza viruses are unknown. The first influenza viruses of the $H1_{av}N1$ 1C.2 cluster analysed (A/sw/Belzig/02/2001) showed an intermediate virulence between viruses from 1998 ($H1_{av}N1$ 1C.1) and 2003 ($H1_{av}N1$ 1C.2), indicating that this process took place between 1998 and 2003. The $H1_{av}N1$ influenza A viruses also evolved towards low virulence, although this process took longer than with the $H1_{pdm}N1$ viruses. Since the analysed $H1_{cl}N1$ influenza A virus also showed a comparably low virulence, it can be assumed that this process also took place in the $H1_{cl}N1$ viruses. In this context, it should be noted that the evolution of $H1_{pdm}N1$ towards lower virulence described above occurred in humans. Apparently, more passages were made in humans in a shorter period of time than in pigs.



Supplement 42: Comparison of the virulence of H1$_{av}$N1 influenza viruses; A, C, D, infection with A/sw/Potsdam/15/1981; B, D, F, infection with A/sw/Haselünne/2617/2003

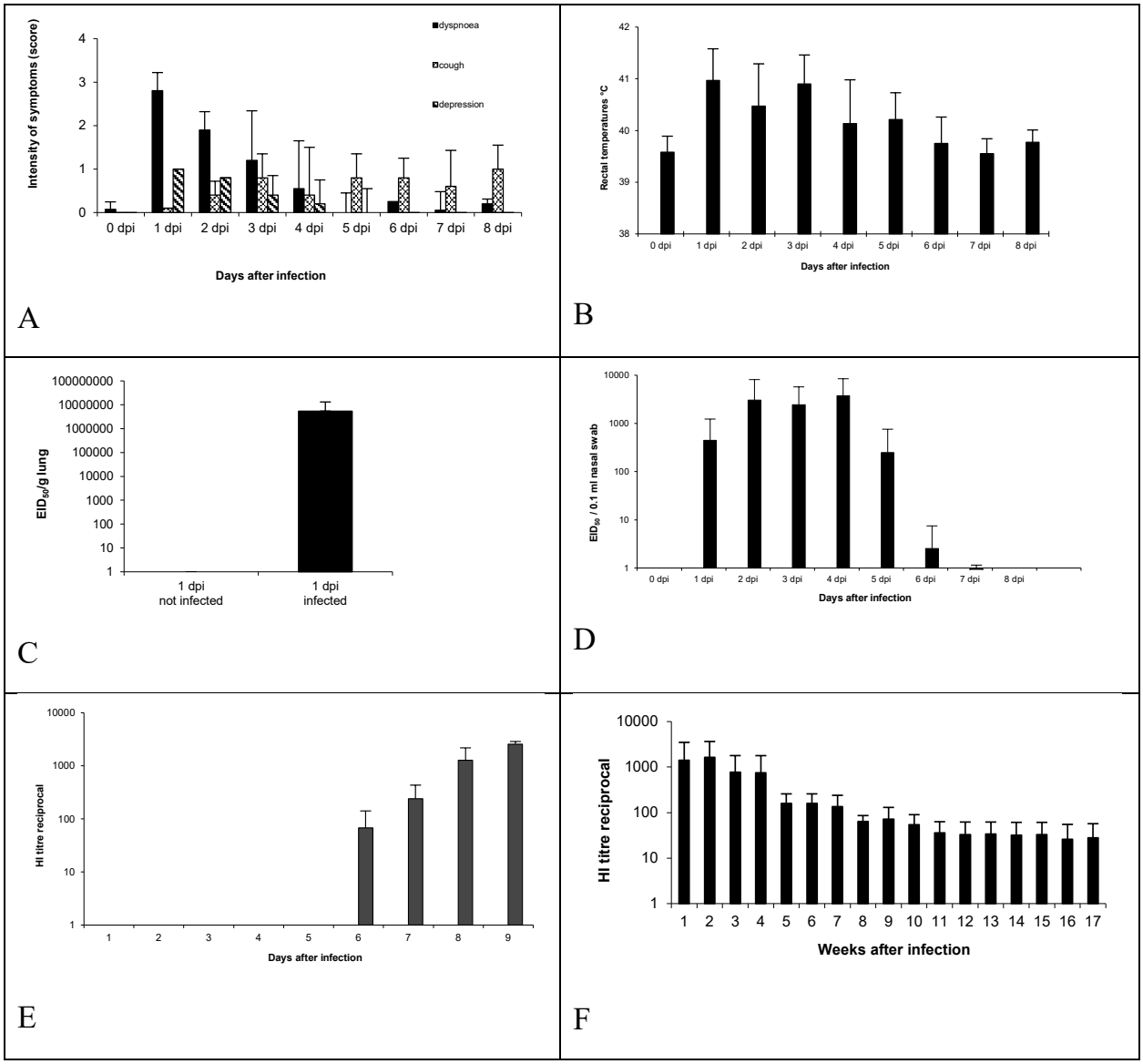
$H1_{AV}N1$ 1C.1 VIRUSES

The data for kinetics of clinical and immunological data were established in 12 weeks old pigs in a basic trial using strain A/sw/Bakum/3543/1998 ($H1_{av}N1$). Marked signs of respiratory disease and an elevated body temperature were observed after just 24 hours. Coughing was only observed in isolated cases. The most prominent sign of disease was dyspnoea reflected by strong pumping and flank and nostril movements accompanied by depression in the form that pigs lay lethargic around in the corners of the infection unit and did not move much. Cough was no prominent sign of the disease and occurred only in a few pigs at the end of the first infection week (Supplement 43). Respiratory frequency was increased in the control pigs but varied much between the individual pigs and was excluded as parameter from most of the following trials because of its time-consuming character (with the exception of the trial comparing different infection models). Depression correlated with dyspnoea. Therefore, further trials mainly focused on dyspnoea and rectal temperature to assess clinical symptoms. The infection induced two peaks of fever on 1 and 3 dpi in pigs.

Antibodies induced by the infection reached the highest level around 9-14 dpi and decreased thereafter weekly until they reached a final level around 1:10 to 1:40.

The viral lung load was high already 1 dpi, Infected pigs had lower body weight gains over the first week after infection in comparison to uninfected; later trials showed that this is only the case when the pigs are still growing and when symptoms had been induced by infection (data not shown).

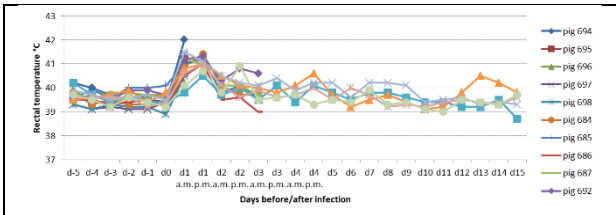
Virus shedding was observed over 7 days and had its peak 2 – 4 dpi (Supplement 43 D). The decrease in virus shedding was accompanied by occurrence of the first antibodies (HI) around day 6. The different subtypes vary in their antigenicity. H3N2 and $H1_{hu}N2$ viruses induce the highest and long-lasting antibodies whereas H1N1 viruses display the lowest antibody induction.



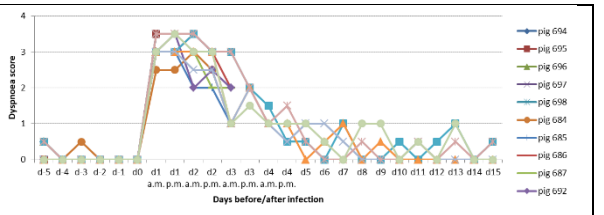
Supplement 43: Establisment of clinical, serological and serological parameters after high dose aerosol infection with strain A/sw/Bakum/3543/1998 ($H1_{av}N1$ 1C.1), n=13 pigs until 1 dpi, thereafter 7 pigs; on 1 dpi 5 pigs were euthanised and lung samples were taken; A, Symptoms; B, Rectal body temperatures; C, Viral lung load 1 dpi; D, Virus excretion; E, HI antibodies in the first week after infection; F, HI antibody kinetics in the first 4 months after infection; arithmetic mean with standard deviation, geometric mean for antibodies

SUPPLEMENT CHAPTER 12 – INVESTIGATION OF CLINICAL CHEMICAL PARAMETERS

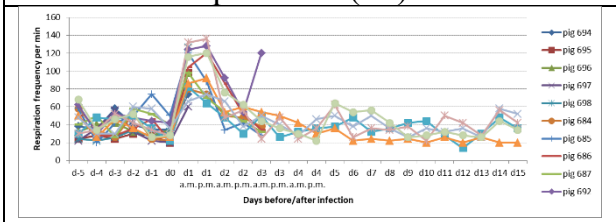
The kinetics of clinical chemical parameters and immune response after high-dose aerosol infection of pigs were investigated following infection with strain A/Jena/VI2688/2010 ($H1_{pdm}N1$). This strain was isolated from a pregnant woman who died of influenza. 15 pigs were included in the study. At 1 and 3 days post infection (dpi), 5 pigs were removed from the study for lung sampling. 5 pigs were retained until the end of the study. None of the pigs in this study died of infection. Serum parameters were measured. Synlab, Leipzig, Germany, was contracted to conduct the clinical chemistry studies. IFNγ, TNFα and IL6 were determined by commercially available ELISAs (Quantikine porcine IFNγ, TNFα and IL6 kits, R&D Systems, Wiesbaden, Germany). Despite the induction of severe influenza, clinical chemistry parameters did not change significantly after infection (although a few individuals had stronger reactions). Antibodies appeared at 5 dpi, but IFNγ, TNFα and IL6 did not increase after infection. The effect of infection on clinical chemistry parameters indicates that infection and the pathogenic process are not limited to the respiratory tract, but affect several organs. There was a large individual variation between pigs, suggesting an influence of host factors. Serum interleukins also reflected individual variation (Supplement 44).



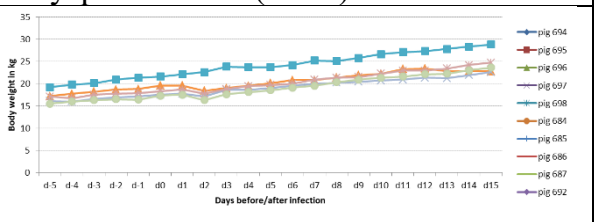
A Rectal Temperatures (°C)



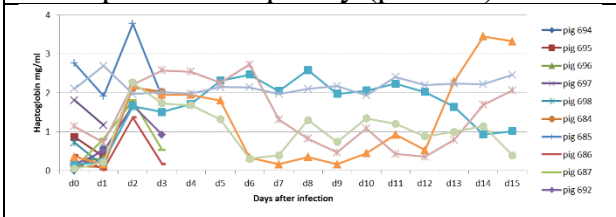
B Dyspnoea score (score)



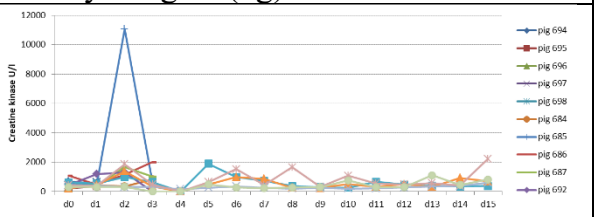
C Respiration frequency (per min)



D Body weights (kg)



E Haptoglobin, VHAPTO (mg/ml)



F Creatine kinase, CK (U/l)

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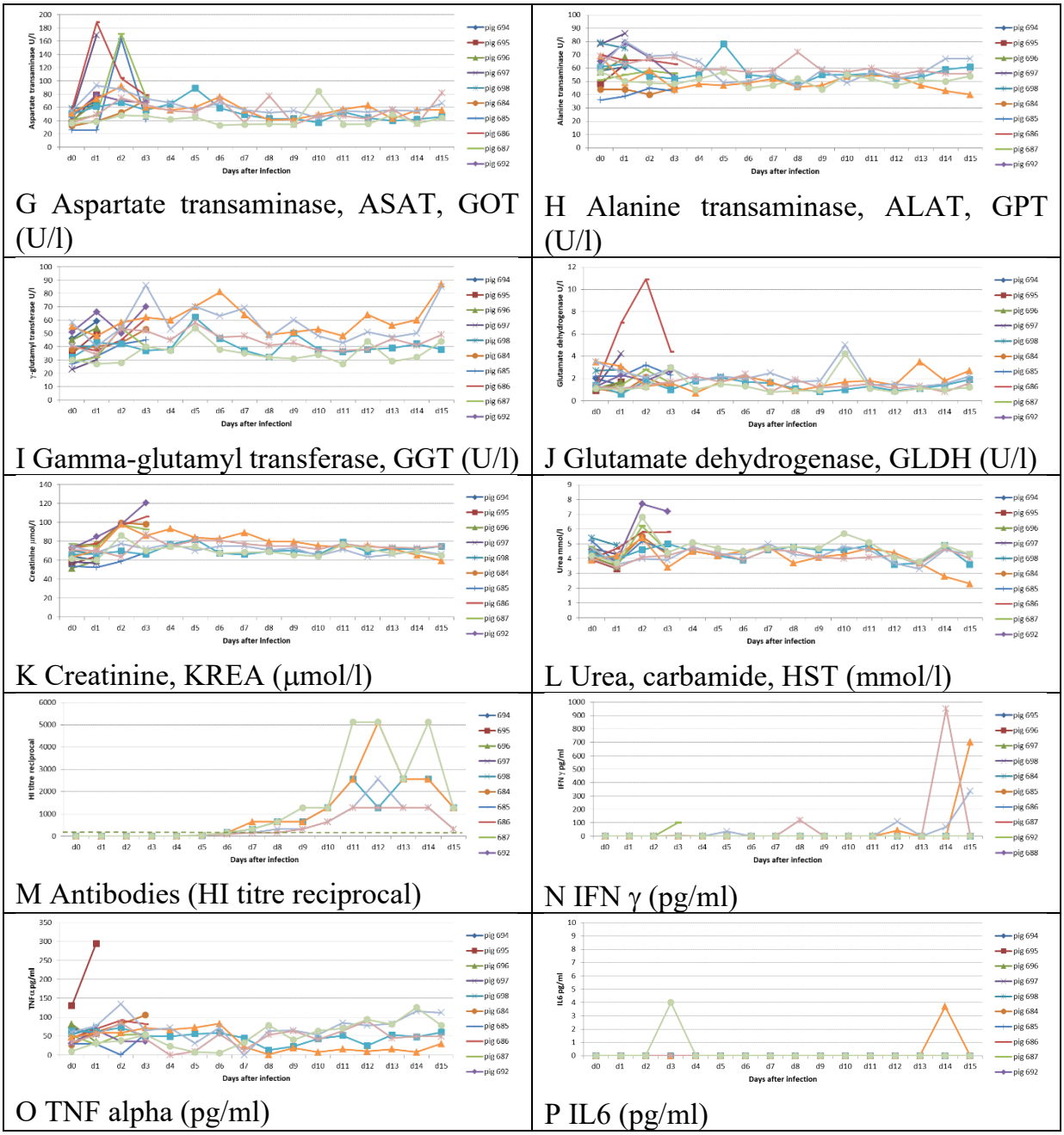


G Aspartate transaminase, ASAT, GOT (U/l)

H Alanine transaminase, ALAT, GPT (U/l)

I Gamma-glutamyl transferase, GGT (U/l)

J Glutamate dehydrogenase, GLDH (U/l)

K Creatinine, KREA (µmol/l)

L Urea, carbamide, HST (mmol/l)

M Antibodies (HI titre reciprocal)

N IFN γ (pg/ml)

O TNF alpha (pg/ml)

P IL6 (pg/ml)

Supplement 44: Kinetics of clinical chemical parameters and immune response after high-dose aerosol infection of pigs with strain A/Jena/VI2688/2010 ($H1_{pdm}N1$). The parameters (E-P) were determined in sera. Despite the induction of severe influenza (A-D) clinical chemical parameters (E-I) did not change significantly after infection (although a few individuals had stronger reactions). At some time points, pigs were were removed from the experiment for examination of lung samples; therefore follow-ups were not available. Antibodies appeared on 5 dpi (M) but IFNγ, TNFα and IL6 did not increase after infection in most of the pigs (N-P). The clinical chemical parameters were investigated under contract by Synlab, Leipzig, Germany. IFNγ, TNFα and IL6 were determined by commercially available ELISAs (Quantikine porcine IFNγ, TNFα and IL6 Kits, R&D Systems, Wiesbaden, Germany)

To further investigate clinical parameters, five three-month-old pigs with different outcomes of A/Hamburg/NY1580/2009 $H1_{pdm}N1$ virus infection (all from the same farrowing event and trial) were selected and serum samples (taken every second day after infection) were analysed for C-reactive protein (CRP), neopterin, haptoglobin and HI antibodies

(Supplementary Table 18). Synlab was commissioned with the analyses. The analyses were carried out using ELISAs (CRP - Life Diagnostics, neopterin, haptoglobin - IBL International).

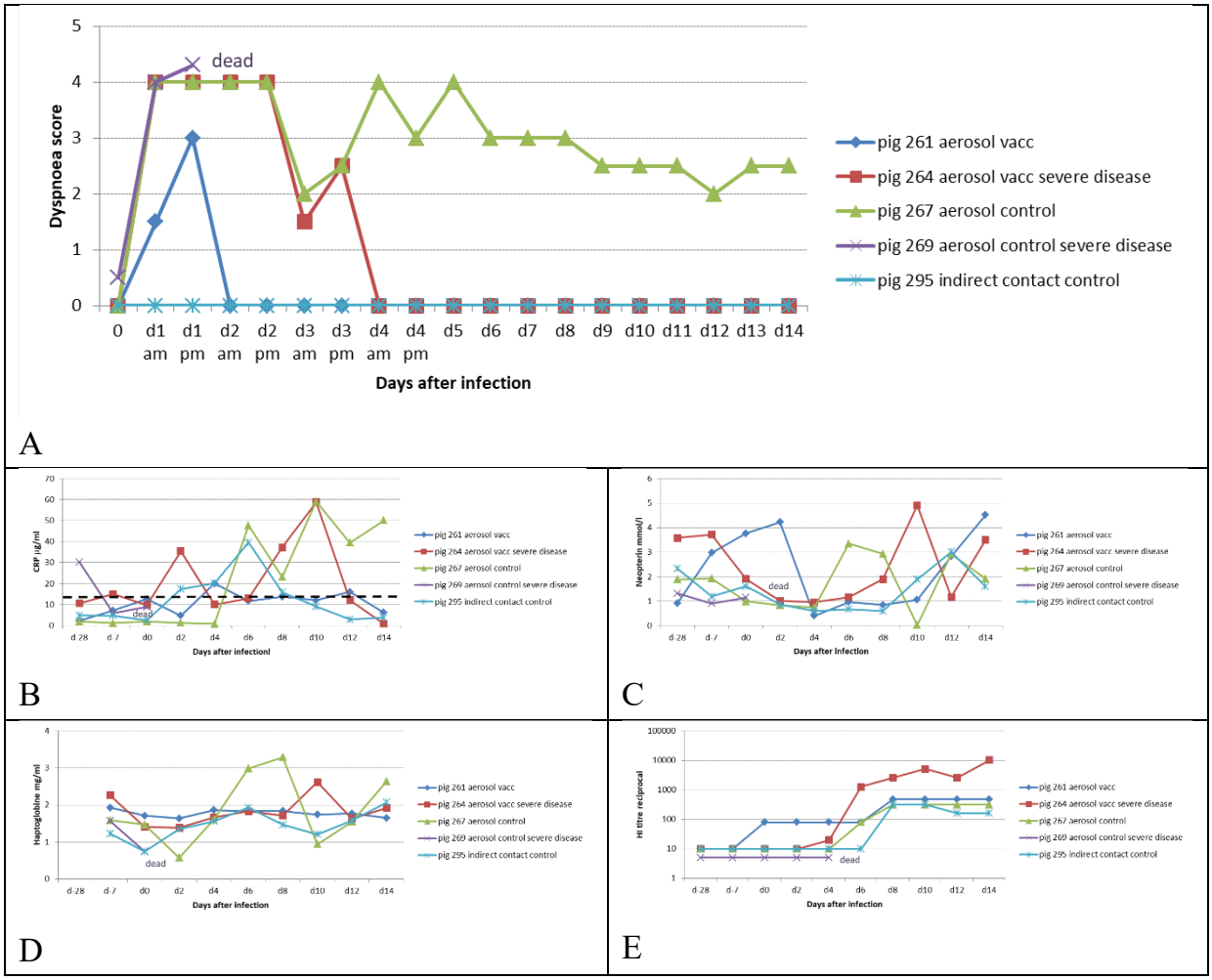
Supplementary Table 18: Overview of selected pigs for analysis of clinical parameters (CRP, neopterin, haptoglobine)

Pig	Vaccination	Infection route	Outcome
261	yes	Aerosol (high dose)	Short period of moderate disease
264	yes	Aerosol (high dose)	Severe disease and recovery
267	no	Aerosol (high dose)	Long-lasting severe disease
269	no	Aerosol (high dose)	Lethal severe disease
295	no	Indirect contact	No disease

In the pig that died of influenza, CRP was already activated before infection (Supplement 45). This may indicate inflammation prior to infection, which may have contributed to the severe course of the disease. All infected pigs developed elevated serum CRP levels. This level was lowest in the vaccinated pig with short and moderate respiratory disease. The vaccinated pig with severe disease due to lack of initial antibodies (vaccination response blocked by maternal immunity) had an initial peak in CRP during the period of severe disease, but developed a second peak after recovery when it showed no symptoms. The pig with prolonged disease showed intermittent peaks in CRP levels that persisted until the end of the study. The indirectly exposed pig also showed CRP activation.
There was only one pig that showed an increase in haptoglobin 1 weakly after infection (dpi 6-8), the pig with prolonged disease (3.0-3.3 mg/ml) (Supplement 45).
At the beginning of the study (first immunisation of two of the pigs), the maternally immune pig (pig from a vaccinated sow) had the highest neopterin level (3.6 mmol/l), while the others were in the range of 0.9 - 2.3 mmol/l (Supplement 45). The vaccine-protected pig (from a sow not vaccinated against influenza) responded with a large increase in neopterin to vaccination (3.8 mmol/l) and infection (4.2 mmol/l) and showed a second peak 14 days after infection (4.5 mmol/l). The aerosol control pig showed a peak in neopterin levels at 6-8 dpi (1.2 and 1.6 mmol/l). All infected pigs showed higher neopterin levels at infection (10-14 dpi). All pigs responded to infection with HI antibodies; the vaccinated pig that recovered from the disease had the highest titres.

Short interpretation of results. The April 2009 $H1_{pdm}N1$ virus repeatedly induced severe disease in 3-month-old pigs in all experiments, causing approximately 20% lethality. C-reactive protein (CRP) activation was observed after infection. CRP levels were high in pigs with severe disease but also after recovery from severe respiratory distress. The vaccinated pig with moderate disease had the lowest level of CRP. CRP is an acute-phase protein of hepatic origin that binds to the surface of dead or dying cells and activates the complement system, promoting phagocytosis of necrotic cells by macrophages. CRP production is stimulated by increasing levels of interleukin-6, which is produced by macrophages in conditions of acute or chronic inflammation. Interferon α inhibits the production of CRP. Increased levels of CRP have been found after H7N9 infection[509] but also after

infection with SARS-CoV-2, where CRP could be correlated with the severity of COVID-19[510]. In pigs infected with $H1_{pdm}N1$ virus in April 2009, CRP was highest in the later stages of infection, not at the peak of symptoms.

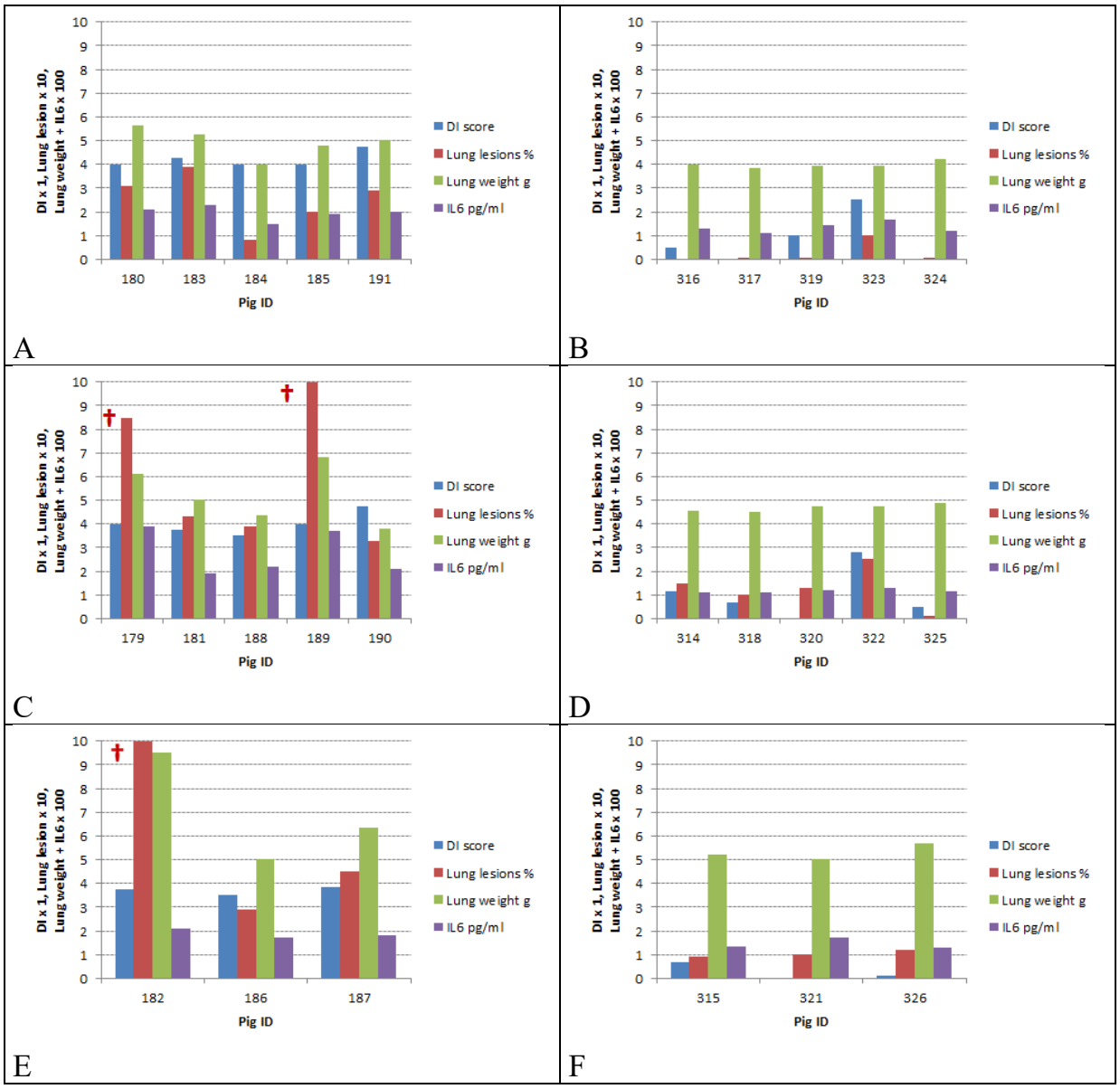


Supplement 45: Kinetics of dyspnoea (A), C-reactive protein (B), neopterin (C), haptoglobine (D), and HI antibodies (E) in pigs selected from different groups (aerosol infection: vaccinated, vaccinated into maternally-derived immunity, not vaccinated with severe disease, not vaccinated with lethal disease; indirect contact infection: not vaccinated) after infection with $H1_{pdm}N1$ April 2009 virus (A/Hamburg/NY1580/2009)

Haptoglobin binds free plasma haemoglobin. It is also an antibacterial component of alveolar fluid. During haemolysis, plasma haptoglobin levels are reduced, whereas during inflammation they are increased. There was only one pig that showed an increase in haptoglobin one week after infection, the pig in the aerosol control group with long-standing disease. The higher haptoglobin levels in this pig - at a time when no infectious virus was detectable - may indicate increased bacterial activity in the lung microbiome (bacterial commensals that were not affected by the antibiotic treatment at the start of the study).

There was always a great individual variation in the size of lung lesions although the pigs originated from the same farrowing event and had been infected at exact the same time with the same dose of virus. If lung lesions extended more than 50% of the lung this was

accompanied by a larger lung weight. Expression of cytokines (demonstrated by IL6) was higher in pigs with larger lesions. Supplement 46 represents the individual reaction patterns of 3-months-old pigs after infection with high virulent and low virulent $H1_{pdm}N1$ viruses.



Supplement 46: Invividual variation in lung lesion size (red), lung weight (green) and cytokine expression (purple, reflected by IL-6) after infection of pigs with (A, C, E) high virulent (A/Hamburg/NY1580/April 2009) and (B, D, F) low virulent (A(sw/Schallern/19989/2014) $H1_{pdm}N1$ viruses (A, B) 1 dpi, (C, D) 3 dpi, and (E, F) 9 dpi; DI, Disease index (average of symptoms within the first 3 days, for 1 dpi within the first day after infection); x1, x10, x100, factors used in order to optimize the scale; † , death of animal due to influenza; the infectious dose in the trials was $10^{8.3}$ (high virulent, strain NY1580) and $10^{9.46}$ (low virulent, strain 19989) $TCID_{50}$ MDCK/m^3, Pig ID, Pig identification (ear tag)

SUPPLEMENT CHAPTER 13 – ENHANCEMENT OF LUNG PATHOLOGY WITHIN AN ANTIGENIC SUPERGROUP ($H1_{PDM}$ VERSUS $H1_{AV}$)

Background. It has been shown that infection with influenza viruses whose haemagglutinin and neuraminidase are not covered by the vaccine strongly increases lung pathology and morbidity. The question was whether such effects could also be observed with homo-subtype infection, when antibodies against the receptor-binding surface glycoproteins are low or absent.

Methods. To this end, pigs were immunised with laboratory batches containing either $H1_{pdm}N1$ or $H1_{av}N1$ virus or both components (together with viruses of other subtypes) and challenged with $H1_{av}N1$ virus (Supplementary Table 19). $H1_{pdm}N1$ and $H1_{av}N1$ are antigenically related: their haemagglutinins do not cross-react at the level of immune sera, but do cross-react at the level of hyperimmune sera, suggesting that they recognise and prime each other.

Supplementary Table 19: Overview of experimental design of the study

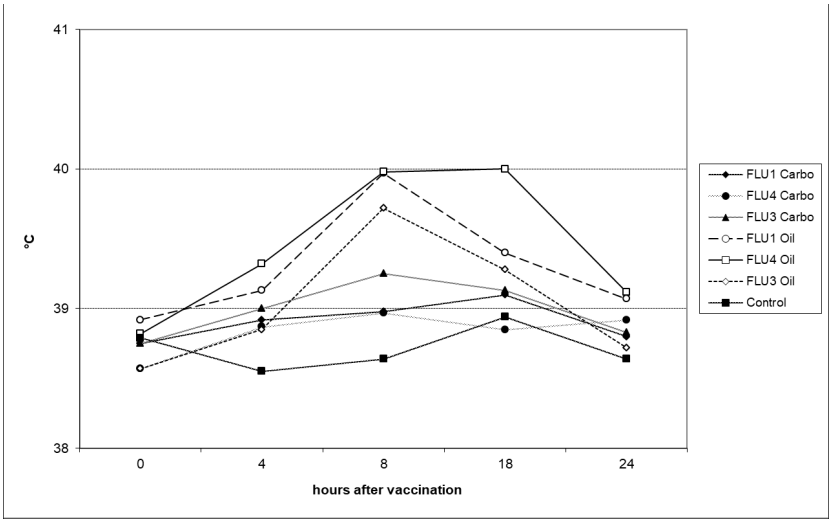
Group	Virus used for immunisation*	Immunogenic characterisation	Number of pigs included
FLU1 Carbo	FLUAV/Hamburg/7/2009 ($H1_{pdm}N1$) Carbopol adjuvant	Different intrasubtypic virus in comparison to infection virus Not strongly adjuvanted	6
FLU4 Carbo	FLUAV/sw/Harlebach/2998/2004 ($H1_{av}N1$) FLUAV/Anderlingen/3507/2004 ($H1_{hu}N2$) FLUAV/sw/Bissendorf/1864/2003 (H3N2) FLUAV/Hamburg/7/2009 ($H1_{pdm}N1$) Carbopol adjuvant	Both intrasubtypic viruses ($H1_{pdm}N1$ + $H1_{av}N1$) contained Not strongly adjuvanted	6
FLU3 Carbo	FLUAV/sw/Harlebach/2998/2004 ($H1_{av}N1$) FLUAV/Anderlingen/3507/2004 ($H1_{hu}N2$) FLUAV/sw/Bissendorf/1864/2003 (H3N2) Carbopol adjuvant	Vaccine $H1_{av}N1$ virus homologous to infection virus Not strongly adjuvanted	6
FLU1 Oil	FLUAV/Hamburg/7/2009 ($H1_{pdm}N1$) ISA25 adjuvant (mineral oil)	Different intrasubtypic virus in comparison to infection virus Strongly adjuvanted	6
FLU4 Oil	FLUAV/sw/Harlebach/2998/2004 ($H1_{av}N1$) FLUAV/Anderlingen/3507/2004 ($H1_{hu}N2$) FLUAV/sw/Bissendorf/1864/2003 (H3N2) FLUAV/Hamburg/7/2009 ($H1_{pdm}N1$) ISA25 adjuvant (mineral oil)	Both intrasubtypic viruses ($H1_{pdm}N1$ + $H1_{av}N1$) contained Strongly adjuvanted	6
FLU3 Oil	FLUAV/sw/Harlebach/2998/2004 ($H1_{av}N1$) FLUAV/Anderlingen/3507/2004 ($H1_{hu}N2$) FLUAV/sw/Bissendorf/1864/2003 (H3N2) ISA25 adjuvant	Vaccine $H1_{av}N1$ virus homologous to infection virus Strongly adjuvanted	6
Control	None (not vaccinated control)	-	7

* the viruses were inactivated; the content of antigen was 16 hemagglutinating units of each virus

Study design. Monovalent and multivalent inactivated laboratory vaccine batches were prepared in two groups: Low adjuvated (Carbopol 971 P NF, Carbomer, Carboxy-polymethylene, Polyacrylic acid, Lubrizol, Wickliffe, USA) and high adjuvated (Mineral oil ISA 25, Seppic, France) vaccines with identical vaccine strain composition. Pigs were vaccinated twice within 21 days. Rectal temperatures were taken every 2 to 24 hours after each

vaccination. Ten days after the second vaccination, a high-dose aerosol challenge with 8.93 lg $TCID_{50}/m^3$ of virus A/sw/Harlebach/2998/2004 ($H1_{av}N1$) was performed. Pigs were observed for dyspnoea and fever twice daily, and later daily. Blood samples were taken before each vaccination, 10 and 11 days after the second vaccination and every 2 days after infection and analysed for antibodies against haemagglutinin and neuraminidase (HI and neuraminidase antibody tests, see Materials and methods in the general part). Half of the pigs were removed from the study at 2 dpi and the other half at 10 dpi to examine the lungs for macroscopic lung lesions.

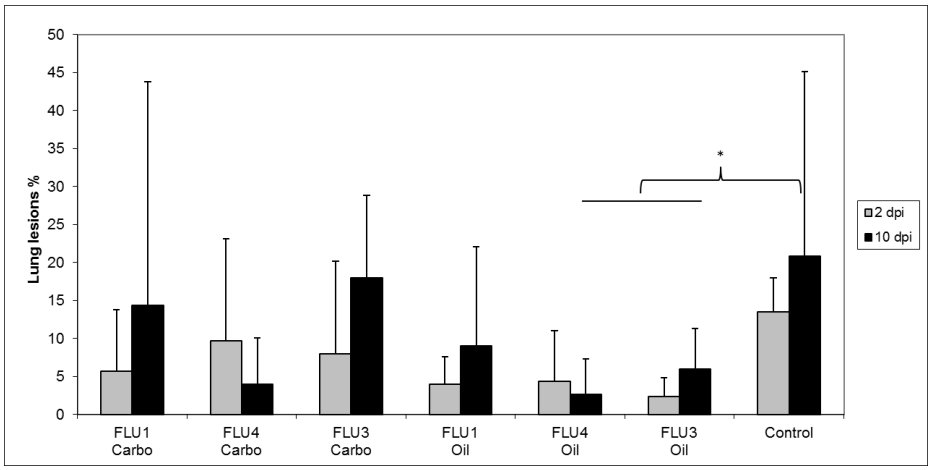
Results. Pigs vaccinated with oil-adjuvanted vaccines responded to vaccination with fever, peaking between 8 and 18 hours after vaccine administration, whereas pigs vaccinated with carbopol-adjuvanted vaccines did not show fever (Supplement 47). Pigs vaccinated with mineral oil-adjuvanted vaccines responded to vaccination with higher antibody titres than pigs vaccinated with carbopol-adjuvanted vaccines (Supplementary Table 20). In addition, the antibody response obtained with vaccines adjuvated with mineral oil was broader and covered a wider intrasubtype range (Supplementary Table 21). There was greater variation in individual immune responses after immunisation with low adjuvated vaccines. Individual pigs with low levels of antibody to the surface proteins showed more extensive lung lesions at 2 dpi and 10 dpi than pigs in the control group. Some pigs with higher lung pathology also had more severe disease.



Supplement 47: Rectal temperatures after administration of experimental vacxcines (here measured after second administration of the vaccine; arithmetic means are displayed

Pigs vaccinated with experimental batches containing a strong adjuvant (mineral oil) did not show the same extension of lung lesions as those observed in pigs vaccinated with vaccines containing a less strong adjuvant (carbopol) (Supplement 48). One vaccinated pig that did not have neuraminidase inhibitory antibodies developed a disease index (average of dyspnoea scores over the first few days after infection) that was almost as high as that observed in unvaccinated pigs. This pig reflected also stronger macroscopic lung lesions in comparison to control pigs (Supplement 49). Two other pigs that did not have

neuraminidase inhibitor antibodies also showed more extensive lung lesions, one of which developed symptoms similar to those in the control group.



Supplement 48: Lung lesions (%) in pigs of vaccinated and not vaccinated groups after experimental aerosol infection with swine $H1_{av}N1$ virus indicate a higher variability in pigs immunised with low-adjuvanted vaccines; arithmetic means with standard deviation

Supplementary Table 20: Antibodies (HI, NI) induced by vaccination with batches of Carbopol-adjuvanted vaccines 10 days after second administration of the vaccine (= at challenge) and lung lesions 2 and 10 dpi as well as disease index in comparison to the not vaccinated control group (lung lesions of vaccinated pigs that exceed the average of the control group are highlighted)

Group	Pig ID	αH1	αH1(N2)	αH3	αH1pdm	αN1	αN2	Lung lesions 2 dpi %	Lung lesions 10 dpi %	DI*
FLU1 Carbo	901	<20	<20	<20	**320**	**8**	<2	n.i.	**5**	**0**
	902	<20	<20	<20	**320**	**8**	<2	n.i.	**3**	**0.33**
	903	<20	<20	<20	**640**	**16**	<2	**2**	n.i.	**0**
	904	**80**	<20	<20	**2560**	**8**	<2	**0**	n.i.	**0**
	905	<20	<20	<20	**160**	<2	<2	n.i.	**35**	**1.67**
	906	<20	<20	<20	**2560**	**8**	<2	**15**	n.i.	0.67
FLU4 Carbo	907	**80**	**40**	**80**	**160**	**8**	**8**	n.i.	**1**	**0.17**
	908	**80**	**40**	**160**	**160**	**8**	**8**	**0**	n.i.	**0.17**
	909	**80**	**40**	**160**	**320**	**16**	**8**	n.i.	**0**	**0.67**
	910	**80**	**80**	**160**	**320**	**8**	**8**	4	n.i.	**0.5**
	911	**320**	**160**	**160**	**1280**	**16**	**16**	n.i.	**11**	**0.17**
	912	**160**	**160**	**160**	**320**	<2	**8**	**25**	n.i.	**0.83**
FLU3 Carbo	913	**640**	**320**	**640**	<20	**8**	**8**	**2**	n.i.	**0.5**
	914	**640**	**320**	**640**	<20	**8**	**4**	n.i.	**30**	**0.17**
	915	<20	<20	<20	<20	<2	<2	**22**	n.i.	**0.17**
	916	**320**	**320**	**640**	<20	**8**	**8**	n.i.	**15**	**0.33**
	917	**80**	**80**	**80**	<20	**8**	**8**	n.i.	**9**	**0**
	918	**640**	**640**	**1280**	<20	**16**	**16**	**0**	n.i.	**0**
Control Not vacc	937	<20	<20	<20	<20	<2	<2	**15**	n.i.	**1.83**
	938	<20	<20	<20	<20	<2	<2	n.i.	**30**	**0.33**
	939	<20	<20	<20	<20	<2	<2	**15**	n.i.	**1,5**
	940	<20	<20	<20	<20	<2	<2	n.i.	**22**	**1**
	941	<20	<20	<20	<20	<2	<2	n.i.	**14**	**0.83**
	942	<20	<20	<20	<20	<2	<2	n.i.	**28**	**2**
	943	<20	<20	<20	<20	<2	<2	**7**	n.i.	**1.33**
	944	<20	<20	<20	<20	<2	<2	n.i.	**10**	**0.67**
	945	<20	<20	<20	<20	<2	<2	**17**	n.i.	**1.67**

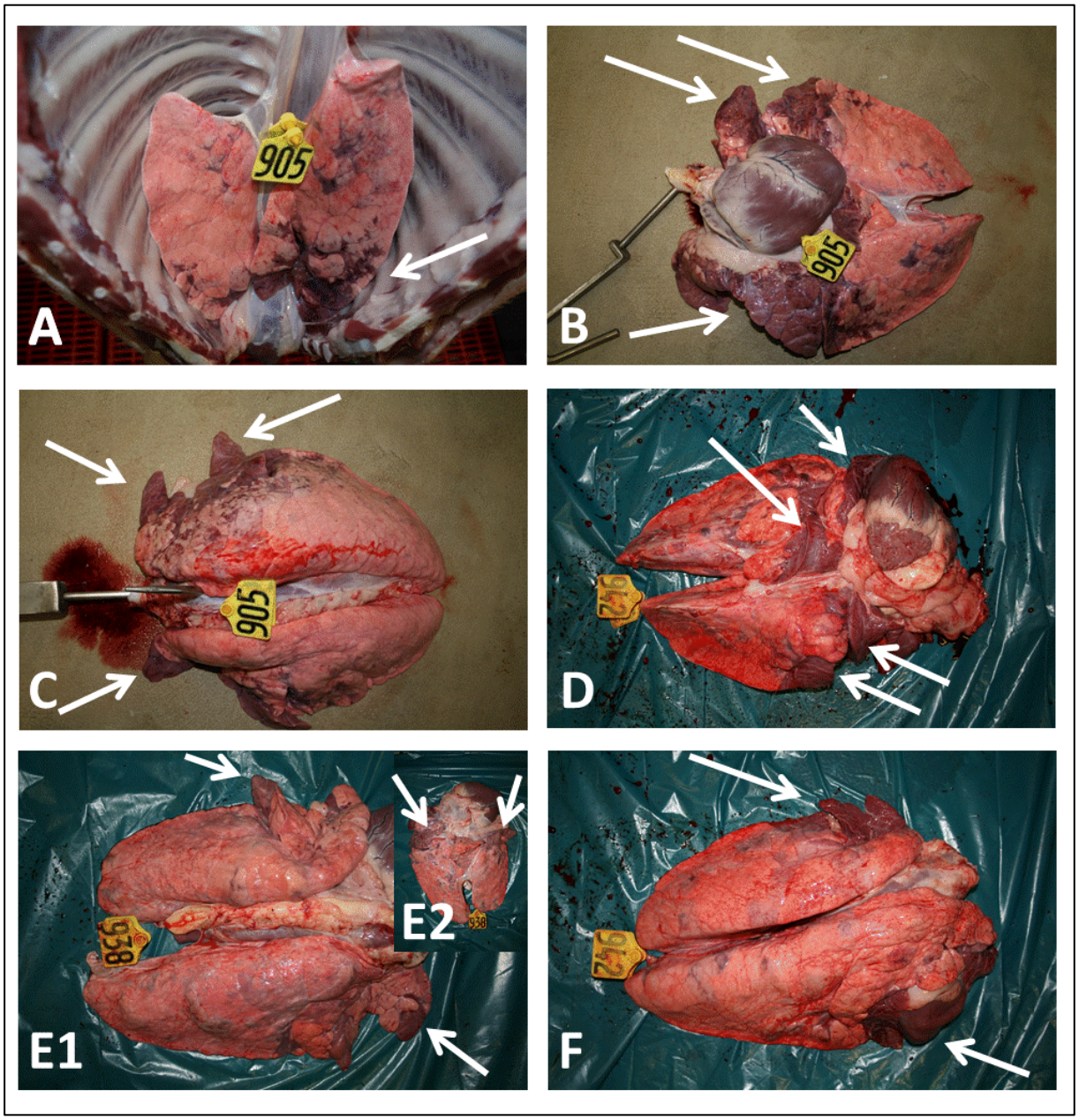
* Disease index, here average of dyspnoea score of the first two days after infection; HI and NI antibodies are shown

Supplementary Table 21: Antibodies induced by vaccination with batches of Mineral-oil-adjuvanted vaccines 10 days after second administration of the vaccine (= at challenge) and lung lesions 2 and 10 dpi and disease index (for comparison to the not vaccinated control group see table above)

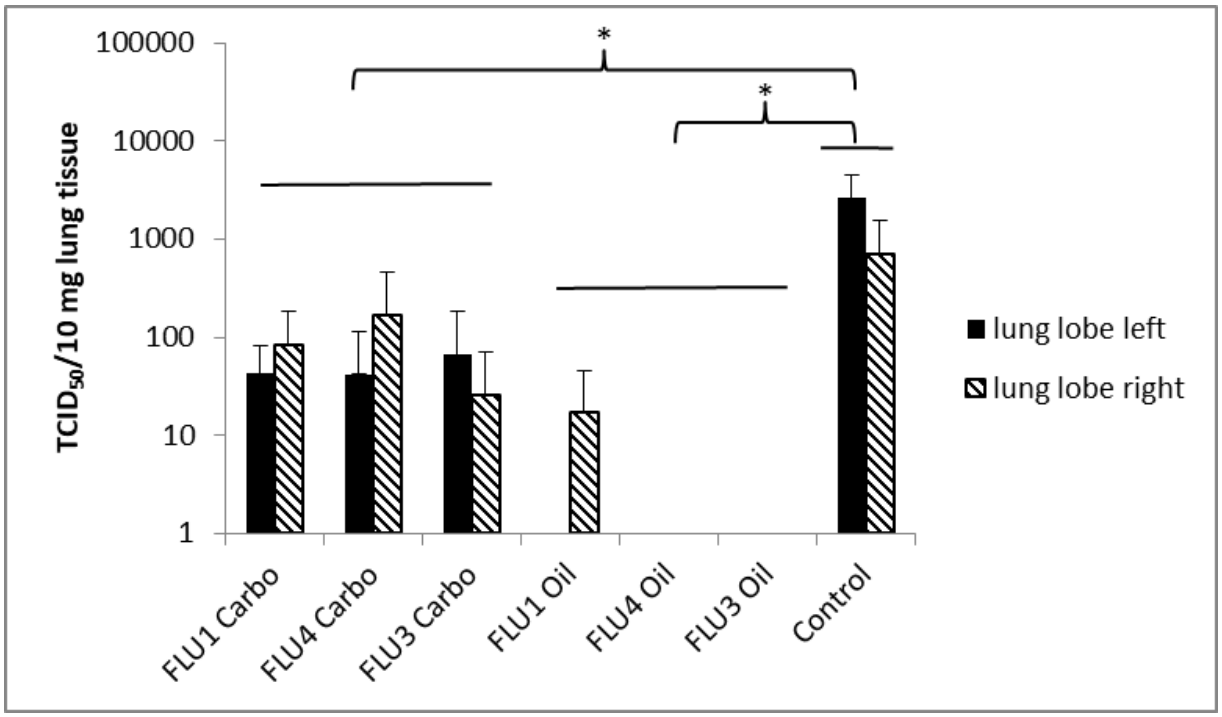
Group	Pig ID	αH1	αH1(N2)	αH3	$\alpha H1_{pdm}$	αN1	αN2	Lung lesions 2 dpi %	Lung lesions 10 dpi %	DI*
FLU1 Oil	919	<20	<20	<20	**10240**	**32**	<2	n.i.	**0**	**0.33**
	920	<20	<20	<20	**10240**	**32**	<2	n.i.	**24**	**0**
	921	<20	<20	<20	**10240**	**32**	<2	**7**	n.i.	**0.17**
	922	**80**	**80**	<20	**20480**	**32**	<2	**5**	n.i.	**0.17**
	923	<20	<20	<20	**5120**	**16**	<2	n.i.	**3**	**0.17**
	924	<20	<20	<20	**20480**	**64**	<2	**0**	n.i.	**0.67**
FLU4 Oil	925	**1280**	**1280**	**1280**	**1280**	**32**	**16**	n.i.	**0**	**0.17**
	926	**2560**	**2560**	**10240**	**20480**	**64**	**32**	**1**	n.i.	**0.67**
	927	**1280**	**1280**	**2560**	**10240**	**32**	**16**	**0**	n.i.	**0.83**
	928	**320**	**640**	**1280**	**1280**	**32**	**32**	n.i.	**0**	**0.17**
	929	**320**	**640**	**1280**	**2560**	**32**	**32**	**12**	n.i.	**0.67**
	930	**2560**	**640**	**2560**	**20480**	**64**	**64**	n.i.	**8**	**1.33**
FLU3 Oil	931	**2560**	**1280**	**1280**	**80**	**16**	**8**	**0**	n.i.	**0.33**
	932	**1280**	**640**	**2560**	**80**	**16**	**16**	n.i.	**5**	**1**
	933	**5120**	**2560**	**10240**	**320**	**32**	**16**	n.i.	**0**	**0**
	934	**5120**	**2560**	**5120**	**1280**	**16**	**8**	**5**	n.i.	**0.67**
	935	**640**	**2560**	**5120**	**80**	**16**	**8**	**2**	n.i.	**0**
	936	**2560**	**1280**	**2560**	**320**	**16**	**16**	n.i.	**10**	**0.67**

***** Disease index, here average of dyspnoea score of the first two days after infection
Antibodies against the M protein were not investigated in this trial

Pigs immunised with Carbopol-based vaccines showed greater individual variation in lung lesions than those immunised with mineral oil-based vaccines

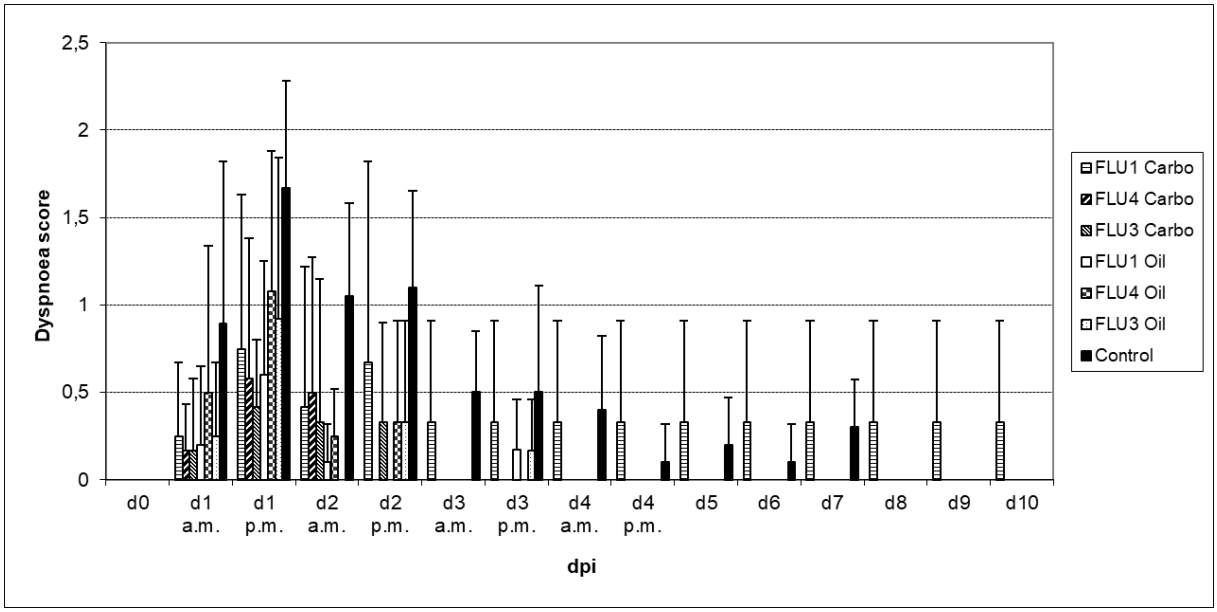


Supplement 49: Enhanced lung lesions in a vaccinated pig 10 days after infection; the extension of the lesion was larger than the lesion size of not vaccinated control pigs and may display vaccine-induced enhancement of disease; (A+B+C) lung of the vaccinated pig, (D+E+F) lungs of not vaccinated pigs with the most severe lung pathology among the not vaccinated pigs; the arrows indicate the lesions



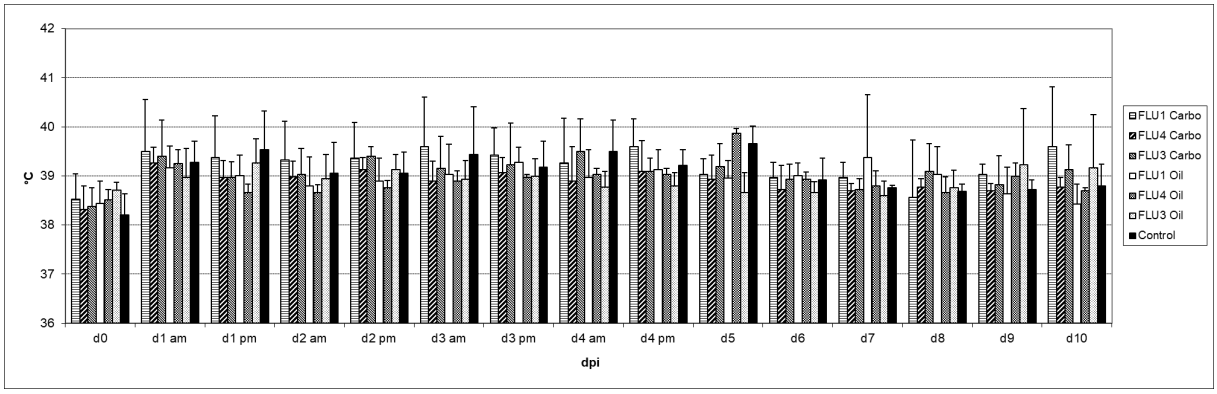
Supplement 50: Viral lung load of pigs of vaccinated and not vaccinated groups after experimental aerosol infection with swine $H1_{av}N1$ virus; arithmetic means with standard deviation

All vaccinated pigs had a significantly lower lung viral load than unvaccinated pigs (Supplement 50). Pigs vaccinated with vaccines containing a mineral oil adjuvant had the lowest lung viral load.



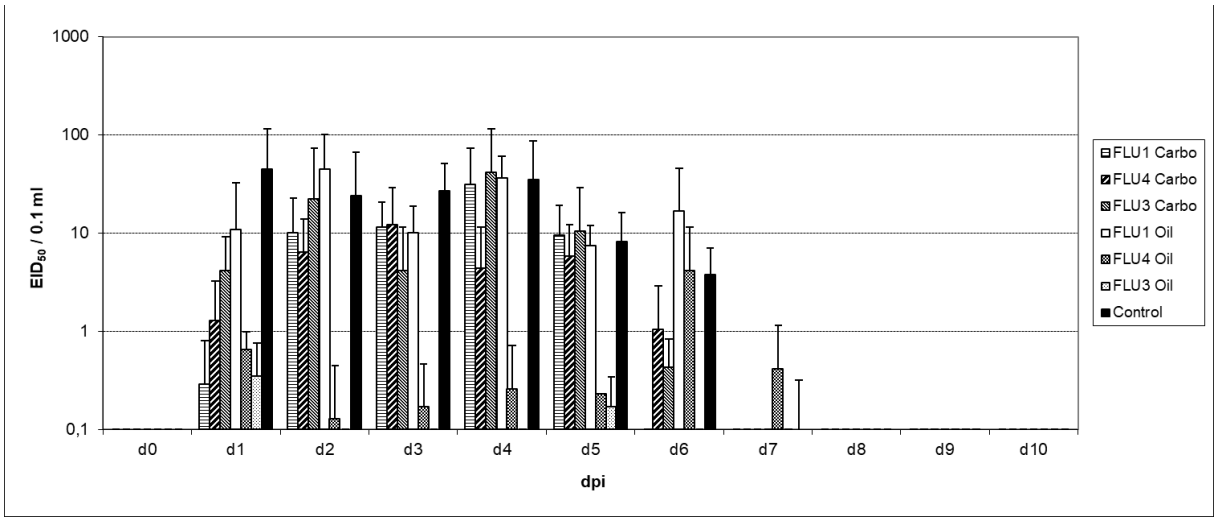
Supplement 51: Dyspnoea in pigs of vaccinated and not vaccinated groups after experimental aerosol infection with swine $H1_{av}N1$ virus; arithmetic means with standard deviation

The infection strain did not cause severe disease. Pigs in the unvaccinated control group showed moderate dyspnoea, not exceeding a dyspnoea score of 2.5. All vaccinated pigs showed only mild symptoms (Supplement 51).



Supplement 52: Rectal temperauture kinetics in pigs of vaccinated groups and one not vaccinated group (control) after experimental aerosol infection with swine $H1_{av}N1$ virus; arithmetic means with standard deviation

The infection strain induced only a slight increase in rectal temperature in pigs in the unvaccinated control group (Supplement 52). There were no significant differences between the vaccinated groups and the control group. Pigs vaccinated with the $H1_{pdm}N1$ virus but not with the $H1_{av}N1$ viruses showed the greatest variation in individual rectal temperatures.



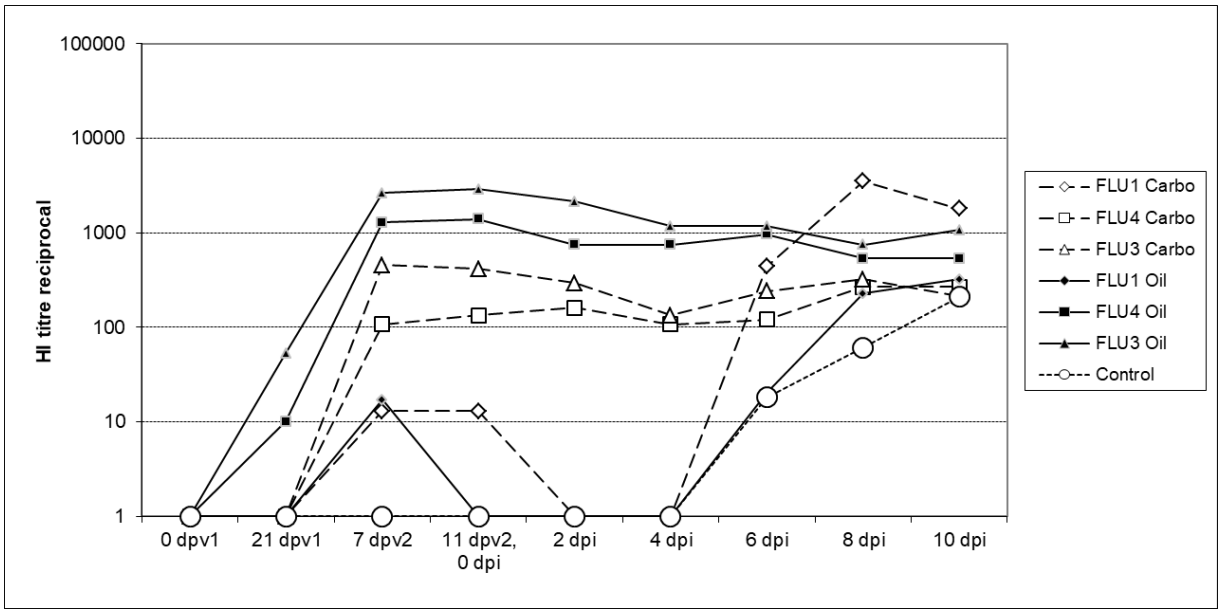
Supplement 53: Virus excretion measured in nasal swabs of in pigs of vaccinated groups and one not vaccinated group (control) after experimental aerosol infection with swine $H1_{av}N1$ virus; arithmetic means with standard deviation

Virus shedding occurred over seven days post-infection. Pigs vaccinated with mineral oil-adjuvanted vaccines containing antigen homologous to the infecting strain had the lowest shedding rates in nasal swabs (Supplement 53).

Only a few of the pigs vaccinated with vaccines containing $H1_{pdm}N1$ virus but not $H1_{av}N1$ virus responded to vaccination with antibodies against $H1_{av}N1$ virus. Pigs in these groups (vaccinated with $H1_{pdm}N1$ only) developed antibodies much faster and at higher titres than pigs in the unvaccinated control group (Supplement 54).

Brief discussion. Some of the vaccinated pigs had more extensive lung lesions than the unvaccinated pigs. Some of these pigs had either no antibodies or low levels of antibodies against the major surface glycoproteins of the influenza virus at the time of infection. This

suggests that an increase in lung pathology (or antibody-dependent enhancement of disease) can occur even when viruses within a supergroup differ between the vaccine virus and the infecting virus, but that these processes are not of concern because they are quickly covered by the rapid immune response in vaccinated pigs.



Supplement 54: HI antibody kinetics in pigs of vaccinated groups and one not vaccinated group (control) after experimental aerosol infection with swine $H1_{av}N1$, dpv1, days after first vaccination, dpv2 days after second administration of the vaccines; dpi, days after infection (HI was carried out using the infection virus $H1_{av}N1$); geometric means are displayed

In some pigs with larger lung lesions, dyspnoea reached the level of unvaccinated pigs, but in general, symptoms in vaccinated pigs were lower than in unvaccinated pigs. Thus, although at an individual level there may be increased lung pathology in vaccinated individuals, this effect is not necessarily associated with more severe disease because the potential increase in disease severity is overcome by the rapid immune response in vaccinated individuals. An important consideration in measuring vaccine efficacy is that despite the protective effect of vaccination, vaccinated pigs may shed virus and develop lung pathology and symptoms shortly after infection, particularly in individuals with low levels of antibodies against the target receptor proteins HA and NA. To avoid this, stable antibodies against the major target glycoprotein should be achieved by vaccination. Thus, in cases of intrasubtype variation of the vaccine antigen and the infecting virus, a few individuals may show signs of disease enhancement, but these are overcome by the more rapid immune response, indicating a beneficial effect of vaccination in general. To achieve better individual protection, greater adjuvantation of vaccines may be beneficial. In the case of mineral oils, these good immunogenic effects are offset by the low safety of the mineral oil adjuvant, which induces fever after vaccination.

SUPPLEMENT CHAPTER 14 – PYROGENIC AND VIRUCIDAL EFFECTS OF MINERAL OILS

Background: Vaccines need to be made safe to use. The following studies were carried out to investigate the safety of strong adjuvants such as mineral oils and less strong adjuvants such as carbomer. The reason for the investigations was that temperature increases were observed in individual pigs 4 hours after injection of commercially available influenza vaccines. For safety tests, temperature measurements are required at 4 hours, 24 hours and 48 hours after vaccine application. In order to clarify the temperature increases 4 hours after injection that did not occur at 24 and 48 hours, temperature kinetics were established. And when clear fever reactions occurred, further investigations were initiated. The investigations with Porcine reproductive and respiratory syndrome virus as a model virus served to test compatibility with live vaccines.

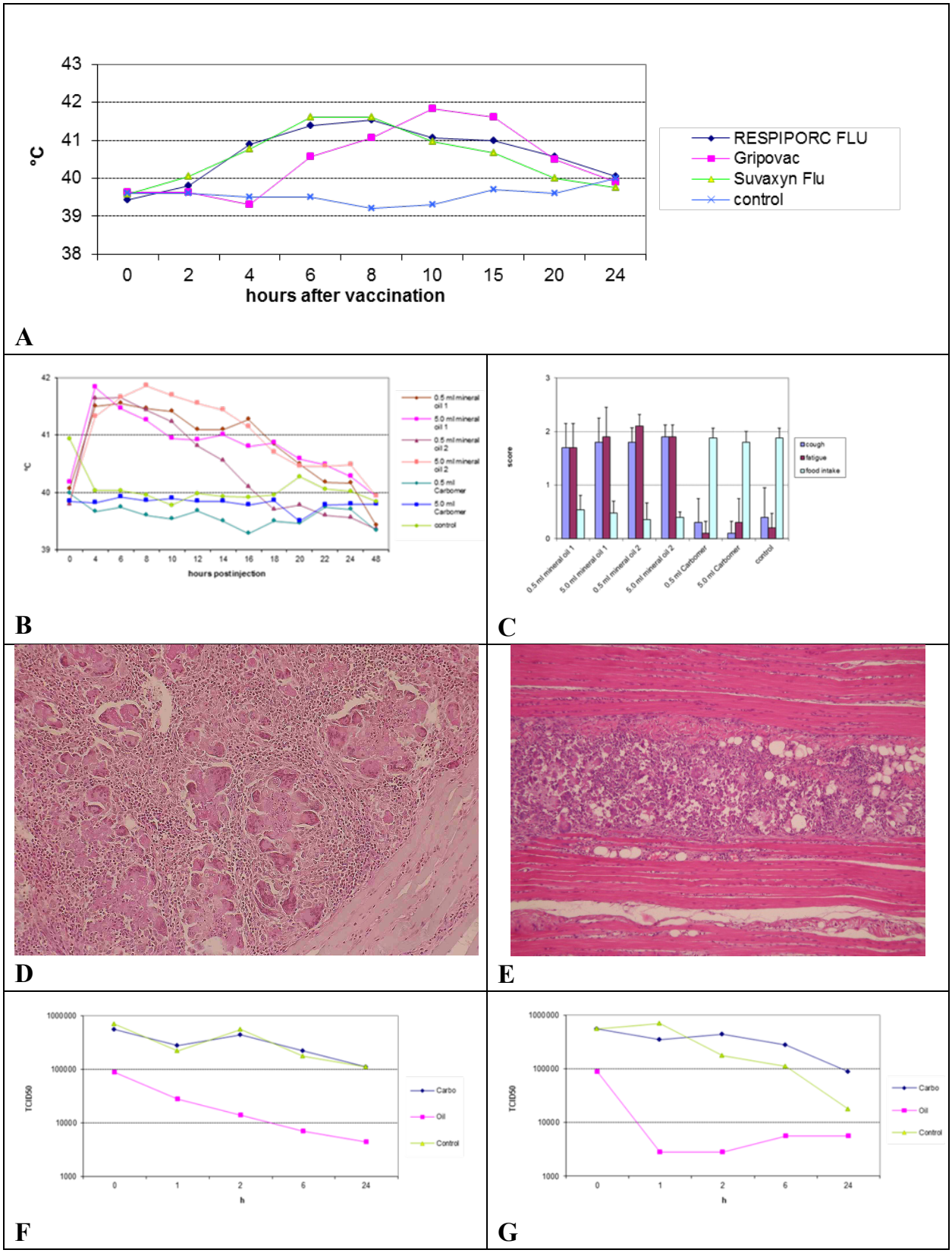
Methods: Fifty-six-day-old pigs (five pigs in each group) were injected with mineral oil-adjuvanted authorised influenza vaccines available in Germany in 2003. Some of the pigs were examined after they were sent to the abattoir. Samples were taken from the injection site for macroscopic and histological examination. In a second study, fifty-six-days-old pigs from a pig herd with low background activity of respiratory disease were injected (five pigs in each group) with pure adjuvants: mineral oil 1 (ISA25, Seppic), mineral oil 2 (ISA206, Seppic) and carbomer (Carbopol 971 P NF, Carbomer, Carboxy-polymethylene, Polyacrylic acid, Lubrizol, Wickliffe, USA). The injection volumes were 0.5 and 5 ml. Temperature kinetics were determined, local reactions and clinical parameters such as anorexia, coughing and feed intake were measured.

The adjuvants were mixed with Porcine reproductive and respiratory syndrome virus and stored at 2 to 8 °C or 24 °C and then titrated on MA-104 cells.

Results. All pigs injected with pure mineral oil adjuvants or vaccines containing mineral oil developed high fever and local swelling at the injection site (Supplement 55). In addition, some of the pigs did not eat, became lethargic and developed a cough, whereas the pigs receiving the Carbomer adjuvant did not develop any side effects (Supplement 55).

Carbopol 971 P NF had no significant effect on Porcine reproductive and respiratory syndrome virus titres in *in vitro* experiments on MA-104 cells compared to positive controls not mixed with adjuvant (Supplement 55). In contrast, there was a strong virucidal effect of mineral oil (ISA25) on PRRSV, which was higher at 24°C than at 2-8°C (Supplement 55).

Short interpretation of results. Mineral oils are potent adjuvants and can induce strong humoral immune responses, but are pyrogenic. These effects may lead to activation of latent or subclinical infections in pig herds, but also to abortions in pregnant sows, and must be considered when such vaccines are used. Ten hours instead of 4 hours after injection of adjuvanted vaccines is the right time for safety testing



Supplement 55: Response of pigs to adjuvants, A, Kinetics of rectal temperatures after administration of mineral-oil adjuvanted influenza vaccines for swine available in 2003, B, Temperature kinetics in pigs after application of pure adjuvants; C, Symptoms after administrations of adjuvants in a pig herd with subclinical respiratory disease; D, Histological picture at injection site after administration of mineral oil (severe inflammation with giant cell migration); E, Histological picture after administration of Carbomer (slight injury to the muscle at the puncture site and slight inflammation); F, Effects of adjuvants on Porcine reproductive and respiratory virus at 2-8°C; G, Effects of adjuvants on Porcine reproductive and respiratory virus at 24°C

SUPPLEMENT CHAPTER 15 – STRESSING THE INNATE IMMUNITY

Background. By acting on toll-like receptors, viral molecules can stimulate the production of cytokines (Il-1, Il-6, TNF-α)[300]. These cytokines can cause changes in the thermostatic set points in the thermoregulatory centre, raising body temperature. They can induce malaise and lethargy and suppress appetite. Over the years of this work, trials of influenza infection in pigs have shown that certain influenza A viruses induce fever, short-term dyspnoea and apathy despite homologous vaccination. Such strong innate immune responses were only induced by $H1_{pdm}Nx$ viruses (Supplementary Table 22). To investigate this in detail, a high-dose challenge study was carried out with nebulised $H1_{pdm}N2$ virus $>10^{10}$ $TCID_{50}/m^3$ and the response was studied in homologously vaccinated pigs (the same virus used for vaccination as for infection) and in pigs vaccinated with an antigenically distant virus of the same antigenic supergroup.

Supplementary Table 22: Overview of high-dose infection trials and viruses that induced fever and dyspnoea despite homologous vaccination (highlighted in ochre)

Virus	Number of trials with high dose infections > 10^9 $TCID_{50}$ /m^3	Induction of strong responses of innate immunity
$H1_{pdm}N1$ April 2009	2	Induction of fever and dyspnoea in vaccinated pigs
$H1_{pdm}N1$ July 2009	2	No reaction in vaccinated pigs
$H1_{pdm}N1$ 2014	1	No reaction in vaccinated pigs
$H1_{pdm}N1$ 2015	1	No reaction in vaccinated pigs
$H1_{pdm}N2$ 2010	1	Induction of fever and dyspnoea in vaccinated pigs
$H1_{av}N3$	3	No reaction in vaccinated pigs
$H1_{hu}N2$	1	No reaction in vaccinated pigs
H3N2	3	No reaction in vaccinated pigs
H3N1	1	No reaction in vaccinated pigs

Study design. Pigs free of antibodies to influenza viruses from the same farrowing event were vaccinated with experimental batches of two whole virus inactivated vaccines 28 and 7 days before challenge (Supplementary Table 23). Some pigs were not vaccinated. These pigs served as unvaccinated controls. FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$) was aerosolized at $10^{10.21}$ $TCID_{50}/m^3$, equivalent to 9 billion virus particles/m^3. Pigs were monitored for HI antibodies, rectal temperatures and dyspnoea, and lung samples were collected from 5 pigs at 1 and 3 dpi and from the remaining 3 pigs at 9 dpi and examined for lung viral load, macroscopic lung lesions and inflammation.

Supplementary Table 23: Overview of the experimental design of the trial

Group	Virus used for immunisation	Virus used for infection	Number of pigs included
1	None (control group)	FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$) $10^{10.21}$ $TCID_{50}/m^3$	13
2	FLUAV/Jena/VI5258/2009 ($H1_{pdm}N1$)	FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$) $10^{10.21}$ $TCID_{50}/m^3$	13
3	FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$)	FLUAV/sw/Papenburg/12653/2010 ($H1_{pdm}N2$) $10^{10.21}$ $TCID_{50}/m^3$	13

Results. The vaccination induced antibodies in all pigs (Supplementary Table 24). There was cross-reactivity between the haemagglutinins of $H1_{pdm}N2$ and $H1_{pdm}N1$ viruses in the haemagglutination inhibition test, but this was not very strong. 8 days after infection, the titres had increased significantly, the titre against the virus of the antigenic first contact was higher, and antibodies against $H1_{cl}N1$ were also formed in the vaccinated animals (Supplementary Table 25). The non-immunised pigs had a much weaker antibody response to infection (Supplementary Table 25).

Pigs in the unvaccinated control group developed severe and prolonged respiratory distress (Supplement 56). The pigs showed fever at 1 dpi and a lower temperature rise at 3 dpi. Pigs in the control group had no antibodies prior to infection. Virus shedding peaked at 1 dpi, as is usual with high-dose aerosol infection. Shedding stopped at 6 dpi. Antibodies started to appear in individual pigs at 6 dpi. In general, antibody titres were low at 9 dpi, the time when antibody titres peak after infection. At 1 dpi, all pigs had very high viral titres in the lungs. These titres were decreasing by 3 dpi. No virus was detected in the lungs at 9 dpi. Inflammation increased from 1 to 9 dpi. Pulmonary pathology was most severe at 3 dpi. 1 pig died at 5 dpi. There was stagnation in the body weight of pigs in the control group.

Pigs in the $H1_{pdm}N1$ vaccinated group also developed dyspnoea, but showed greater individual variation in symptoms and recovered much faster than the unvaccinated controls (Supplement 56). There were also two peaks in rectal temperature in the vaccinated pigs. There was no difference in virus shedding compared to the control group. This can be explained by the low prevalence of antibodies at infection. However, there was a lower lung viral load at 1 dpi ($p=0.056$ left lung; $p=0.032$ right lung) and 3 dpi ($p=0.016$ left lung; $p=0.016$ right lung) compared to the control group. This was associated with less lung inflammation and a lower degree of macroscopic lung lesions.

Supplementary Table 24: Antibodies 7 days after second administration of the vaccine

Pig ID	HI a$H1_{pdm}N2$	HI $\alpha H1_{pdm}N1$	HI $\alpha H1_{cl}N1$	NI $\alpha N2$	NI $\alpha N1$	ELISA αM
$H1_{pdm}N2$ vaccinated pigs						
27	1280	40	<20	4	<2	160
28	640	20	<20	2	<2	80
29	160	<20	<20	2	<2	80
30	160	20	<20	4	<2	80
31	640	20	<20	8	<2	320
32	320	40	<20	8	<2	40
33	80	<20	<20	4	<2	160
34	1280	20	<20	64	<2	160
35	160	20	<20	16	<2	320
36	1280	20	<20	32	<2	320
37	2560	160	<20	32	<2	640
38	2560	<20	<20	64	<2	640
$H1_{pdm}N1$ vaccinated pigs						
14	<20	40	<20	<2	16	20
15	<20	40	<20	<2	2	20
16	20	80	<20	<2	32	80
17	<20	160	<20	<2	4	80
18	<20	40	<20	<2	4	40
19	<20	80	<20	<2	8	80
20	40	320	<20	<2	4	160
21	40	320	<20	<2	16	160
22	<20	40	<20	<2	4	80
23	40	160	40	<2	16	640
24	<20	80	<20	<2	8	80
25	40	40	<20	<2	8	80
26	160	320	160	<2	16	80
Unvaccinated control pigs						
1	<20	<20	<20	<2	<2	<20
2	<20	<20	<20	<2	<2	<20
3	<20	<20	<20	<2	<2	<20
4	<20	<20	<20	<2	<2	<20
5	<20	<20	<20	<2	<2	<20
6	<20	<20	<20	<2	<2	<20
7	<20	<20	<20	<2	<2	<20
8	<20	<20	<20	<2	<2	<20
9	<20	<20	<20	<2	<2	<20
10	<20	<20	<20	<2	<2	<20
11	<20	<20	<20	<2	<2	<20
12	<20	<20	<20	<2	<2	<20
13	<20	<20	<20	<2	<2	<20

HI, hemaggluination inhibition assay, antigens used A/sw/Papenburg12652/2010 ($H1_{pdm}N2$), A/Jena/5258/2009 ($H1_{pdm}N1$), A/sw/England117316/1986 ($H1_{cl}N1$); NI, neuraminidase inhibition assay: A/sw/Papenburg12652/2010 ($H1_{pdm}N2$), A/Jena/5258/2009 ($H1_{pdm}N1$), αM ELISA: recombinant influenza A M2 protein, red, fatal courses of disease after infection

Supplementary Table 25: Antibodies 9 days after infection in the remaining pigs

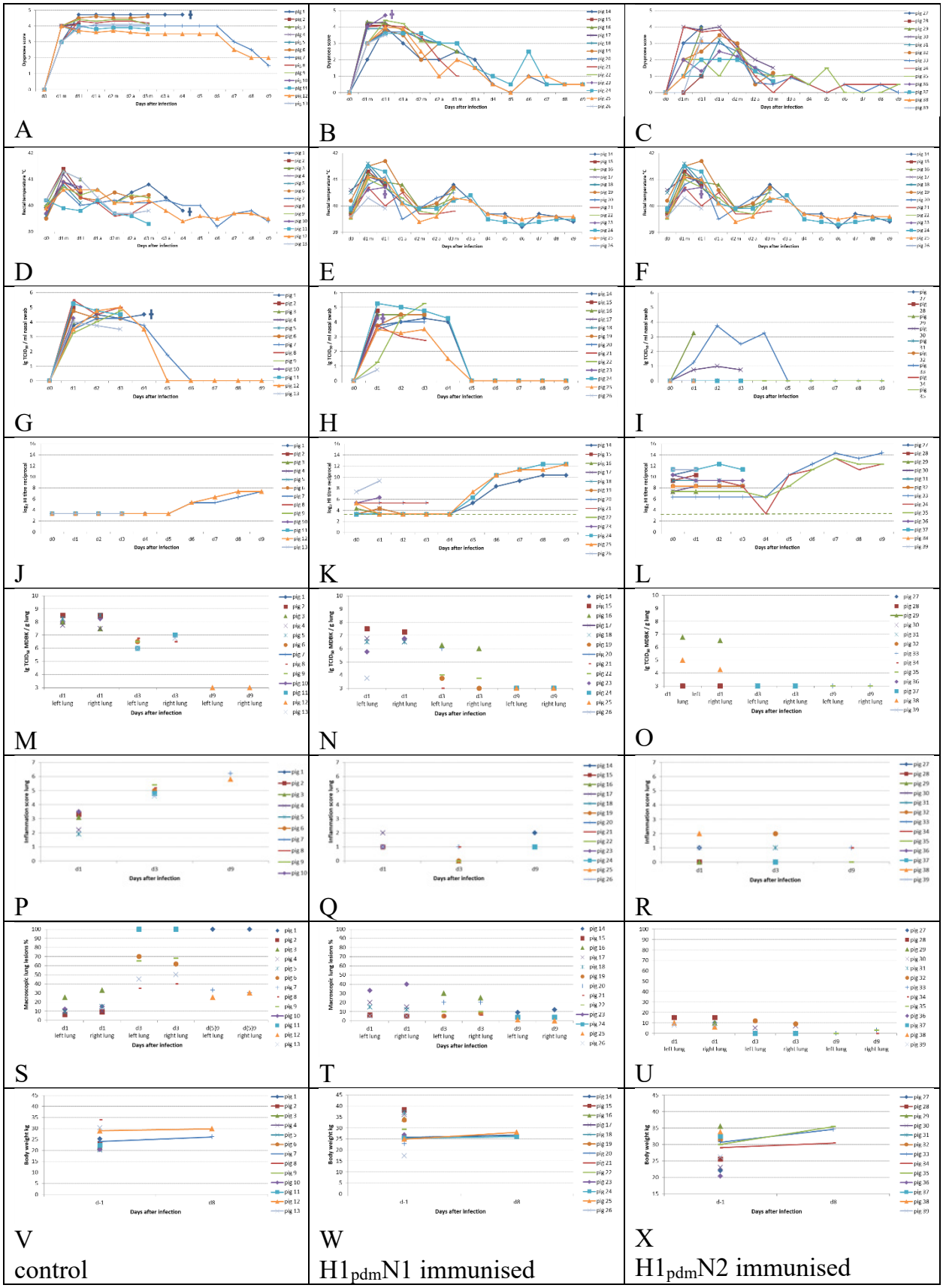
Pig ID	HI a$H1_{pdm}N2$	HI $\alpha H1_{pdm}N1$	HI $\alpha H1_{cl}N1$	NI αN2	NI αN1	ELISA αM
$H1_{pdm}N2$ vaccinated pigs						
33	20240	2560	320	128	<2	640
34	10240	320	160	256	<2	1280
35	10240	320	80	128	<2	320
$H1_{pdm}N1$ vaccinated pigs						
14	640	2560	320	16	32	320
24	2560	5120	2560	32	16	320
25	2560	5120	1280	16	16	320
Unvaccinated control pigs						
6	40	<20	<20	4	<2	80
12	80	20	<20	4	<2	40
13	80	<20	<20	8	<2	40

HI, hemaggluination inhibition assay, antigens used A/sw/Papenburg12652/2010 ($H1_{pdm}N2$), A/Jena/5258/2009 ($H1_{pdm}N1$), A/sw/England117316/1986 ($H1_{cl}N1$); NI, neuraminidase inhibition assay: A/sw/Papenburg12652/2010 ($H1_{pdm}N2$), A/Jena/5258/2009 ($H1_{pdm}N1$), αM ELISA: recombinant influenza A M2 protein

The $H1_{pdm}N2$ vaccine group developed dyspnoea and fever despite high antibody titres in all pigs at infection. However, pigs in this group showed prevention of virus shedding (in 10 out of 13 pigs), low lung viral loads, low levels of inflammation and lung pathology, and normal weight gain (Supplement 56, Supplement 57). The lung viral loads at 1 dpi and 3 dpi were significantly lower compared to the control group (1 dpi: p=0.008 left lung; p=0.016 right lung; 3 dpi: p=0.008 left lung; p=0.008 right lung).

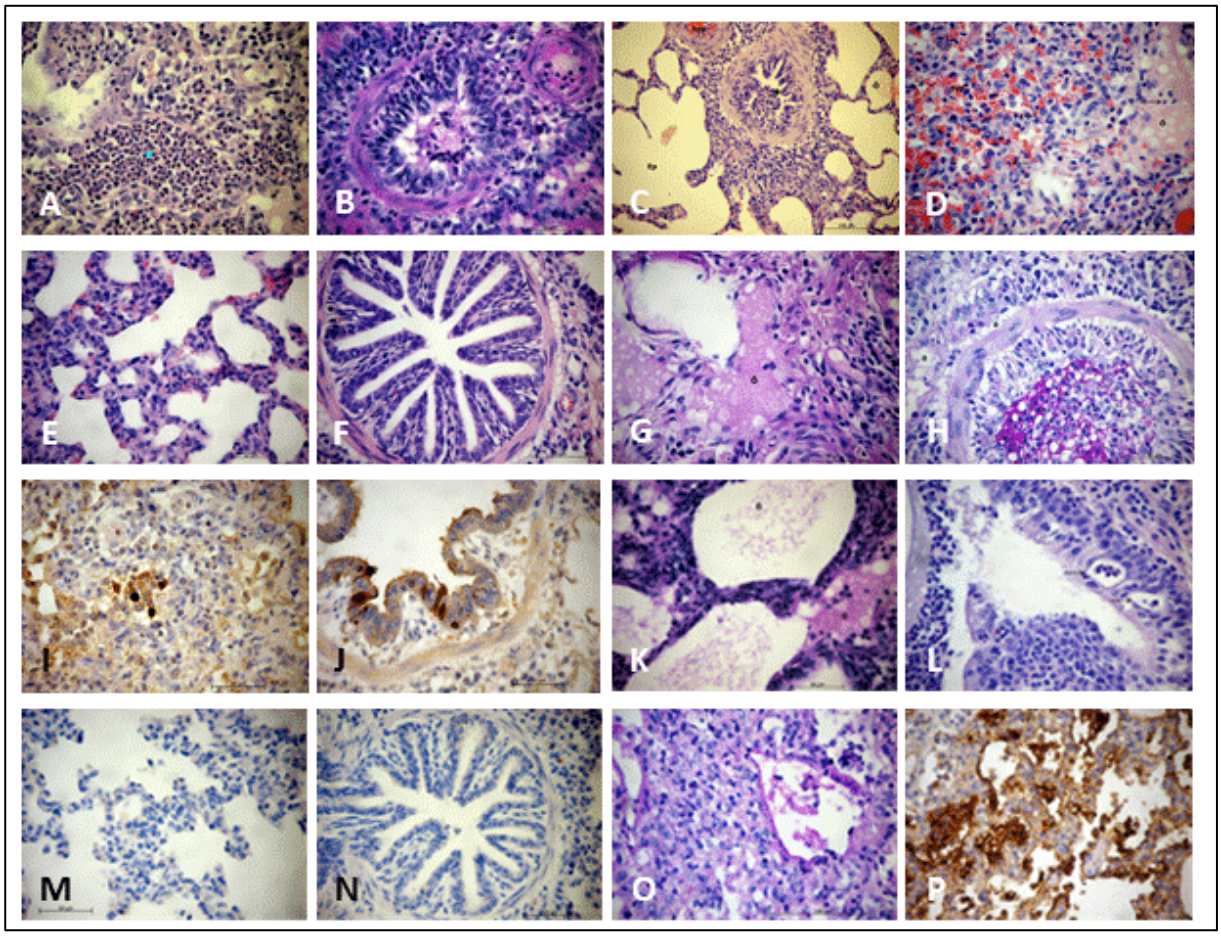
In the $H1_{pdm}N1$ immunised group, one pig died 24 hours after infection. This pig (pig 23) did not have a high viral lung load, nor did it have more severe inflammation or lung pathology than the other pigs in the group. The reciprocal HI antibody titer at infection was 1:40. While most pigs in the group had antibodies to $H1_{pdm}N2$ virus below the detection limit at infection, this pig had a moderate antibody titer to $H1_{pdm}N2$ virus. The macroscopic lung lesions in pig 23 were moderate, but histology revealed massive haemolysis and severe alveolar oedema (Supplement 58). Some of the unvaccinated pigs had severe lung lesions and severe inflammation in the lung on day 5 after infection (Supplement 59).

Short interpretation of results. The data show that under conditions of high-dose infection with newly emerged $H1_{pdm}Nx$ viruses, short-term induction of dyspnoea and fever is possible despite a homologous vaccine that reduces virus shedding and prevents pulmonary pathology in most pigs. This may indicate activation of innate immune mechanisms despite antibody protection and may be due to the high initial viral lung load. This results in the release of cytokines that stimulate an increase in body temperature. Since there was almost no inflammation and lung pathology in the homologous vaccinated pigs, the data suggest that the respiratory symptoms were also caused by innate immune mechanisms.

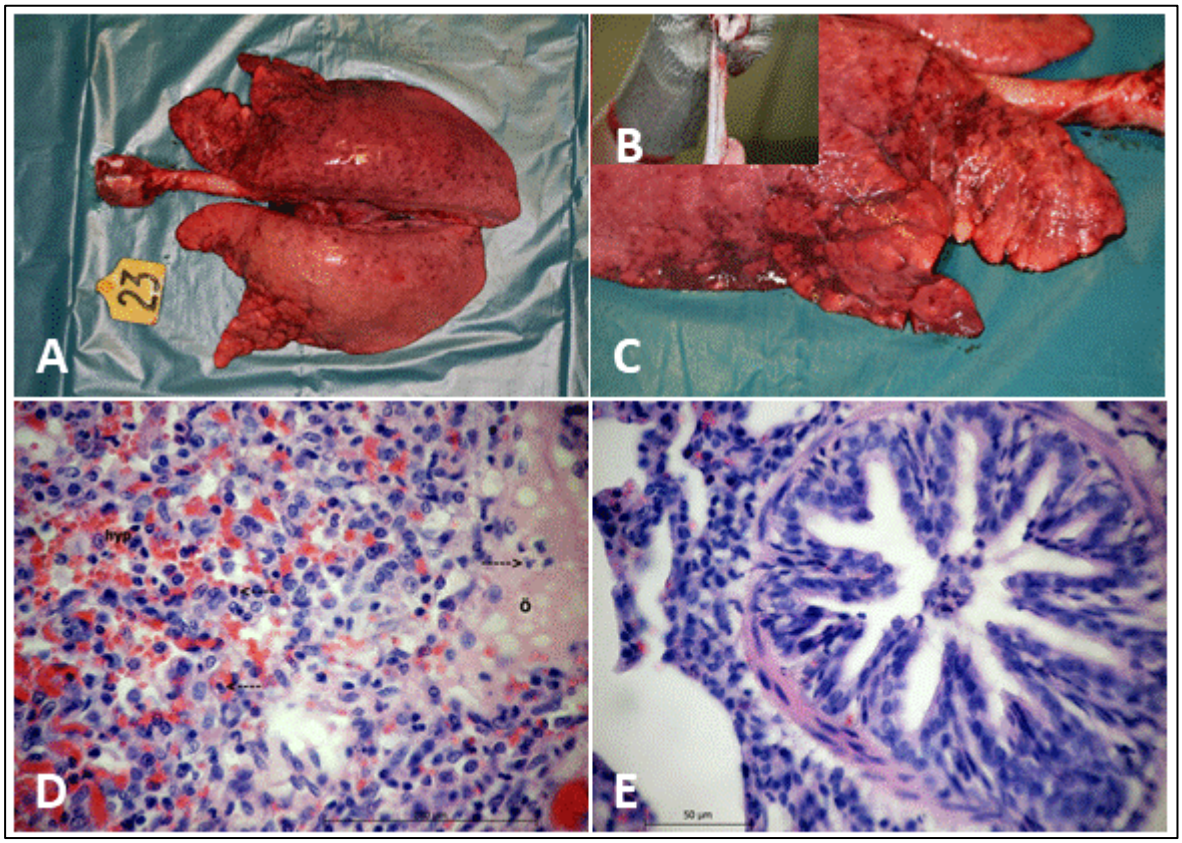


Supplement 56: Summary of the results of the trial on the investigation of the responses of the innate immune system in spite of vaccination; individual data are shown; A, symptoms in pigs of the control group; B, symptoms in pigs of the $H1_{pdm}N1$-vaccinated group; C, symptoms in pigs of the homologously vaccinated group ($H1_{pdm}N2$); D; rectal temperatures control group; E; rectal temperatures $H1_{pdm}N1$ group; F, rectal temperatures $H1_{pdm}N2$ group; G, virus excretion (nasal swabs) control group; H, virus excretion (nasal swabs) $H1_{pdm}N1$ group; I, virus excretion (nasal swabs) $H1_{pdm}N2$ group; J, antibody kinetics against

infection virus H1$_{pdm}$N2 in pigs of the control group (note the low antibody response after infection despite high-dose approach); K, antibody kinetics H1$_{pdm}$N1 group (note that the majority of the pigs has no antibodies at infection); L, antibodies H1$_{pdm}$N2 group (note the high antibodies at infection and the strong booster reaction); M, viral lung load control group; N, viral lung load H1$_{pdm}$N1 group; O, viral lung load H1$_{pdm}$N2 group; P, inflammation determined by histological investigation control group; R, inflammation H1$_{pdm}$N1 group; R, inflammation H1$_{pdm}$N2 group; S, lung pasthology (lesions macroscopically visible) control group; T, lung pathology H1$_{pdm}$N1 group; U, lung pathology H1$_{pdm}$N2 group; V, body weights control group; W, body weights H1$_{pdm}$N1 group; X, body weights H1$_{pdm}$N2 group



Supplement 57: Histologic and immunohistochemical profile after infection with A/sw/H1$_{pdm}$N2 virus, (A) Massive accumulation of interstitial alveolar macrophages (asterisk) after high-dose H1$_{pdm}$N2 infection; HE staining; (B) Large accumulation of interstitial alveolar macrophages and bronchluminal obstruction with alveolar macrophages, alveolar and interstitial edema in a pig afer high-dose H1$_{pdm}$N2 infection; (C) Beginning inflammation, peribronchitis, hyperaemia (hyp), alveolar emphysema (Ep), alveolar edema (Ö), HE staining; (D) Massive hyperaemia (hyp), interstitial and alveolar edema (O), eosinophil granulocytes (arrows) in a vaccinated pig that died after infection, HE staining; (E) lung alveolae of strict negative control, HE staining, (F) bronchiolus of strict negative control, HE staining; (G) Massive alveolar edema (Ö) in a high-dose H1$_{pdm}$N2-infected pig, PAS staining; (H) Large areas of interstitial alveolar macrophages, peribonchial edema (Ö), broncholuminal mucus and alveloae macrophages in a high-dose H1$_{pdm}$N2-infected pig, PAS stainng; (I) Virus-specific staining of alveolar macrophages, SABC method; (J) Specific staing of bronchoepithelial cells, SABC method; (K) Peribronchial infiltrates and strong alveolar edema in a high-dose H1$_{pdm}$N2-infected pig, PAS staining; (L) Exocytosis of neutrophil granulocytes (arrow), HE staining; (M) Alveoli of strict negative control, SABC method; (N) Bronchoilus of strict negative control, SABC method; (O) Massive hyperaemia, edema and infiltration in the lung of a pig that died after high-dose H1$_{pdm}$N2 infection, PAS staining; (P) Virus-specific staining of interstial and intraluminal alveolar macrophages in a high-dose H1$_{pdm}$N2-infected pig, SABC method



Supplement 58: Pig of the $H1_{pdm}N1$ immunised group which died suddenly on 1 dpi; A, lung dorsal view; B, mucus in the trachea; C, lung apical view, note diffuse lesions; D, massive hyperaemia (hyp) and alveolar oedema (Ö), interstitial alveolar macrophages (HE staining), arrows indicate eosinophil granulocytes; E, moderate peribronchitis and few broncholuminal alveolar macrophages (HE staining)

Thus, cytokines and other components are likely to affect respiratory function. This is supported by the fact that there was only moderate peribronchitis and no accumulation of cellular debris in the bronchioli in these vaccinated pigs. Possible mechanisms could be bronchoconstriction. The data highlight the limitations of vaccination.

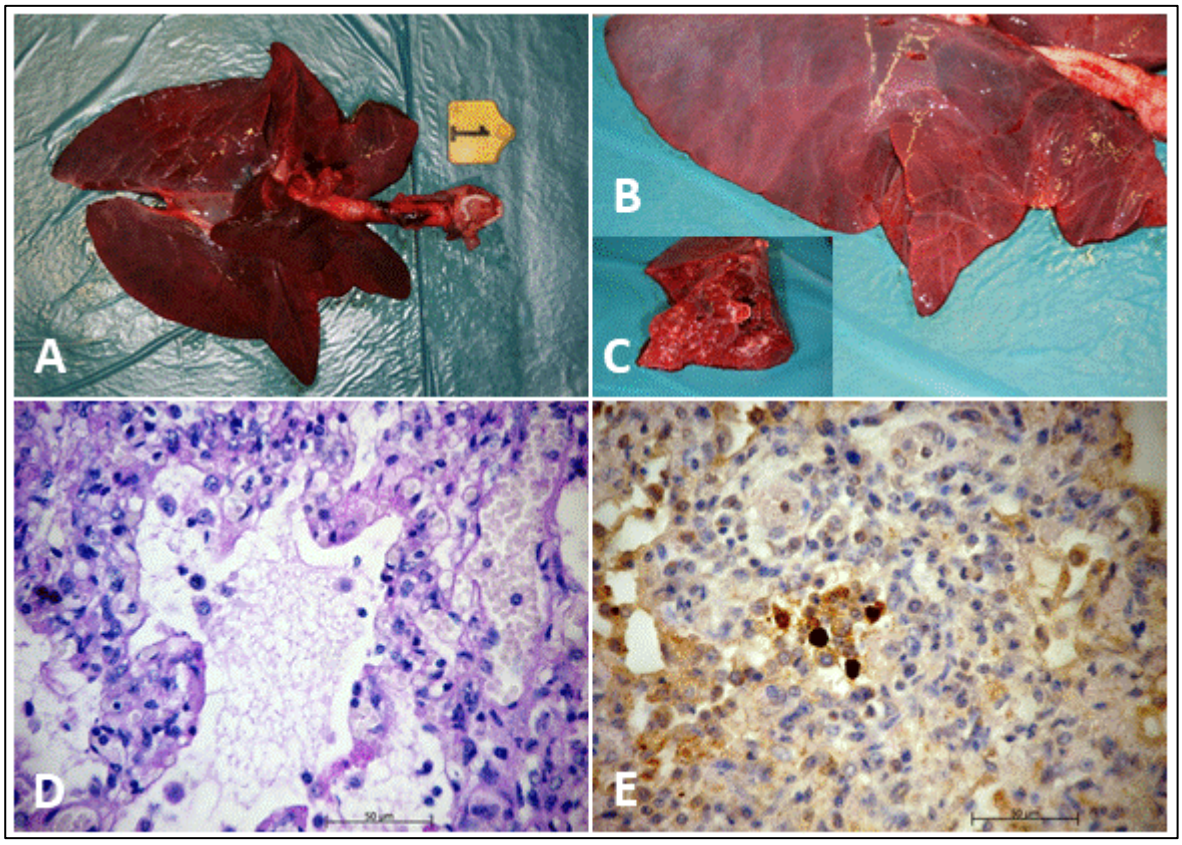
Although this was an experimental approach, it should be noted that the artificial virus shedding curve, with a peak at 1 dpi, is similar to that observed in some human volunteers after infection. It is possible that influenza viruses replicate much more rapidly in humans and may have similar effects as under high-dose infection conditions in pigs. The results of this study reflect the limitations of vaccination under high-dose infection conditions, where viruses replicate to very high lung titres shortly after infection.

The data reflect that homologous vaccination provides the best protection against $H1_{pdm}N2$ infection, but effects of $H1_{pdm}N1$ vaccination were also seen, resulting in less lung pathology and shorter duration of dyspnoea. Despite this protection, 1 pig in the $H1_{pdm}N1$ vaccinated group died 1 dpi. This may have been a result of vaccine-induced exacerbation of the disease. In this case, the lung changes were not yet so severe. Presumably the cytokine

release was so strong that a peracute course occurred as a sign of antibody-dependent enhancement of disease before the lung changes could become macroscopically manifest.

Two processes are discussed as possible reasons for this phenomenon of vaccine-induced disease enhancement: i) ADE (antibody-dependent enhancement of disease): increased uptake of antibody-decorated viruses via Fcψ receptor-bearing cells and ii) ERD (enhancement of respiratory disease): primed T-helper cells more rapidly change the programming of macrophages to a pro-inflammatory phenotype, resulting in increased release of cytokines, stimulation of IL4 and IL13 and others, and increased infiltration of eosinophilic granulocytes. The data suggest that the latter process may have occurred in this vaccinated pig, as the histology reflects eosinophils, oedema and hyperemia.

As shown by the pig dying at 5 dpi, the process takes longer in non-vaccinated pigs. In contrast to the dead vaccinated pig, very severe lung pathology was observed. There was consolidation throughout the lungs. Microscopically, the profiles were similar to those of the dead vaccinated pig: hyperemia and oedema and severe inflammation, but in the control pig this extended to the entire lung.



Supplement 59: Pig of the not immunised control group which died on 5 dpi, A, lung ventral view; B, lung apical view; C, the entire lung tissue is affected and bronchioli are full of foam; D, strong inflammation, large areas with alveolar macrophages, hyperaemia and oedema in the lung (PAS staining); E, virus-specific staining of alveolar macrophages (SABC)

SUPPLEMENT CHAPTER 16 – TESTING THE RELATIONSHIP BETWEEN $H1_{PDM}N1$ AND $H1_{CL}N1$ INFLUENZA VIRUSES

Background. The aim of this experiment was to test the antigenic relationship between $H1_{pdm}N1$ and $H1_{cl}N1$ viruses.

Methods. A laboratory batch of a vaccine with vaccine strain A/Jena/5258/2009 ($H1_{pdm}N1$) was prepared (# 003 02 10, inactivated, adjuvanted with Carbopol). 8-weeks-old pigs were divided into two groups, one group was vaccinated at 8 and 11 weeks of age (Supplementary Table 26).

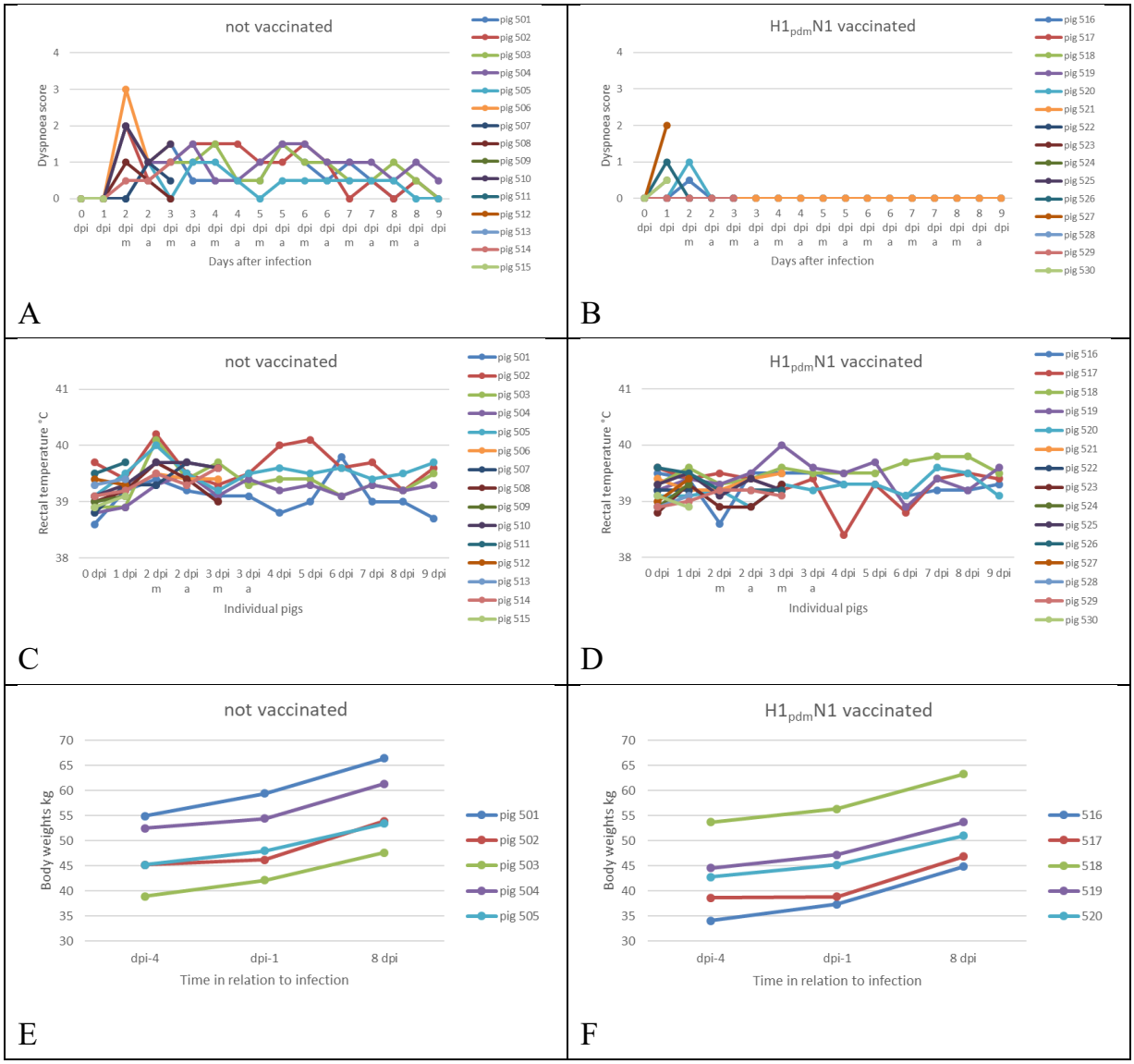
Supplementary Table 26: Overview of the study design

Groups	Number of pigs	Ear tags
Not vaccinated	15	501-515
$H1_{pdm}N1$ vaccinated	15	516-530

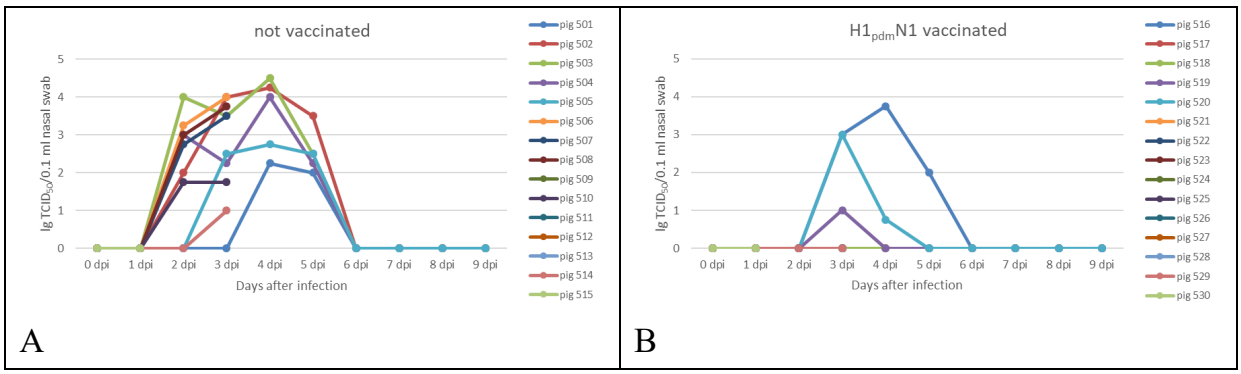
One week later, an infection trial with strain A/sw/England/117316/1986 ($H1_{cl}N1$) was carried out. The virus was concentrated before nebulisation and a very high infectious dose of lg 9.26 $TCID_{50}/m^3$ was nebulised. Symptoms were recorded daily, sometimes even twice daily, as were rectal body temperatures. Nasal swabs were taken daily and the virus content was determined. On days 1, 3 and 9 after infection, the lungs of 5 pigs from each group were examined (macroscopic and microscopic lung lesions, virus content). Body weights were determined 4 and 1 day before infection and 8 days after infection. Blood samples were taken 7 days after the second vaccination and then daily until the end of the trial. The antibody titres against various influenza viruses were determined

Results: Despite the high levels of nebulised virus, only minor symptoms were induced in the control animals (Supplement 60). The respiratory symptoms were mild and soon subsided. The vaccinated animals showed no symptoms (Supplement 60). Rectal temperatures were slightly elevated in the majority of the unvaccinated animals 36 hours after infection, but not in the others (Supplement 60). The low virulence of the nebulised virus was also demonstrated by the fact that body weight development was not affected by the experimental infection (Supplement 60). The unvaccinated animals excreted virus over 7 days, whereas only a few of the vaccinated pigs excreted virus (Supplement 61). This was also reflected in the viral load of the lungs. While unvaccinated pigs had high virus titres in the entire lung on day 1 after infection, only some of the vaccinated pigs showed virus in the lung (Supplement 62). Macroscopic and microscopic lung lesions were low in the

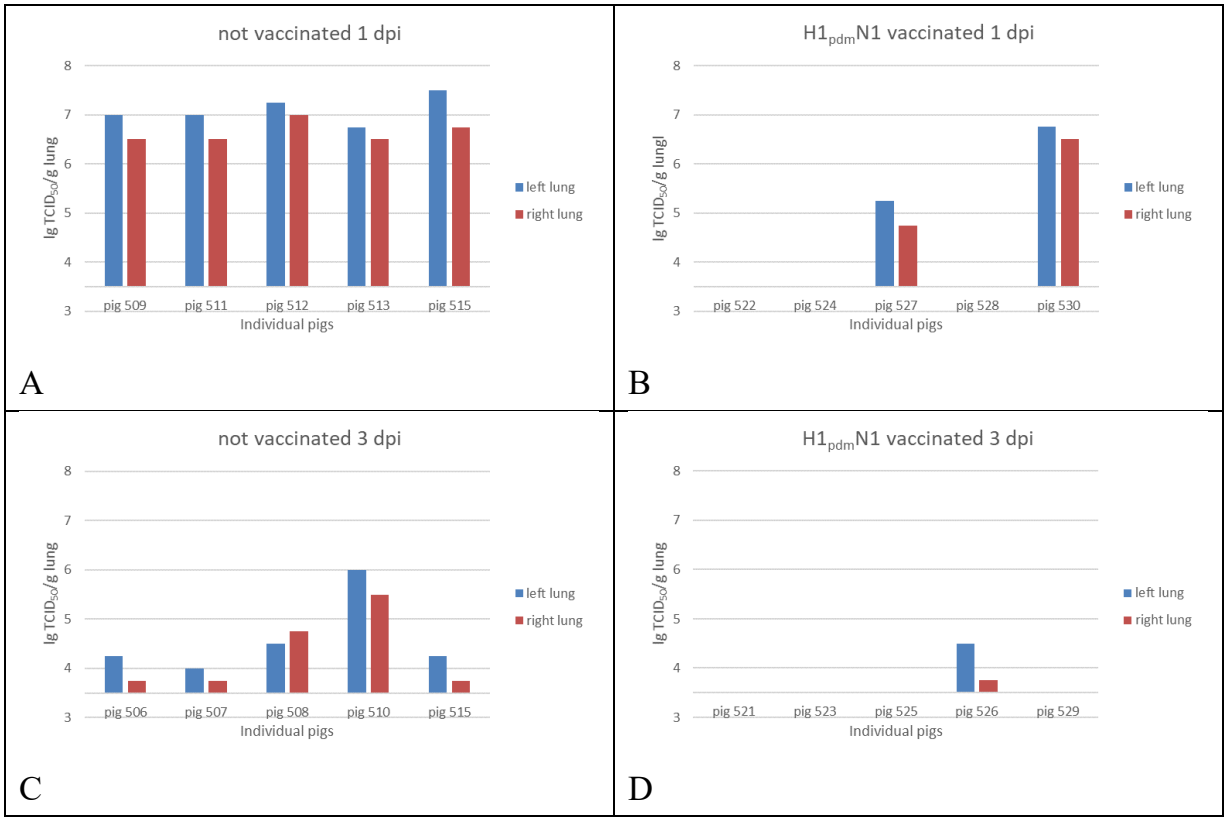
unvaccinated animals and significantly lower in the vaccinated animals (Supplement 63, Supplement 64).



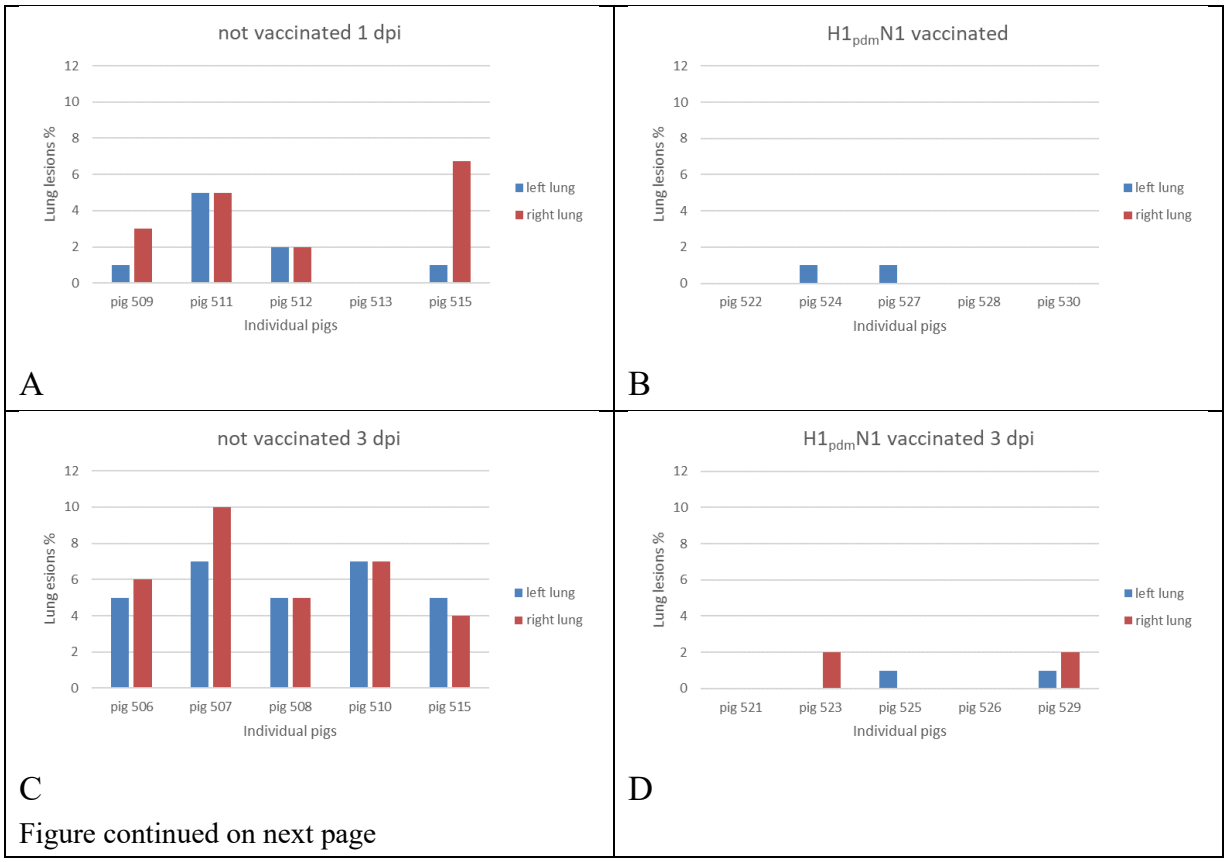
Supplement 60: Effects of infection with A/sw/England/117316/1986 ($H1_{cl}N1$) on unvaccinated and $H1_{pdm}N1$ 2009 vaccinated pigs, A, B, dyspnoea score, C, D, rectal temperatures, E, F, body weights



Supplement 61: Virus shedding in nasal swabs after infection with A/sw/England/117316/1986 ($H1_{cl}N1$) in unvaccinated and $H1_{pdm}N1$ 2009 vaccinated pigs, A, unvaccinated, B, vaccinated pigs



Supplement 62: Viral lung load after infection with A/sw/England/117316/1986 ($H1_{cl}N1$) in unvaccinated and $H1_{pdm}N1$ 2009 vaccinated pigs, A, B, 1 dpi, C, D, 3 dpi
On 9 dpi no virus was detected in the lungs of all pigs



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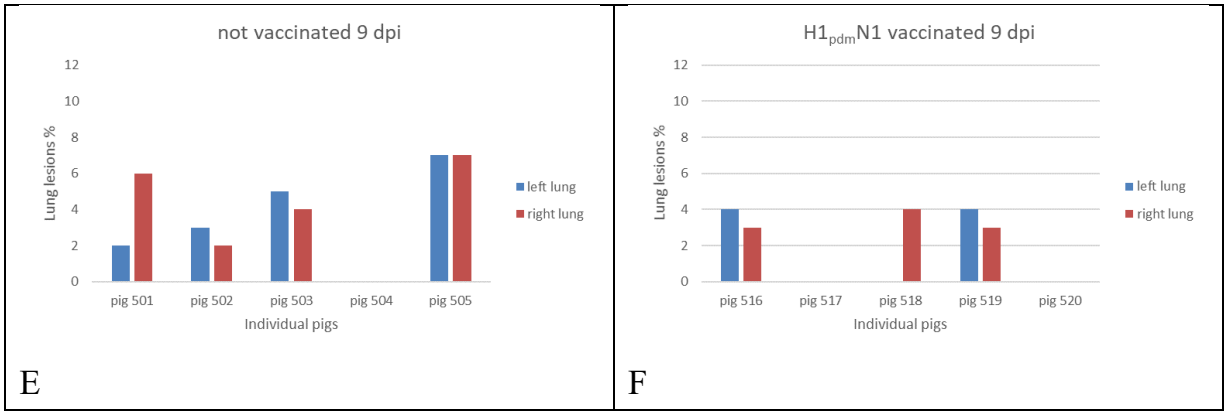
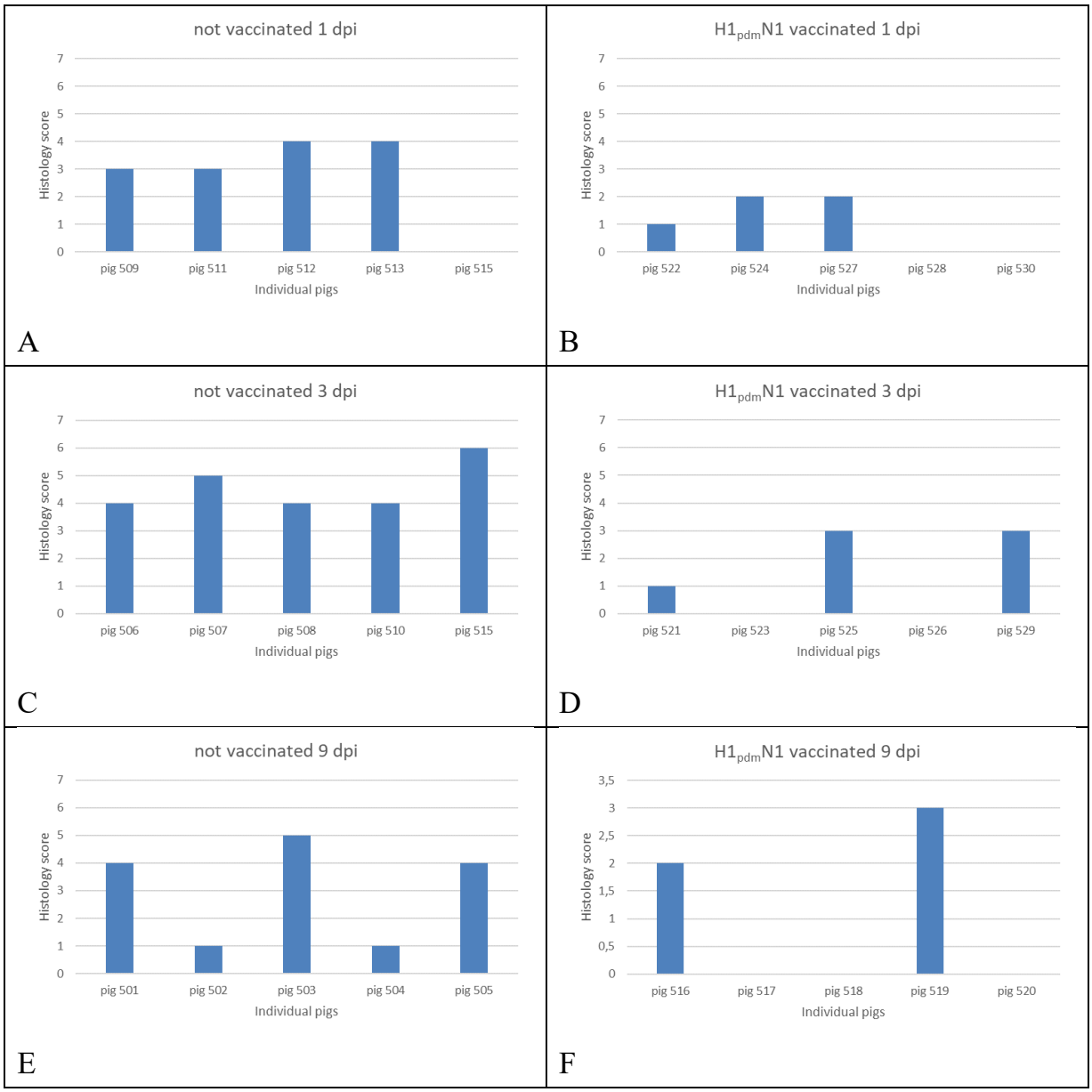
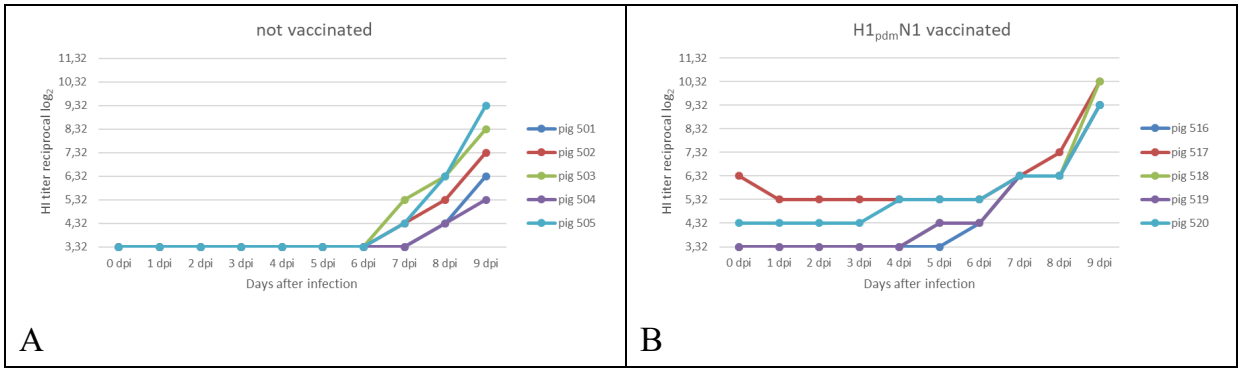


Supplement 63: Macroscopic lung lesions after infection with A/sw/England/117316/1986 ($H1_{cl}N1$) in un-vaccinated and $H1_{pdm}N1$ 2009 vaccinated pigs, A, B, 1 dpi, C, D, 3 dpi, E, F, 9 dpi



Supplement 64: Microscopic lung lesions after infection with A/sw/England/117316/1986 ($H1_{cl}N1$) in unvaccinated and $H1_{pdm}N1$ 2009 vaccinated pigs, A, B, 1 dpi, C, D, 3 dpi, E, F, 9 dpi

After infection, vaccinated animals responded to infection with higher HI titres than unvaccinated pigs (Supplement 65).



Supplement 65: Antibody kinetics against infecting strain A/sw/England/117316/1986 ($H1_{cl}N1$) within the first nine days following infection, A, unvaccinated, B, vaccinated pigs; geometric means are displayed

After vaccination, vaccinated animals had high HI titres against the vaccine virus, other $H1_{pdm}N1$ viruses and lower titres against $H1_{pdm}N2$ viruses. Only 40% of the pigs seroconverted against $H1_{cl}N1$. The vaccinated pigs did not react against $H1_{av}N1$, $H1_{hu}N2$ and H3N2 (Supplementary Table 27).

Supplementary Table 27: HI antibody titres 7 days after second vaccination of the vaccination group

$H1_{cl}N1$ 1986	$H1_{pdm}N1$ 2009	$H1_{pdm}N1$ vacc	$H1_{pdm}N2$ 2010	$H1_{pdm}N1$ 2009	$H1_{av}N1$ 2003	$H1_{hu}N2$ 2000	H3N2 2003
Not vaccinated							
<20	<20	<20	<20	<20	<20	<20	<20
<20	<20	<20	<20	<20	<20	<20	<20
<20	<20	<20	<20	<20	<20	<20	<20
<20	<20	<20	<20	<20	<20	<20	<20
<20	<20	<20	<20	<20	<20	<20	<20
$H1_{pdm}N1$ vaccinated							
<20	40	40	<20	<20	<20	<20	<20
80	80	320	40	<20	<20	<20	<20
<20	80	80	40	<20	<20	<20	<20
40	80	160	40	<20	<20	<20	<20
<20	160	160	40	<20	<20	<20	<20

Viruses used in HI: $H1_{cl}N1$ 1986: A/sw/England/117316/1986 ($H1_{cl}N1$); $H1_{pdm}N1$ 2009: A/Hamburg/NY1580/2009 ($H1_{pdm}N1$), $H1_{pdm}N1$ vacc: A/Jena/5258/2009 ($H1_{pdm}N1$), $H1_{pdm}N1$ 2011: A/sw/Störmede/13124/2011 ($H1_{pdm}N1$), $H1_{av}N1$: A/sw/Haselünne/2617/2003 ($H1_{av}N1$), $H1_{hu}N2$: A/sw/Bakum/1832/2000 ($H1_{hu}N2$), H3N2: A/sw/Bakum/1769/2003 (H3N2)

After infection, the vaccinated pigs reacted earlier and with higher titres against the infectious strain than the unvaccinated pigs. While the unvaccinated pigs had antibodies against $H1_{cl}N1$ and low antibodies against $H1_{pdm}N1$ 9 days after infection, the vaccinated pigs reacted against all viruses of the $H1_{pdm}$+$H1_{cl}$ group (Supplementary Table 28). Only a few

vaccinated pigs showed antibodies against $H1_{av}N1$. There were no signs of antibody-dependent enhancement of disease

Supplementary Table 28: HI antibody titres 9 days after infection

$H1_{cl}N1$ 1986	$H1_{pdm}N1$ 2009	$H1_{pdm}N1$ vacc	$H1_{pdm}N2$ 2010	$H1_{pdm}N1$ 2009	$H1_{av}N1$ 2003	$H1_{hu}N2$ 2000	H3N2 2003
Not vaccinated							
80	80	<20	<20	<20	<20	<20	<20
160	80	<20	<20	<20	<20	<20	<20
160	<20	<20	<20	<20	<20	<20	<20
40	40	<20	<20	<20	<20	<20	<20
320	60	80	<20	<20	<20	<20	<20
$H1_{pdm}N1$ vaccinated							
320	640	640	320	<20	<20	<20	<20
640	640	1280	320	<20	<20	<20	<20
640	320	640	160	<20	<20	<20	<20
320	640	1280	80	20	80	<20	<20
320	320	640	160	<20	<20	<20	<20

Viruses used in HI: $H1_{cl}N1$ 1986: A/sw/England/117316/1986 ($H1_{cl}N1$); $H1_{pdm}N1$ 2009: A/Hamburg/NY1580/2009 ($H1_{pdm}N1$), $H1_{pdm}N1$ vacc: A/Jena/5258/2009 ($H1_{pdm}N1$), $H1_{pdm}N1$ 2011: A/sw/Störmede/13124/2011 ($H1_{pdm}N1$), $H1_{av}N1$: A/sw/Haselünne/2617/2003 ($H1_{av}N1$), $H1_{hu}N2$: A/sw/Bakum/1832/2000 ($H1_{hu}N2$), H3N2: A/sw/Bakum/1769/2003 (H3N2)

Short evaluation. The data show that $H1_{pdm}$ and $H1_{cl}$ influenza viruses form a separate genetic cluster within the $H1_{pdm+cl+av}$ antigenic supergroup and are cross-reactive. This is emphasised by the optimal protective effect in the $H1_{pdm}$-vaccinated pigs against $H1_{cl}$ infection, but also by the absence of increased lung changes in the vaccinated pigs (no ADE). $H1_{cl}$ and early $H1_{pdm}N1$ influenza viruses are antigenically closer to each other than $H1_{pdm}N2$ influenza viruses. As early as 2011, the first $H1_{pdm}N1$ influenza viruses appeared in pigs, which did not react very well with the original $H1_{pdm}N1$ influenza viruses of 2009.

SUPPLEMENT CHAPTER 17 – INFLUENZA AND LETHALITY

Background. Infection studies in pigs are required for vaccine development and are guided by EP Monograph 0963. All studies reported here were performed as part of vaccine development and included vaccination groups to investigate efficacy. Results of routine vaccine development studies are not reported here.

The lethality ('deadliness' of a disease) describes the number of deceased cases as a proportion of the number of actually infected cases - in contrast to mortality, which refers to the entire population.

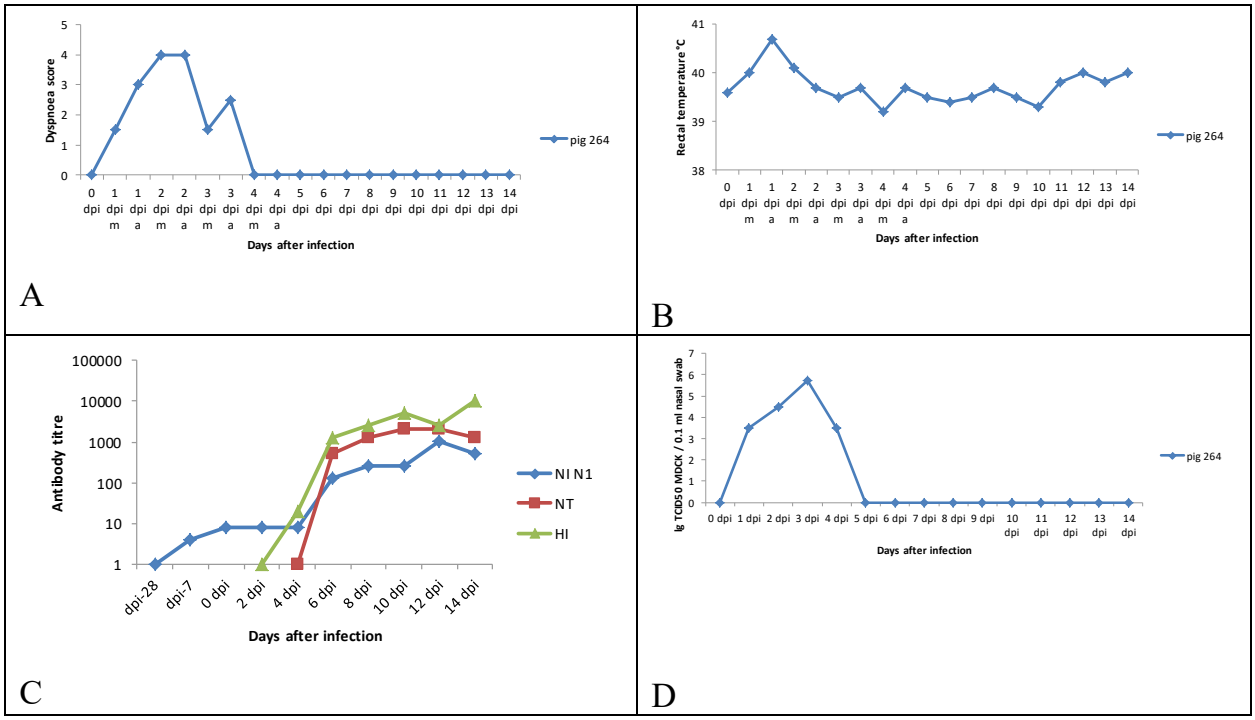
Mortality is a measure of mortality for a specific disease. It describes the number of patients who die from a disease in a certain period of time in relation to the total population or specific groups.

Case fatality rate, in epidemiology, is the proportion of people who die from a particular disease out of all people diagnosed with the disease in a given period. The case fatality rate is usually used as a measure of the severity of the disease.

In the experimental influenza system, all individuals are infected; the risk of severe cases is highest in the first week after infection; this period was covered by all experiments; therefore lethality and case-fatality rate are identical and can both be used as parameters to determine the severity of the infection.

Over the years, 18 out of 3131 pigs (0.57%) died after experimental high-dose influenza A infection: 1 unvaccinated pig after $H1_{av}N1$ 1980s virus infection (HA cluster 1C.1), 1 unvaccinated pig after H3N2 virus infection, 9 unvaccinated pigs after $H1_{pdm}N1$ 2009 virus infection, 3 unvaccinated pigs after H3N1 infection, 1 unvaccinated pig after $H1_{pdm}N2$ infection, 2 $H1_{pdm}N2$-vaccinated pigs after H3N1 infection and 1 $H1_{pdm}N1$-vaccinated pig after $H1_{pdm}N2$ virus infection (in the vaccinated cases, the infection strain was distant to the vaccine strain and antibodies against the matrix protein were dominant over those against HA and NA). The majority of fatal cases were caused by newly reassorted HxN1 viruses ($H1_{pdm}N1$ 2009 and H3N1 viruses). While lethality was low even under high-dose conditions in the experimental system, it cannot be neglected for the freshly reassorted HxN1 influenza viruses. In relation to the group size, a lethality rate of around 20% was achieved here. This figure is probably higher than that for humans in the 1918 pandemic[511], but it should be borne in mind that the initial exposure dose is crucial for the course (i.e. virus that reaches the lungs immediately before immunological processes occur), and this will vary greatly in pandemics. Therefore, such data are difficult to compare. In general, influenza virus infection induced disease, but pigs recovered rapidly within 3-7 dpi. Infections with high doses of pandemic viruses made it necessary to re-evaluate the ethics of animal welfare and to establish criteria for the euthanasia of pigs in respiratory distress.

Study design. All cases of severe disease were subjected to analysis and evaluation of clinical parameters.



Supplement 66: Recovery from severe influenza in pig 264 after infection with FLUAV/Hamburg/NY1580/2009 ($H1_{pdm}N1$); A, Symptoms (dyspnoea score); B, Rectal temperatures (°C); C, Antibodies (NI N1, anti neuraminidase N1 antibodies, neuraminidase inhibition dose 50), NT, neutralising antibodies, neutralisation dose 50; HI, hemagglutination inhibiting antibodies, HI titre reciprocal); D, Virus excretion via nasal swabs (lg $TCID_{50}$ MDCK/0.1 ml nasal swab); arithmetic means are shown, for antibodies geometric mean

Results. Mortality after infection with swine influenza viruses followed two progressions: i) After infection with H3N2 and $H1_{av}N1$ viruses, very few individual pigs died suddenly without respiratory symptoms, as in the case of collapse. Such unexpected sudden deaths due to influenza virus infection are also reported by farmers in practice. ii) This was different for pandemic viruses ($H1_{pdm}N1$, $H1_{pdm}N2$) and newly reassorted viruses (H3N1). Pigs developed severe respiratory disease with signs of severe pneumonia and had to be euthanised. In severe cases, body temperatures dropped. One pig, which developed the most severe disease of all pigs in the trials, recovered rapidly within 48 hours after showing the most severe dyspnoea score peak of all pigs in the trials (Supplement 66). This was due to a strong immune response. This pig had been vaccinated but did not respond to vaccination with antibodies due to the presence of maternal immunity. Priming by vaccination allowed rapid induction of antibodies and the pig recovered and remained healthy like the other healthy pigs. This is something that needs to be taken into account when making an ethical decision about close handling. This case of severe disease expression with recovery shows that disease severity may not be a criterion for euthanasia of pigs. Analysis of all cases showed that the criterion for euthanasia could be a drop in rectal temperature below 38.7 °C.

The analyses show that influenza virus infections should not be underestimated at the individual level. On the one hand, severe courses can occur due to newly reassorted $H1_XN1$ viruses in initial high-dose exposure or under conditions of antibody-dependent enhancement of disease (these factors could have been combined in the 1918 influenza). Also, the expression of antigens outside the respiratory tract can induce inflammation and severe courses, sometimes fatal, in predisposed individuals. On the other hand, high-dose exposures under natural conditions are highly variable and even under the high-dose exposures demonstrated here, the majority of the animals recovered quickly from the infection, so that a lethality of more than about 25% cannot be induced by influenza viruses. This view is based on the consideration that the most virulent virus ($H1_{pdm}N1$ April 2009) was able to induce a lethal course in 20% of the three-months-old pigs, but that the virus was not concentrated in these experiments, so that it can be assumed that the full potential of approximately 25% lethality would have unfolded at higher doses (corresponding to the presumed genetic predisposition in recessive inheritance). Concentrated material was nebulised with $H1_{pdm}N2$ and $H1_{cl}N1$ (concentrated to approximately 9 billion virus particles/m^3, see previous supplementary chapters). This resulted in severe symptoms after $H1_{pdm}N2$ infection, but these were less severe than with the $H1_{pdm}N1$ April 2009 virus, suggesting that reassortment with N2 had weakened virulence. The 1986 $H1_{cl}N1$ virus was extremely mild, suggesting that after 68 years of circulation in pigs, the virus has little pathogenic potential.

SUPPLEMENT CHAPTER 18 – INFECTION OF PIGS OF DIFFERENT AGE

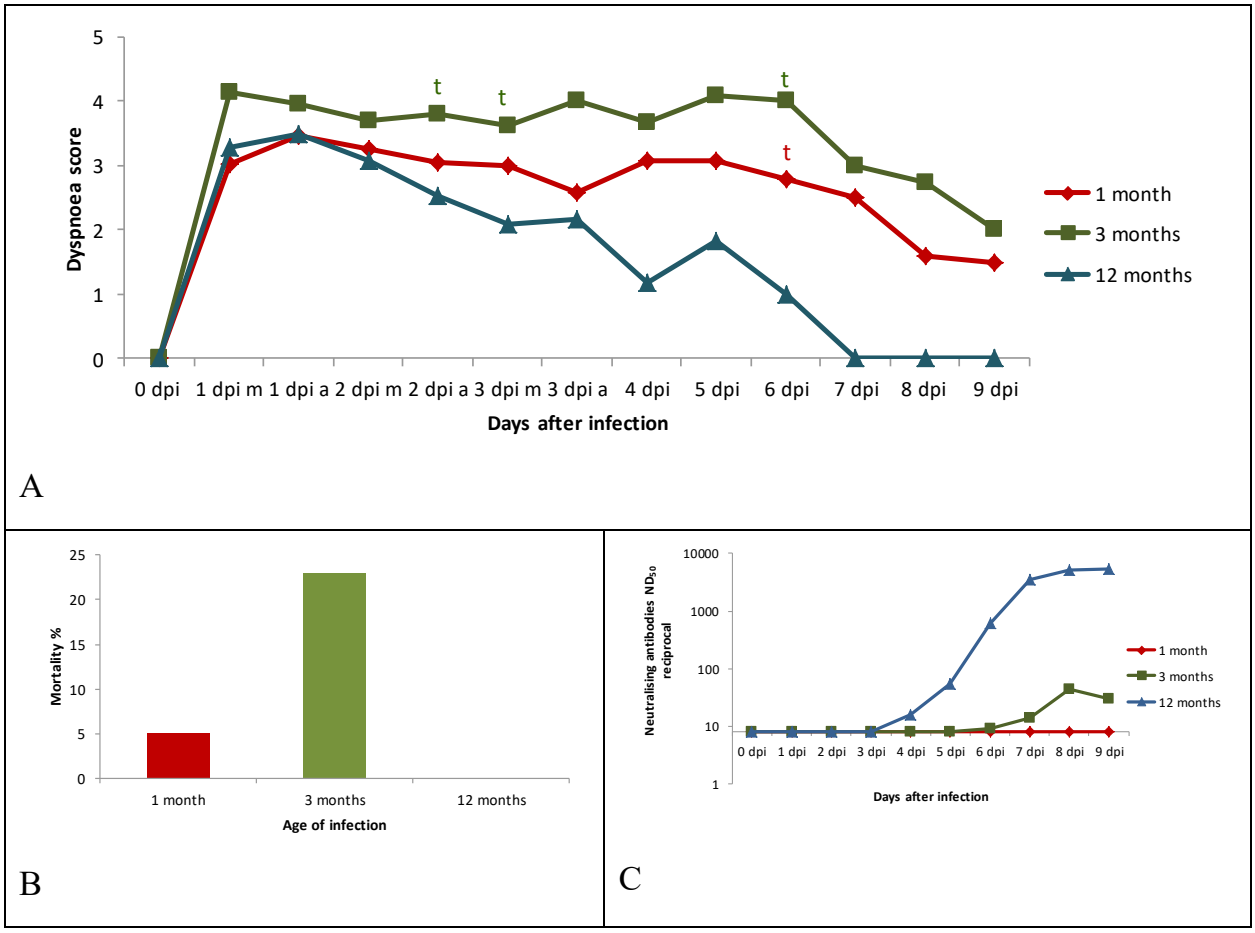
Background. The outcome of influenza may be different in pigs of different ages. Therefore, infection trials were compared in different age groups using the same infection strain.

Study design. Three studies were compared in which pigs were infected at 1, 3 and 12 months of age (Supplementary Table 29). The pigs were infected by high-dose aerosol; the dose of infection was almost identical (±0.25 lg $TCID_{50}$). Sampling was identical (lung samples were collected from 5 pigs; documentation of clinical signs was performed twice daily from 1-3 dpi and daily thereafter; blood samples and nasal swabs were collected daily; body weights were measured).

Supplementary Table 29: Overview of the trials analysed for investigation of age-dependent virulence (in all trials strain A/Hamburg/NY1580/2009 $H1_{pdm}N1$ had been nebulised)

Group	Age at infection (months)	Number of pigs	Infection dose (lg $TCID_{50}/m^3$)
1	1	19	6.17
2	3	13	5.92
3	12	18	5.92
4	12	13	6.38

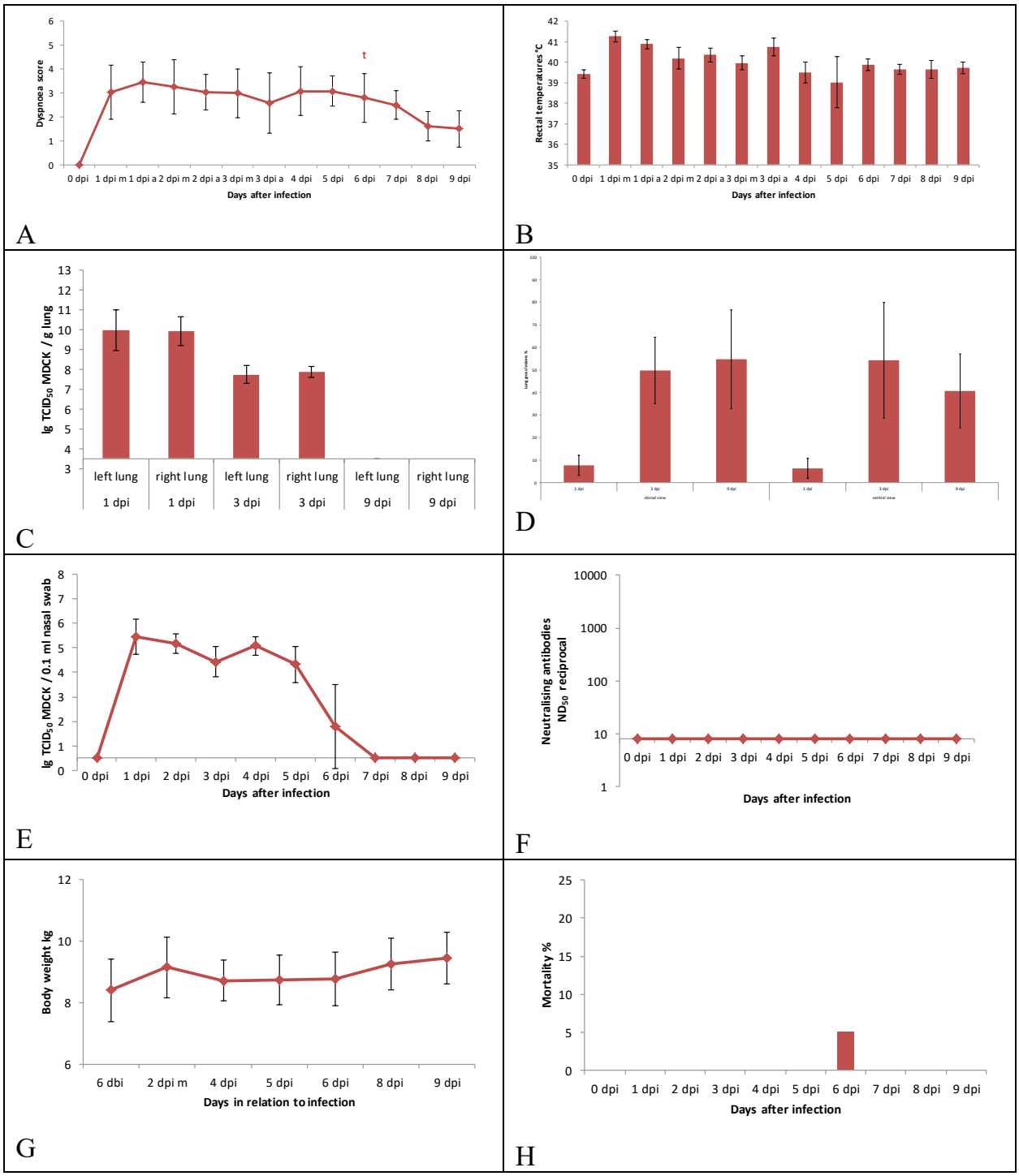
Results. Pigs infected at 3 months of age had the most severe disease course and the highest mortality, despite the fact that the antibody response was stronger than in pigs infected at 1 month of age (Supplement 67 A, B, C). This group also had the most severe gross lung lesions and the greatest weight loss. Pigs infected at 12 months of age had significantly fewer symptoms and the strongest immune response to infection, as reflected by neutralising antibodies, compared with the other groups (Supplement 68, Supplement 69, Supplement 70, Supplement 71). Gross lung lesions were significantly lower at this age compared to the other age groups. Pigs in all groups had very high viral titres of around 9.5 lg $TCID_{50}$/g lung tissue in their lungs at 1 dpi, reduced to 2 lg $TCID_{50}$/g lung tissue at 3 dpi; the shedding profiles were identical; all pigs responded with fever at 1 dpi.



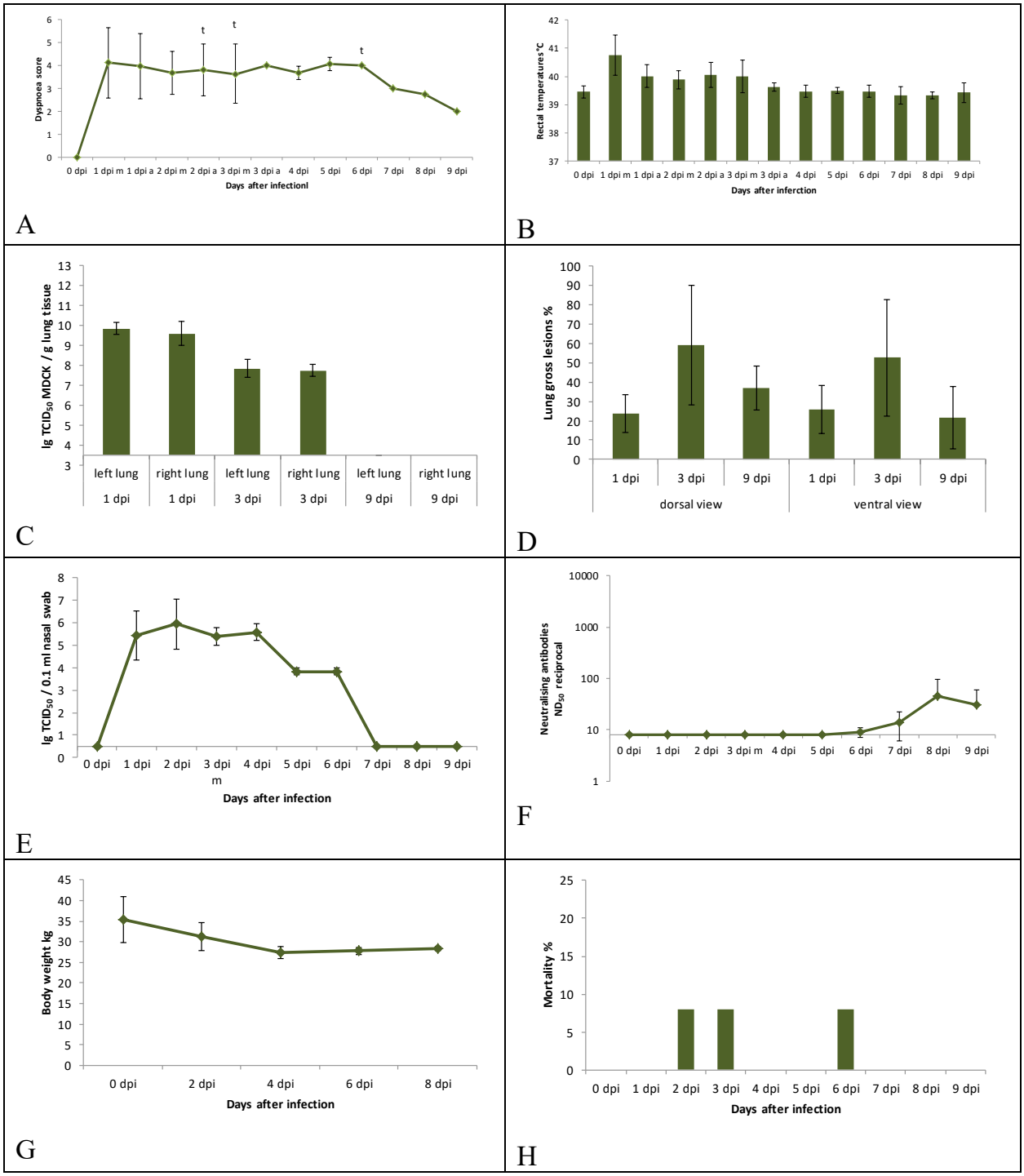
*Supplement 67: Comparison of course of disease in different groups of age at infection with the same influenza A virus strain Hamburg/NY1580/2009 $H1_{pdm}N1$; A, Symptoms (mean dyspnoea score); the differences between the groups were significant (1 months vs. 3 months: * from 1-8 dpi; 3 months vs. 12 months * 1-2 dpi; ** 3 dpi; *** 4-9 dpi, 3 months vs. 1 months: * 2a-8 dpi, 1 months vs. 12 months: * 4-5 dpi, ** 6-9 dpi); B, Mortality in pigs of different age groups at infection with the same influenza A virus strain Hamburg/NY1580/2009 $H1_{pdm}N1$ (%); Induction of neutralizing antibodies in pigs of different age groups at infection with the same influenza A virus strain Hamburg/NY1580/2009 $H1_{pdm}N1$, *, $p<0.05$, **, $p<0.01$, ***, $p<0.005$, for details see Supplement 68, Supplement 69, Supplement 70*

Short interpretation of results. The trials used the most virulent strain available ($H1_{pdm}N1$ April 2009 virus, i.e. A/Hamburg/NY1580/2009). There were marked differences in disease expression between the groups. While pigs at 12 months of age coped best with the infection, pigs at 3 months of age performed worst. Antibody induction was best in pigs aged 12 months and worst in the youngest group. Despite the poor antibody response in pigs at 1 month of age, pigs at 3 months of age developed more severe disease. The reason for this is unknown. All pigs were from the same herd, which had been free of influenza for years and was regularly monitored. The sows of all pigs had been tested for the absence of antibodies against influenza A viruses in their serum and colostrum. All pigs had also been tested in their first week of life and were free of antibodies to influenza A viruses. It is possible that non-specific components of the colostrum had been delivered to the piglets and were still active in the younger pigs, supporting the better performance of this group. Based on the better performance of the 3-months-old pigs in antibody response, it is unlikely that cellular immunity was worse than in the younger pigs. Pigs at 3 months of age had significantly more severe lung lesions 3 dpi than pigs at 1 month of age but recovered

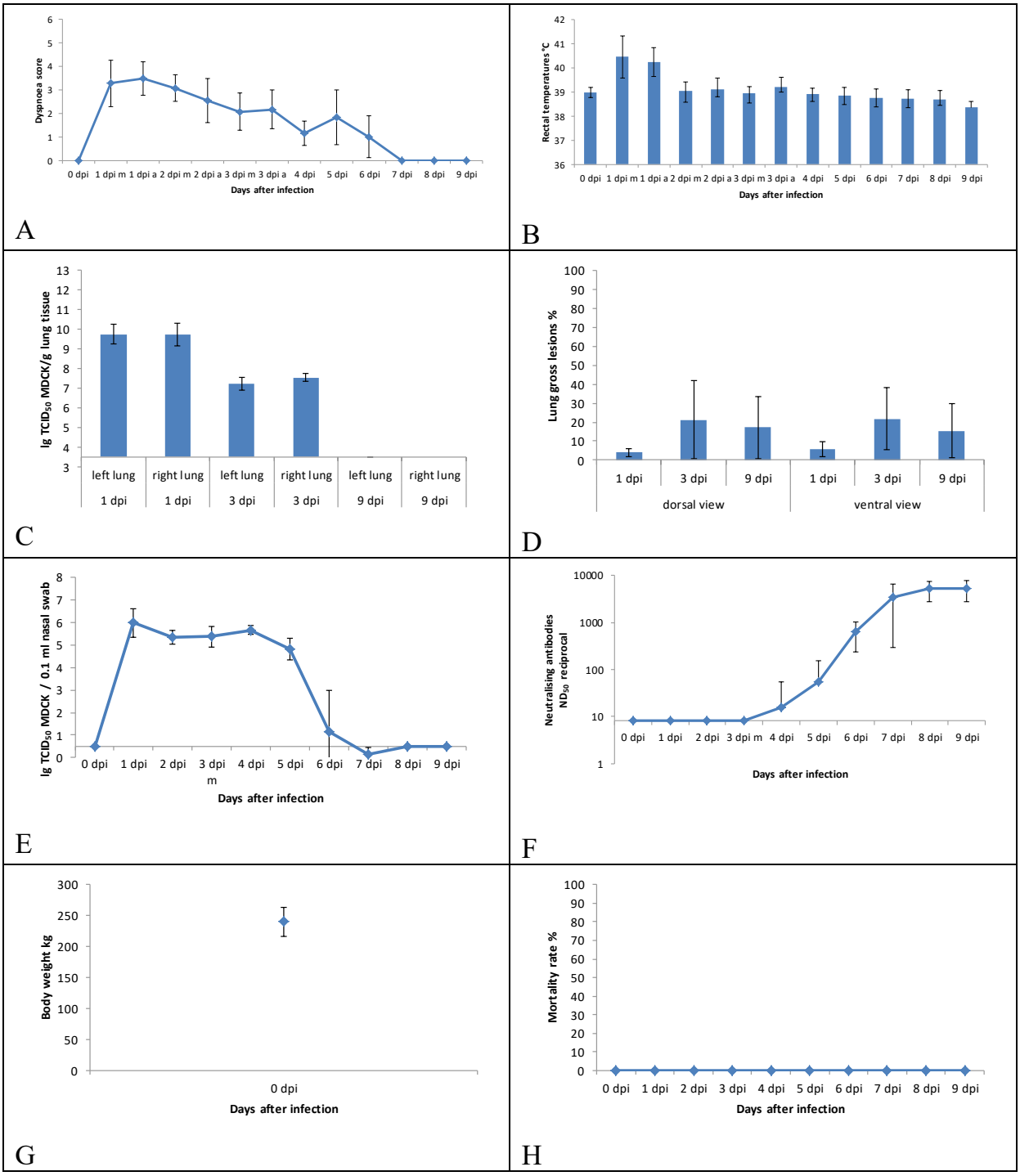
more quickly; lung lesions in 12 months-old-pigs were significantly less pronounced than in the other two groups (data not shown), suggesting that innate immunity may be more developed than in 1 month old pigs and may interact with the virus more than in the younger pigs.



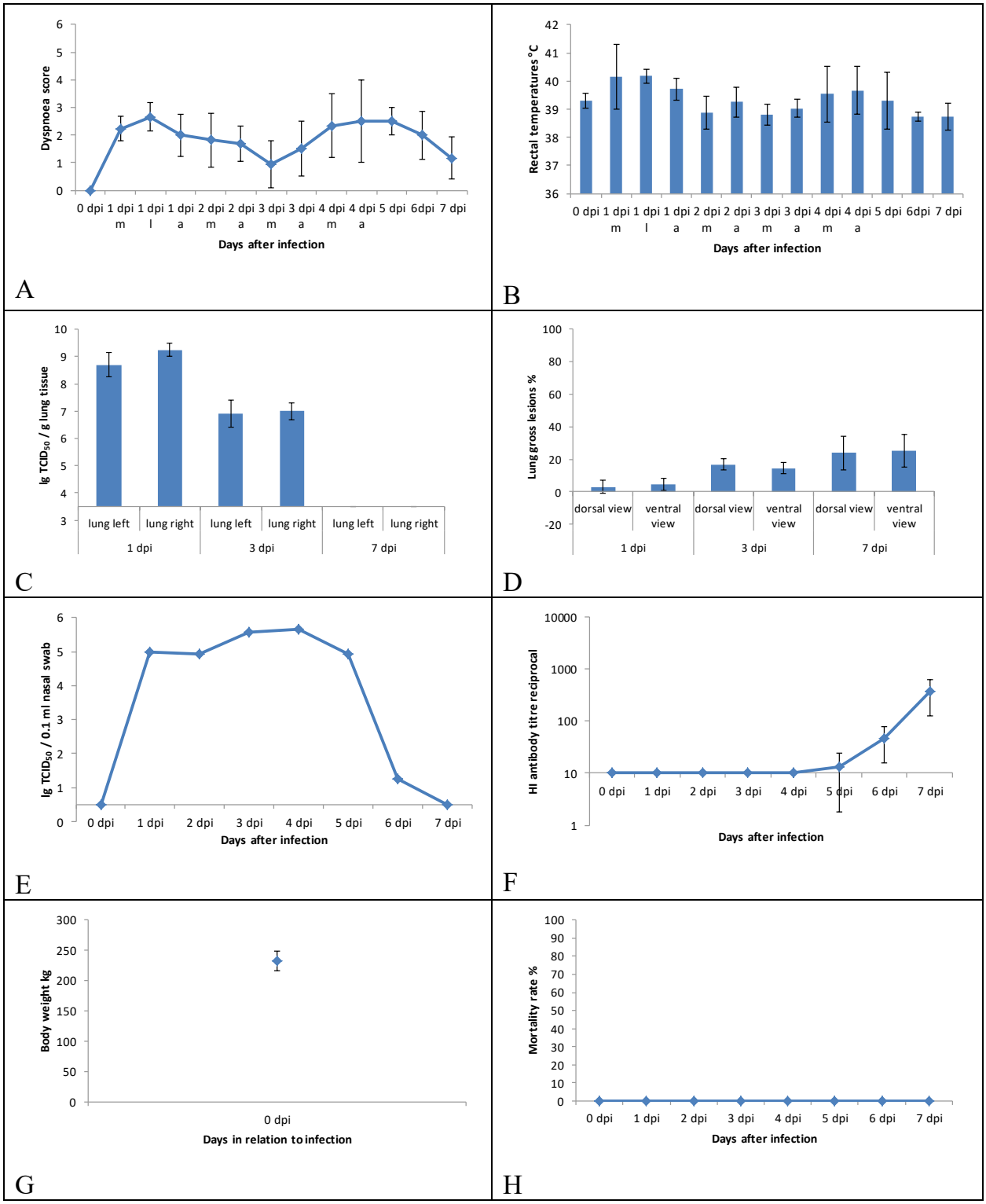
Supplement 68: Infection of pigs at an age of 1 month with strain A/Hamburg/NY1580/2009 ($H1_{pdm}N1$); A, Dyspnoea; B, Rectal temperatures; C, Viral lung load; D, Lung lesions; E, Virus excretion; F, NT antibody kinetics (NT); G, Body weights; H, Mortality rate (%); arithmetic means are shown, geometric means for antibodies; t = one pig that died



Supplement 69: Infection of pigs at an age of 3 months with strain A/Hamburg/NY1580/2009 ($H1_{pdm}N1$); A, Dyspnoea; B, Rectal temperatures; C, Viral lung load; D, Lung lesions; E, Virus excretion; F, NT Antibody kinetics (NT); G, Body weights; H, Mortality rate (%); arithmetic means are shown, geometric means for antibodies; t = corresponds to one pig that died

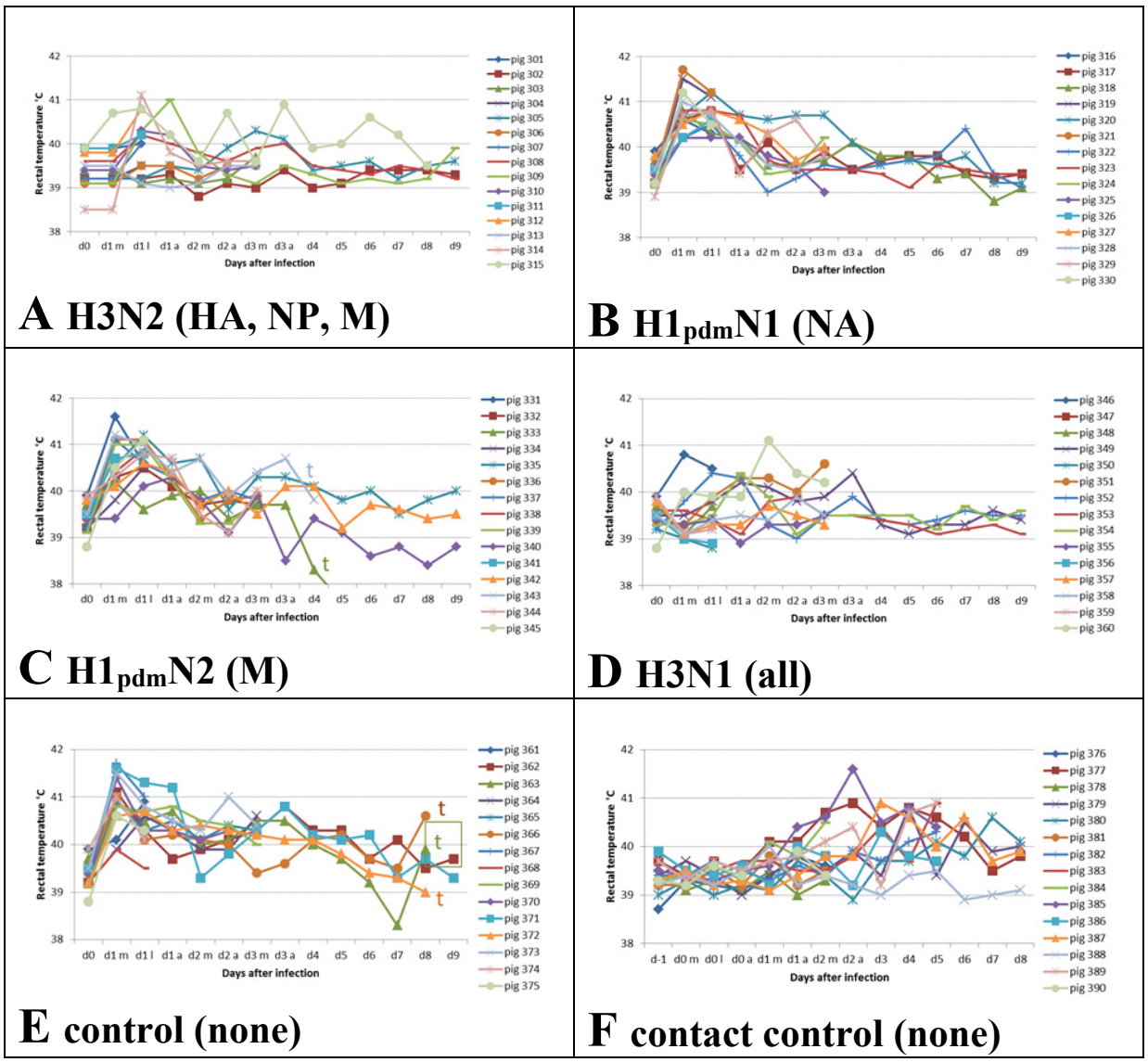


Supplement 70: Infection of pigs at an age of 12 months with strain A/Hamburg/NY1580/2009 ($H1_{pdm}N1$); A, Dyspnoea; B, Rectal temperatures; C, Viral lung load; D, Lung lesions; E, Virus excretion; F, NT aantibody kinetics (NT); G, Body weight (weighing was only performed before infection); H, Mortality rate (%); arithmetic means are shown, geometric means for antibodies

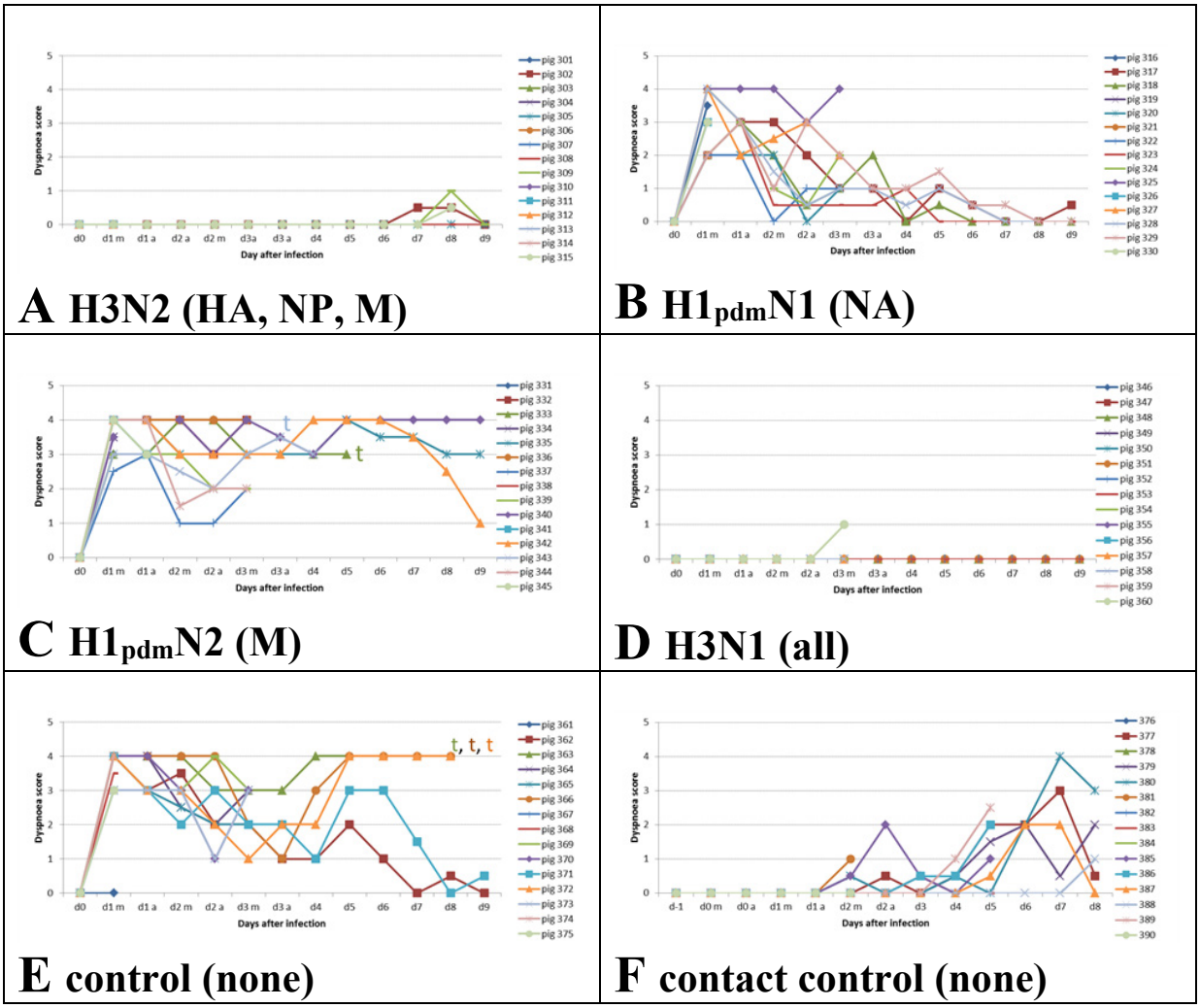


Supplement 71: Parameters of the second trial performing infection of 12 months old pigs with FLUAV/Hamburg/NY1580/2009 H1$_{pdm}$N1; A, Symptoms (dyspnoea score, arithmetic mean); B, Rectal temperatures (°C, arithmetic mean); C, Viral lung load (lg TCID$_{50}$ MDCK/g, arithmetic mean); D, Lung lesions (%, arithmetic mean); E, Virus excretion (lg TCID$_{50}$ MDCK/0.1 ml, arithmetic mean); F, Antibody kinetics (HI titre, geometric mean); G, Body weight (kg, arithmetic mean; weighing was only performed before infection); H, mortality rate (%)

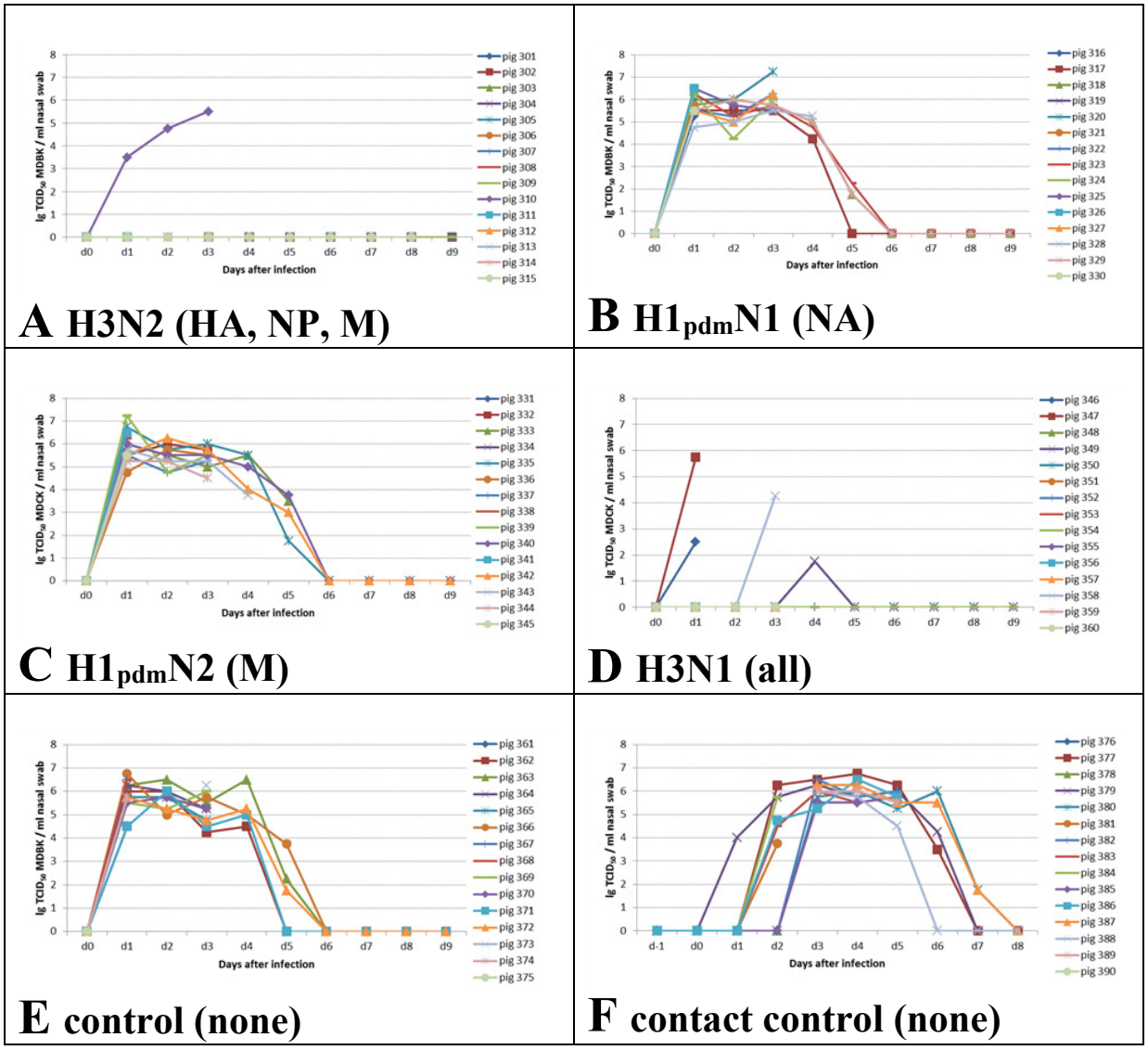
SUPPLEMENT CHAPTER 19 – ANALYSIS IMMUNOGENIC EFFECTS OF VIRAL PROTEINS



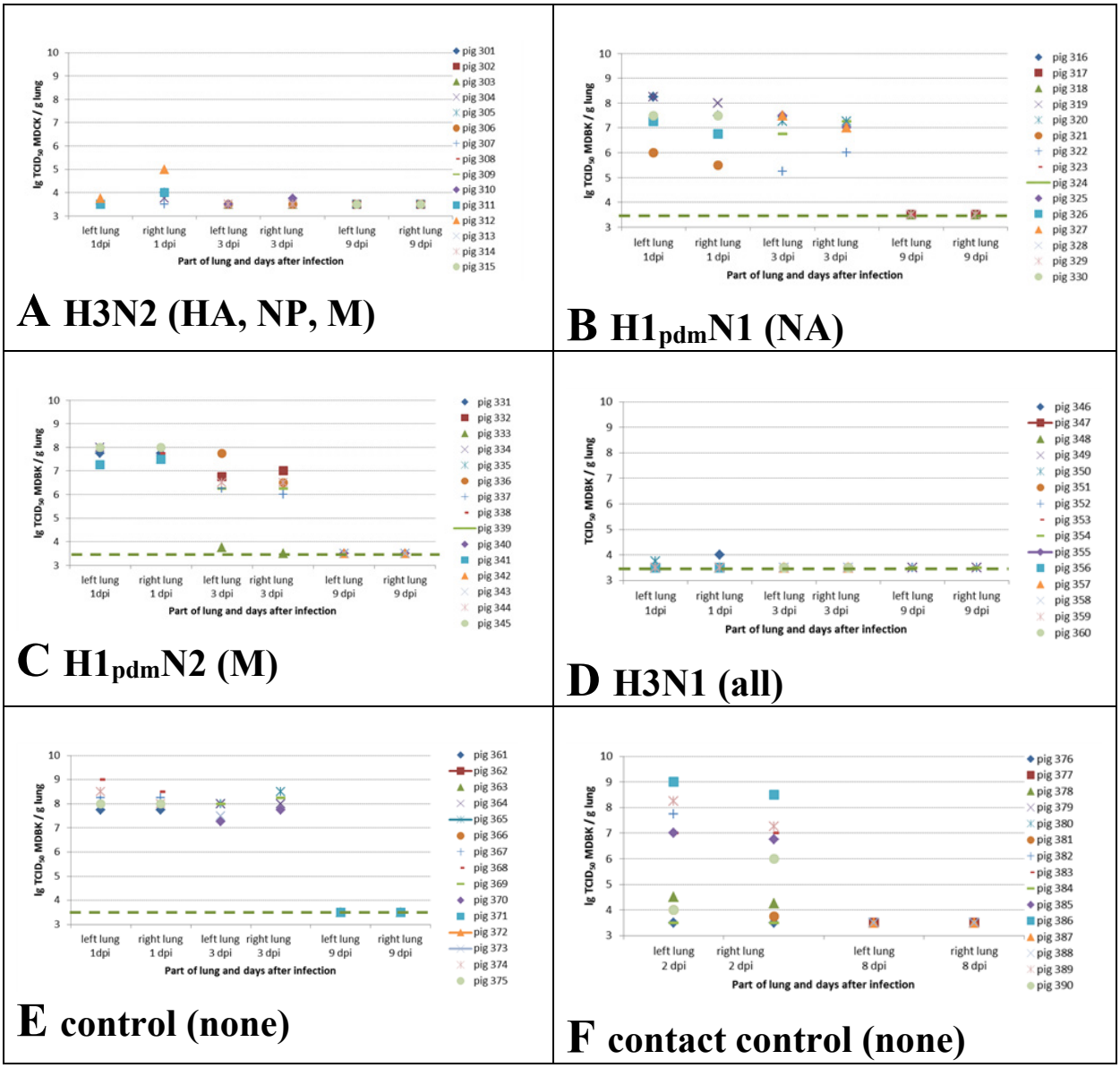
Supplement 72: Rectal temperatures. Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of rectal temperatures in pigs; A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, $H1_{pdm}N1$ immunisation (NA is related to the challenge virus); $H1_{pdm}N2$ immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, pigs removed for the investigation of lung pathology



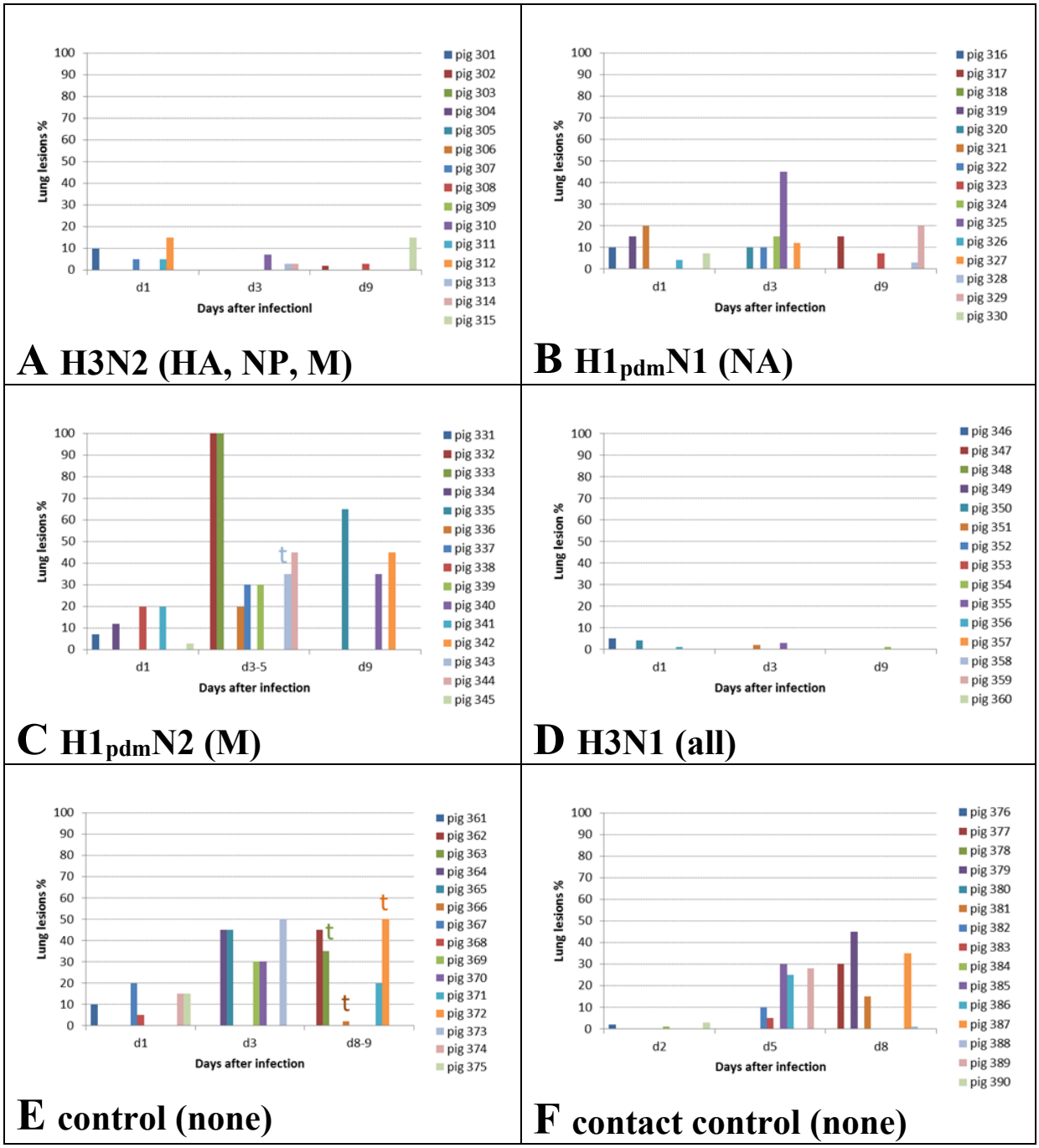
Supplement 73: Dyspnoea score. Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of dyspnoea in pigs; A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, H1$_{pdm}$N1 immunisation (NA is related to the challenge virus); H1$_{pdm}$N2 immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, pigs removed for the investigation of lung pathology; m, morning; a, afternoon



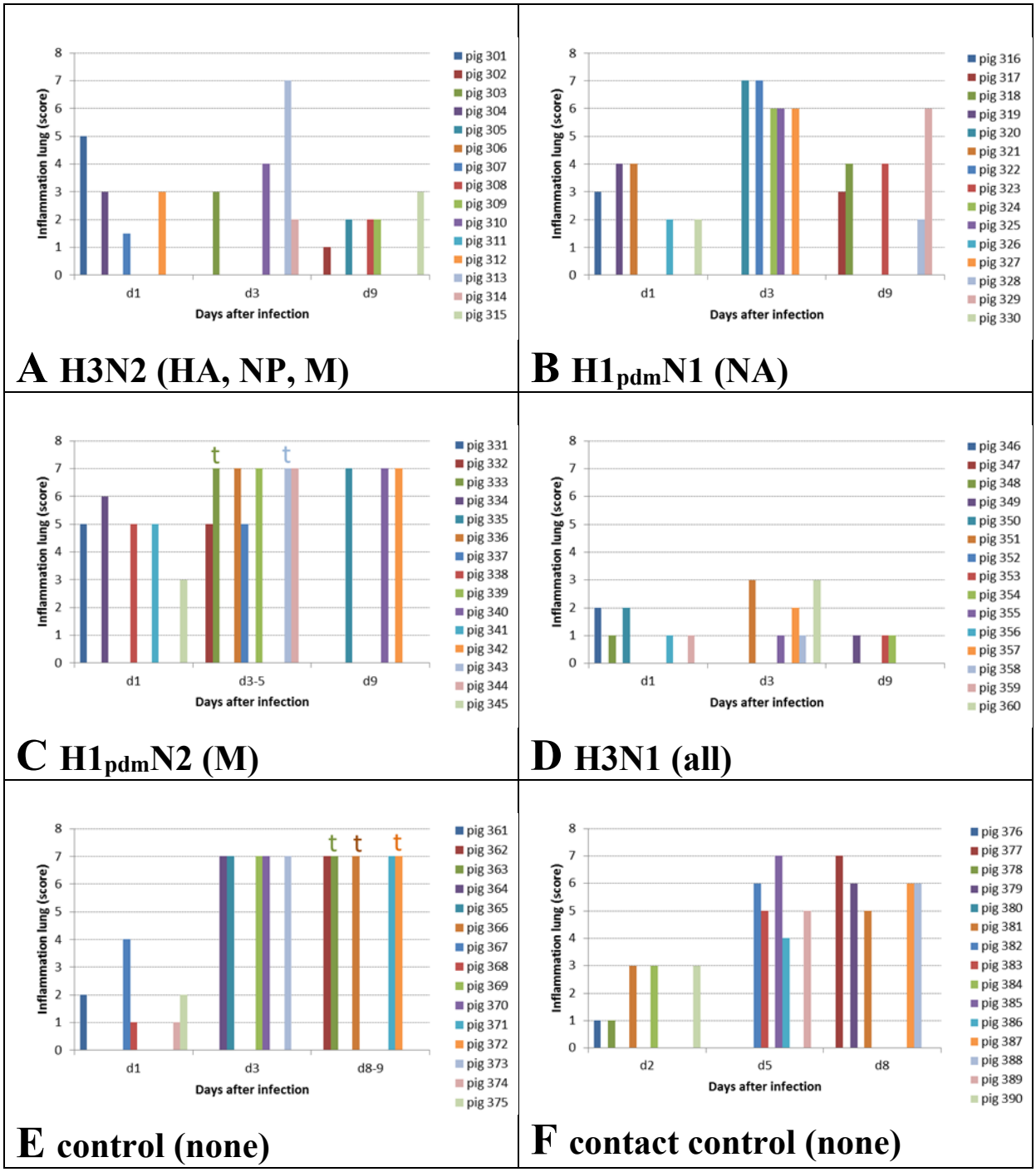
Supplement 74: Virus excretion. Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of virus shedding in nasal swabs; A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, $H1_{pdm}N1$ immunisation (NA is related to the challenge virus); $H1_{pdm}N2$ immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, pigs removed for the investigation of lung pathology



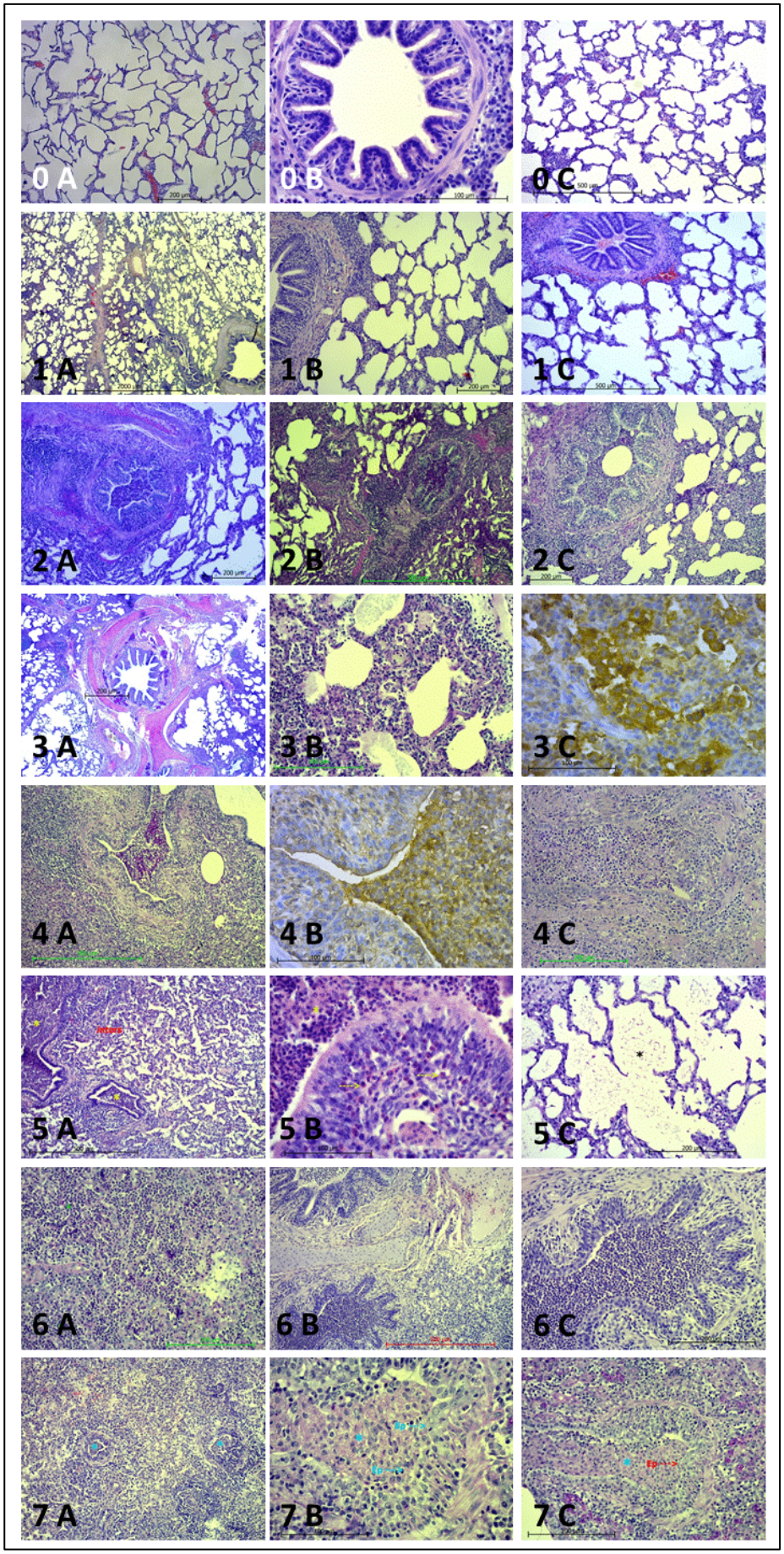
Supplement 75: Viral lung load. Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of viral lung load; A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, H1$_{pdm}$N1 immunisation (NA is related to the challenge virus); H1$_{pdm}$N2 immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, pigs removed for the investigation of lung pathology



Supplement 76: Lung lesions. Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of lung pathology (macroscopically visible lung lesions); A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, H1$_{pdm}$N1 immunisation (NA is related to the challenge virus); H1$_{pdm}$N2 immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, pigs removed for the investigation of lung pathology

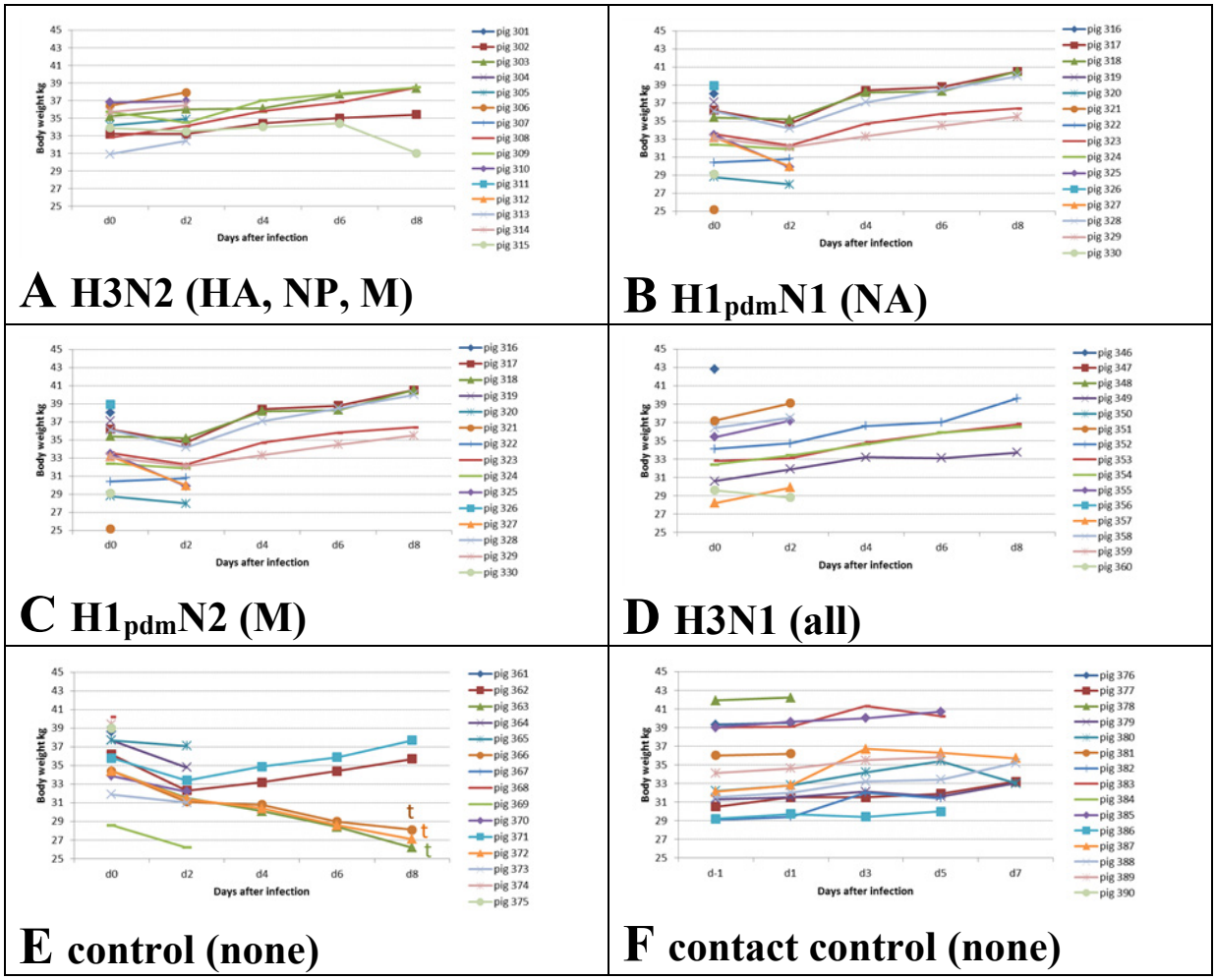


Supplement 77: Lung inflammation. Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of lung histology (inflammation score, for examples see next supplement); A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, H1$_{pdm}$N1 immunisation (NA is related to the challenge virus); H1$_{pdm}$N2 immunisation (only M is related to the virus used for experimental infection; D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection

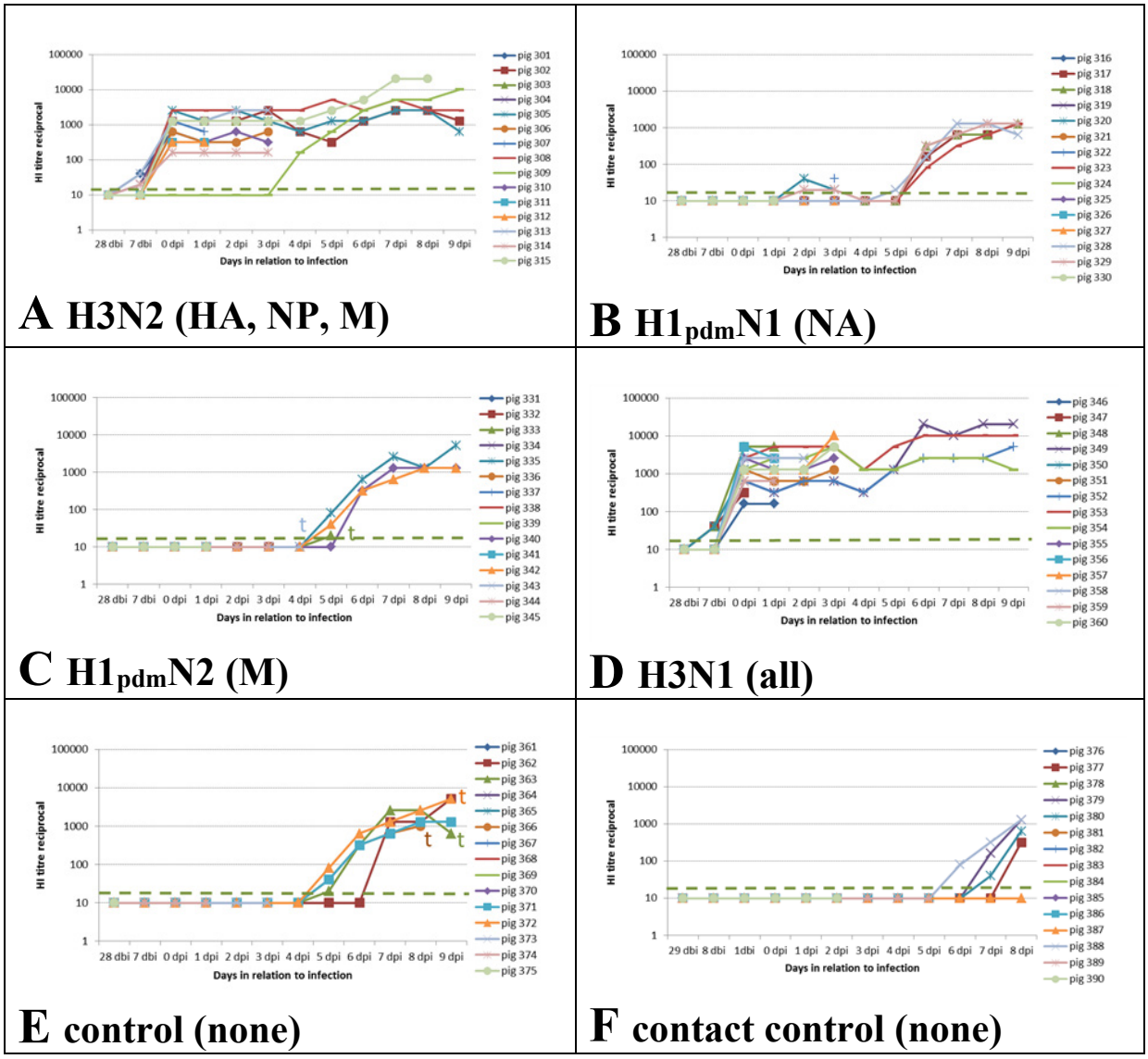


Supplement 78: Inflammation scores 0 – 7, examples, legend continued on next page

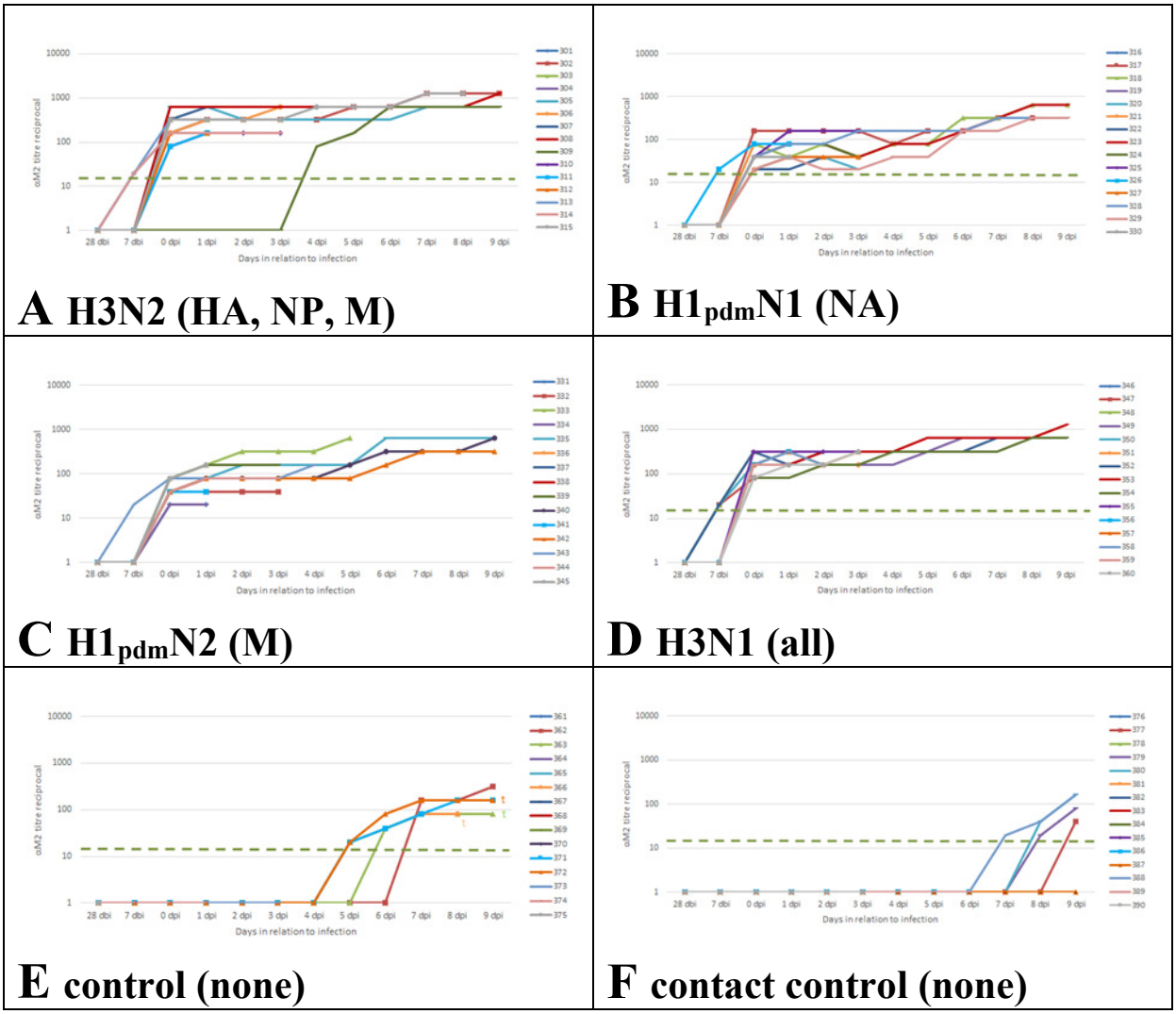
0 A-C, lung score 0, 0 A pg 311 1dpi H3N2 group, HE, good structured parenchyma, few interstial alveolar mnacrophages, slight edema; 0 B, the same animal HE, free lumen of bronchioli; 0 C, pig 901 1 dpi control HE good structured lung parenchyma with slight interstitial accumulation of interstitial alveolar macrophages, slight edema, areas with eosinophile granulocytes; 1 A-C, inflammation score 1, 1 A, pig 348 1 dpi H3N1 group, decent interstitial macrophages, free lumen of bronchioli, 1 B, the same pig as before HE, 1 C, pig 932 contact control 2 dpi HE, moderate mononuclear peribronchitis; 2 A-C, inflammation score 2, 2 A, pig 326 H1$_{pdm}$N1 group 1 dpi PAS interstitial and broncholuminal macrophages, some neutropils and eosinophils, 2 B, pig 350 H3N1 group 1 dpi PAS interstitial and broncholuminal macrophages, 2 C, pig 367 control group 1 dpi HE, BALT hyperplasia, larger areas of interstitial and broncholuminal macrophages, some eosinophils and neutrophils, 3 A-C, inflammation score 3, 3 A, pig 304 H3N2 group 1 dpi HE, areas of interstitial and broncholuminal macrophages, lumen of bronchioli free, good structured microvilli, 3 B, pig 316 H1$_{pdm}$N1 group 1 dpi PAS, 3 C, same pig as before SABC virus-specific staining of macrophages; 4 A-C, inflammation score 4, 4 A, pig 319 H1$_{pdm}$N1 group 1 dpi PAS larger areas of interstitial and broncholuminal macrophages, 4 B, the same pig SABC, specific staining of macrophages, 4 C, pig 331 H1$_{pdm}$N1 group PAS, massive macrophage infiltration with adhesion of macrophages onto epithelial cells, 5 A-C, inflammation score 5, 5 A, pig 301 H3N2 group 1 dpi HE interstitial (Inters) and broncholuminal (star) macrophages, 5 B, the same pig, mixed infiltration (star) and eosinophil granulocytes (arrows), 5 C the same pig as before PAS moderate edema (star); 6 A-C, inflammation score 6, 6 A, pig 325 H1$_{pdm}$N1 group 1 dpi PAS large areas of macrophages and massive interstitial neutrophil granulocytes (star), 6 B pig 327 1 dpi HE massive areas of macrophage accumulation, 6 C, the same pig as before HE, alveolar edema, eosinophil granulocytes, BALT hyperplasia; 7 A-C, inflammation score 7, 7 A, pig 322 H1$_{pdm}$N1 group 1 dpi HE massive areas of inflammation with fibrin exsudation (fribrin = star), 7 B and 7 C, the same pig as before PAS massive obstructive bronchitis with adhesion of macrophages onto epithelial cells (Ep→) and fibrin accumulation (star), alveolar edema and some eosinophile granulocytes



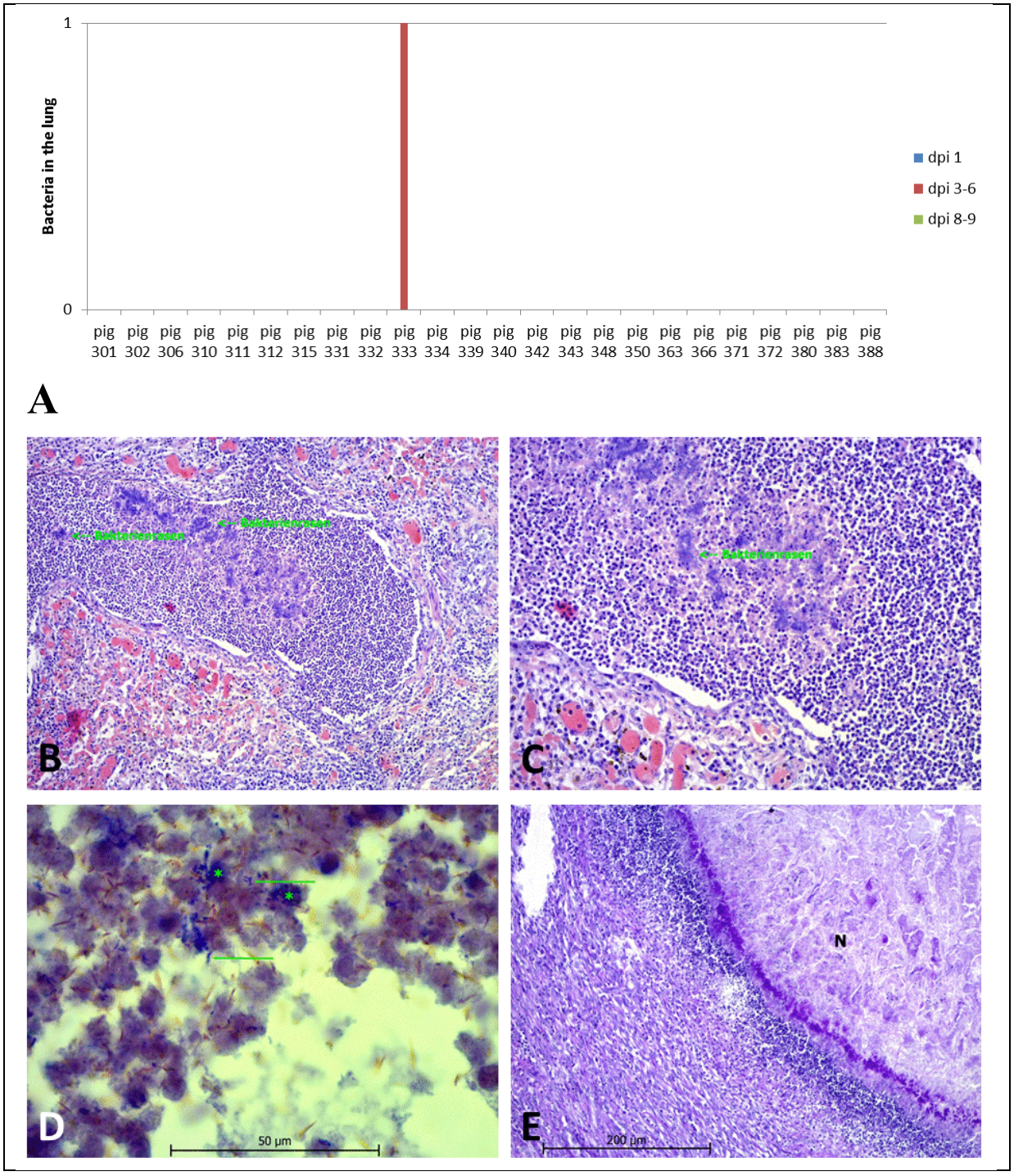
Supplement 79: Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of body weights; A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, H1$_{pdm}$N1 immunisation (NA is related to the challenge virus); H1$_{pdm}$N2 immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, animals were removed from the experiment to analyse the lung samples



Supplement 80: Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of H3N1 HI antibody kinetics; A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, H1$_{pdm}$N1 immunisation (NA is related to the challenge virus); H1$_{pdm}$N2 immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, animals were removed from the experiment to analyse the lung samples; note that in group H1$_{pdm}$N2 two pigs died the time around when antibodies appear; 28 dbi, first immunisation of the immunisation groups, 7 dbi, second immunisation of the immunisation groups; dotted line, detection limit

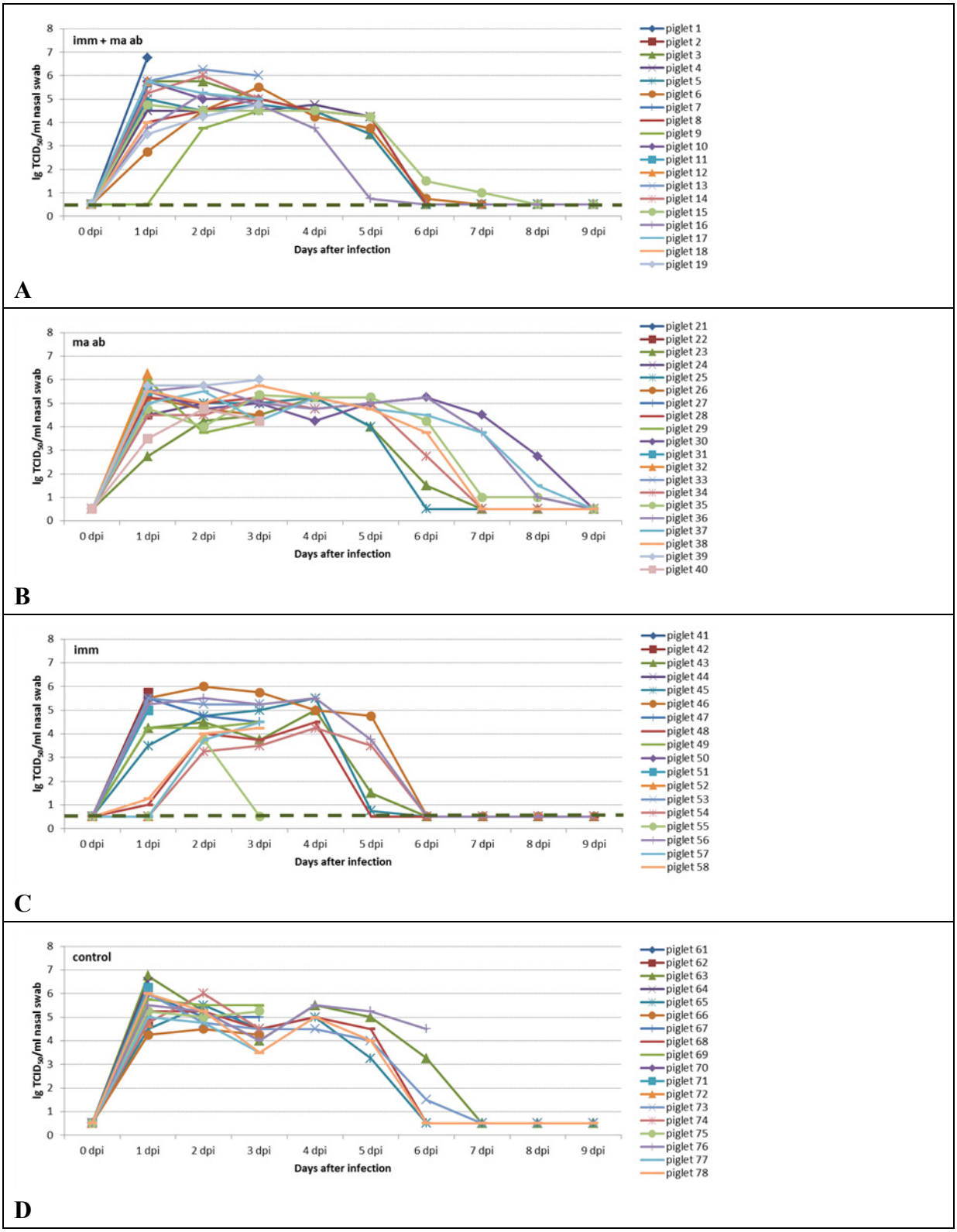


Supplement 81: Investigation of the protective components of whole virus immunisation after aerosol infection with H3N1 virus; individual data of αM2 antibody kinetics; A, H3N2 immunisation (NA, NP and M are related to the infection virus); B, $H1_{pdm}N1$ immunisation (NA is related to the challenge virus); $H1_{pdm}N2$ immunisation (only M is related to the virus used for experimental infection); D, H3N1 homologous immunisation (best protection achievable because all immunogenic components are identical to the challenge virus); E, control (immunisation with placebo, no antigens); F, contact control (infected by direct contact to pigs of the control E); t, pigs that died due to infection; abruptly ending lines, animals were removed from the experiment to analyse the lung samples; note that in group $H1_{pdm}N2$ two pigs died the time around when antibodies appear; 28 dbi, first immunisation of the immunisation groups, 7 dbi, second immunisation of the immunisation groups; dotted line, detection limit

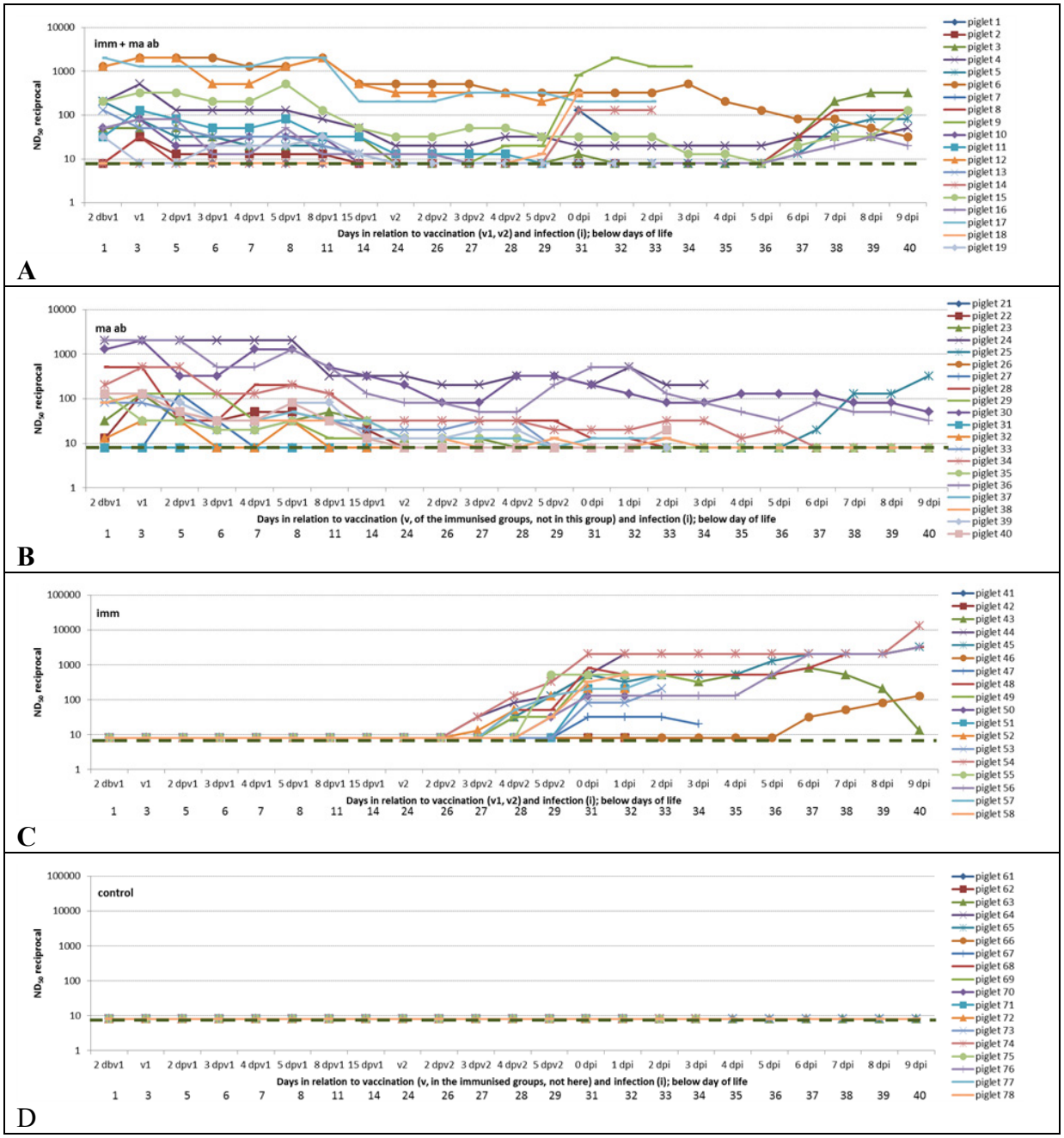


Supplement 82: Detection of bacteria as sign of secondary bacterial infection; A, Overview of pigs whose lungs were investigated for bacteria by staining according to Gram (1 bacteria detected, 0 no bacteria detected by Gram-staining), only one of the pigs was positive; this pig had 100% lung lesions and died 5 dpi, another pig (pig 343) with 100% lung lesions died on 4 dpi and no bacteria were detected indicating that bacteria were not the reason for the strong lung pathology,; B + C, lung of pig 333, HE + Gram staining shows Gram-positive bacteria, arrows; D, lung of pig 333 at larger magnification, HE and Gram staining: Gram-positive cocci (stars) and chopsticks (arrow); E, lung of pig 343 of the same infection group ($H1_{pdm}N2$ group) which died 4 dpi and showed 100% lung lesion despite absence of bacteria; note large numbers of macrophages, neutrophil granulocytes, congestive hyperaemia, fibrinoid-obstructive bronchitis, BALT hyperplasia, alveolar and interstitial edema, eosinophil granulocytes and expanded obstructive-necrotic pneumonia (N = necrosis)

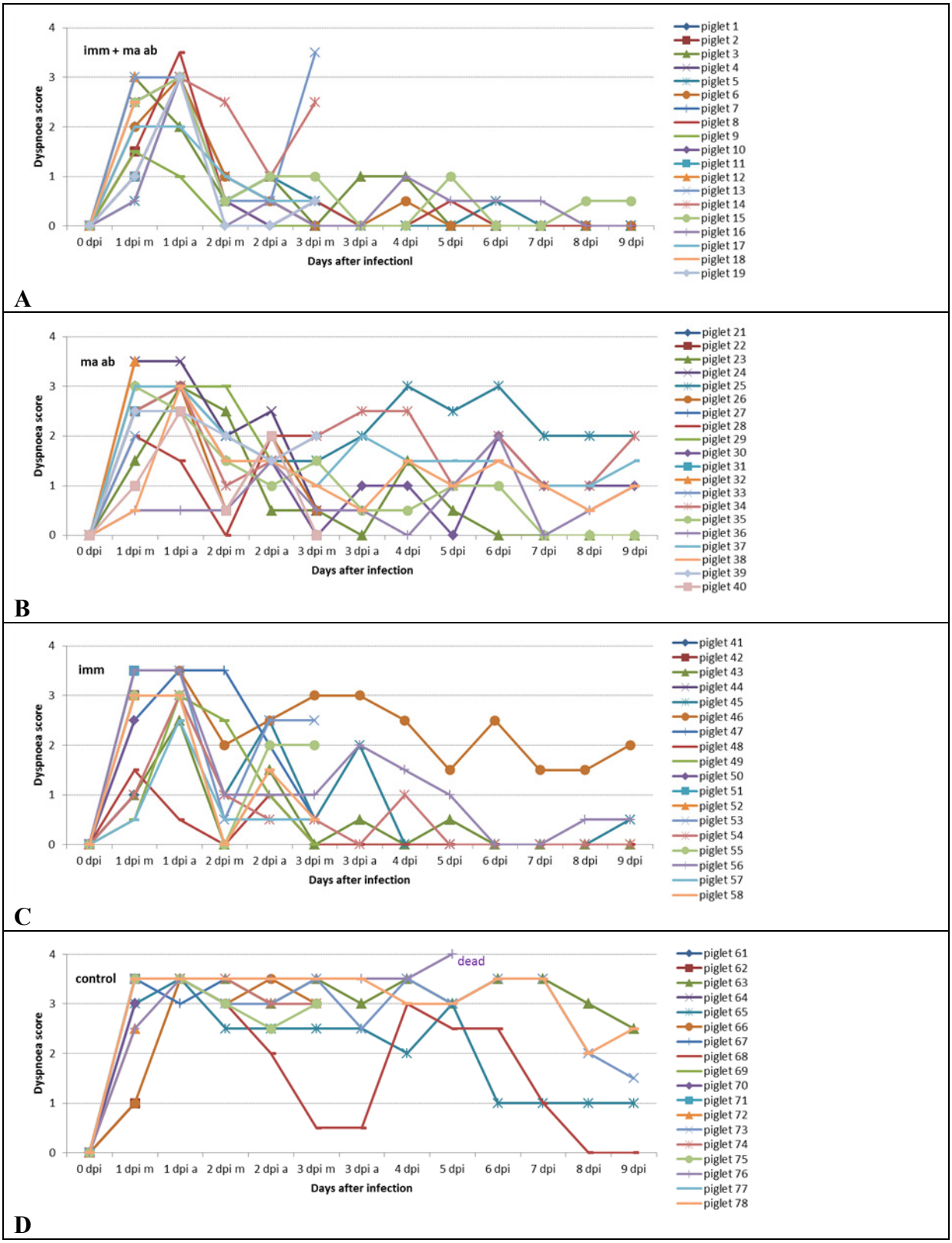
SUPPLEMENT CHAPTER 20 – MATERNAL IMMUNITY



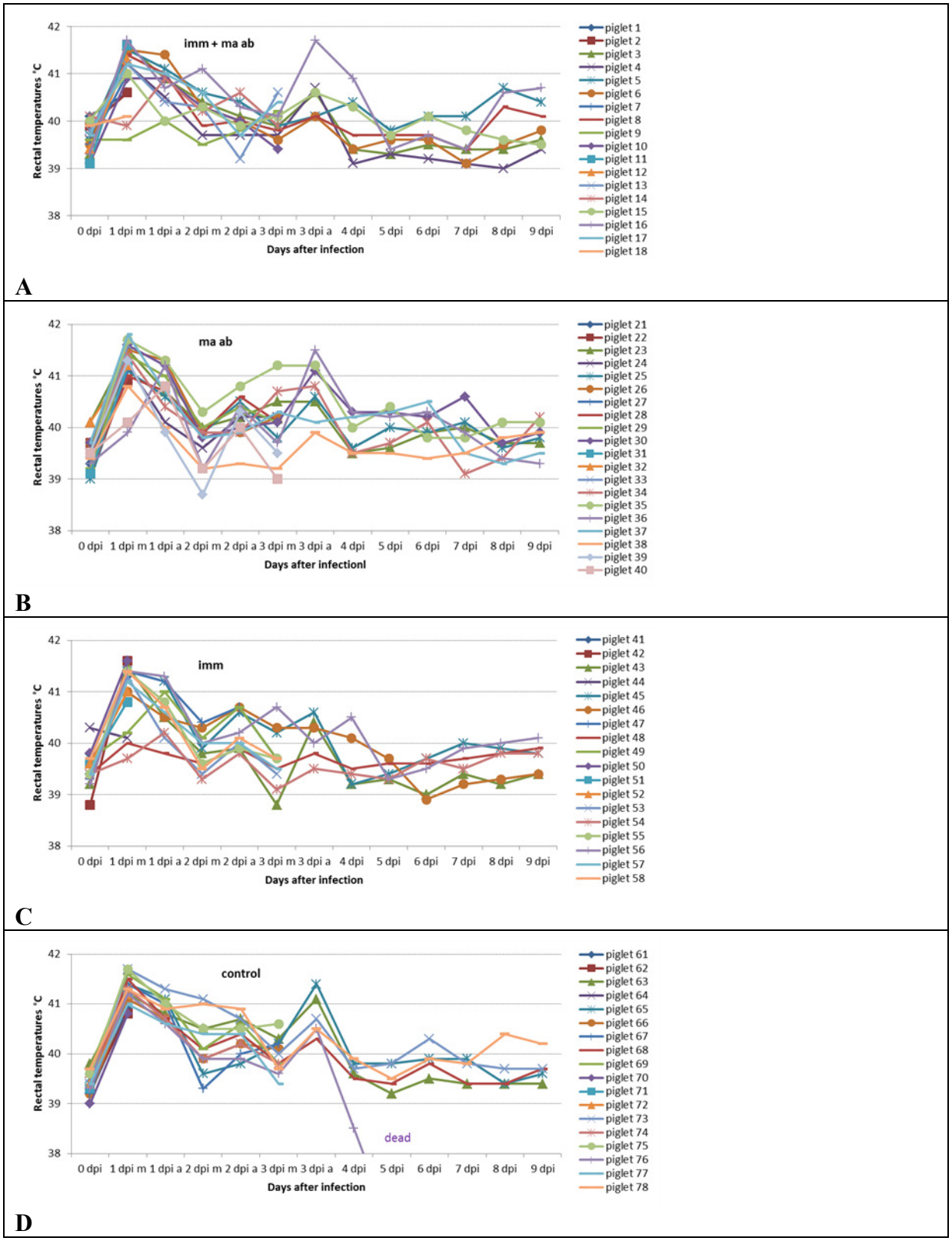
Supplement 83: Individual virus excretion profils of piglets immunised within existing maternal immunity after high-dose infection with A/Hamburg/NY1580/2009 H1$_{pdm}$N1 April 2009 virus (imm + ma ab), with maternally-derived antibodies (ma ab), immunised without maternal immunity (imm) and not immunised without maternally-derived immunity (control); the data show an extension of virus shedding in piglets with maternally-derived antibodies after infection most probably due to an delay caused by locking of blocking factors; this extension in shedding can be overcome by vaccination into maternally-derived immunity; in contrast to older piglets only a few piglets show lower virus excretion indicating that age-dependent factors may influence reduction in virus shedding (maturity of the immune system); see also following supplements



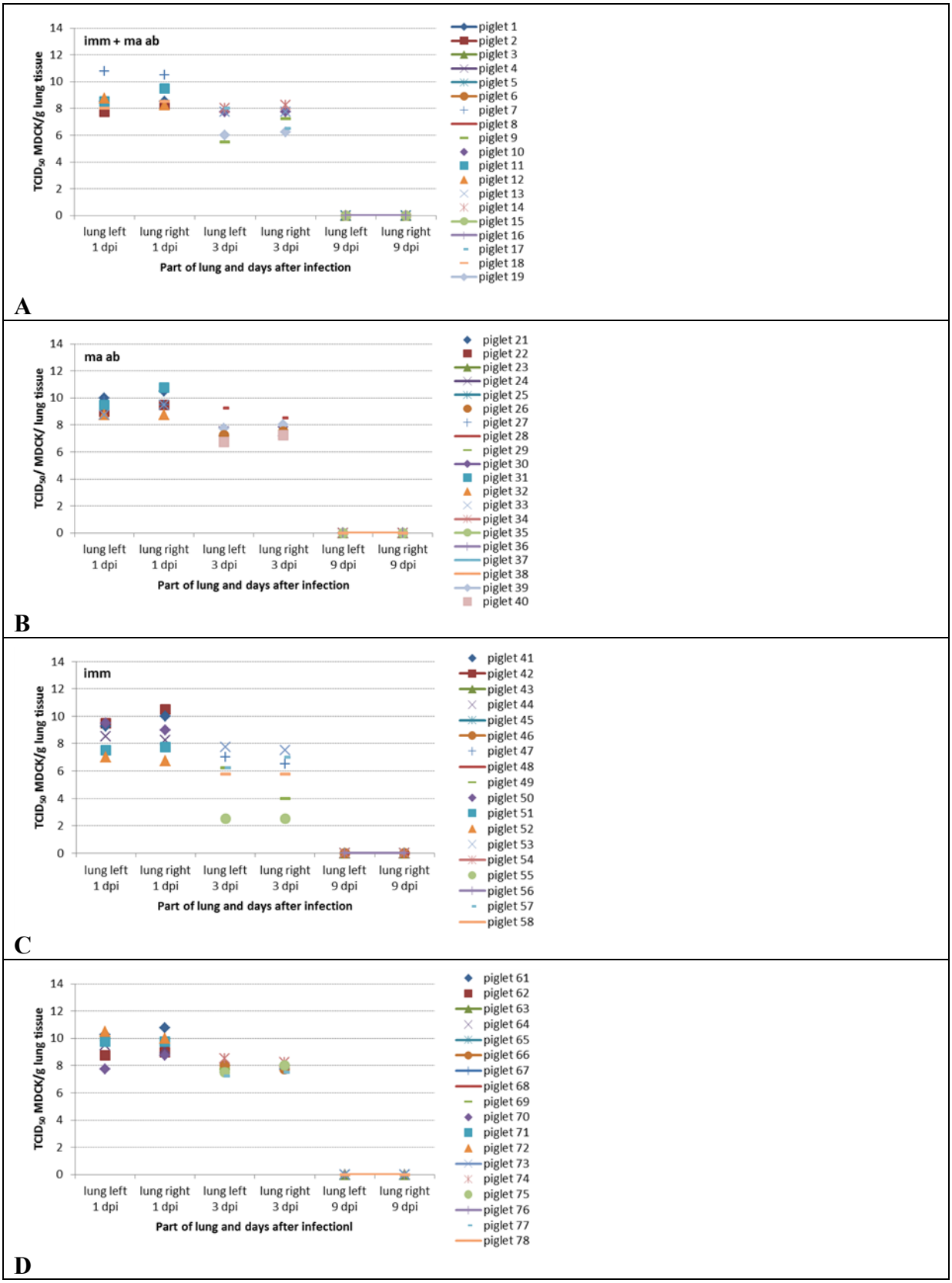
Supplement 84: Individual kinetics of neutralizing antibodies against $H1_{pdm}N1$ virus in piglets immunised within existing maternal immunity after high-dose infection with A/Hamburg/NY1580/2009 $H1_{pdm}N1$ April 2009 virus (imm + ma ab), with maternally-derived antibodies (ma ab), immunised without maternal immunity (imm) and not immunised without maternally-derived immunity (control); after vaccination of sows 5 and 2 weeks before forrowing more than two thirds of the piglets have neutralizing antibodies on day 1 after birth which decrease in the following days; piglets with low or lacking titres of neutralizing antibodies response to second immunisation and or infection by the induction of neutralizing antibodies whereas piglets with high titres of neutralizing antibodies do not respond either; piglets with maternally-derived antibodies do not or only late respond to infection with neutralizing antibodies which can explain the prolonged virus shedding in this group; the response to vaccination and infection is similar to that observed in adult pigs whereas the control group does not show neutralizing antibodies until 9 dpi indicating that response to infection in pigs within their first month of life is impaired in comparison to that of older pigs (in which neutralizing antibodies appear around 5-7 dpi)



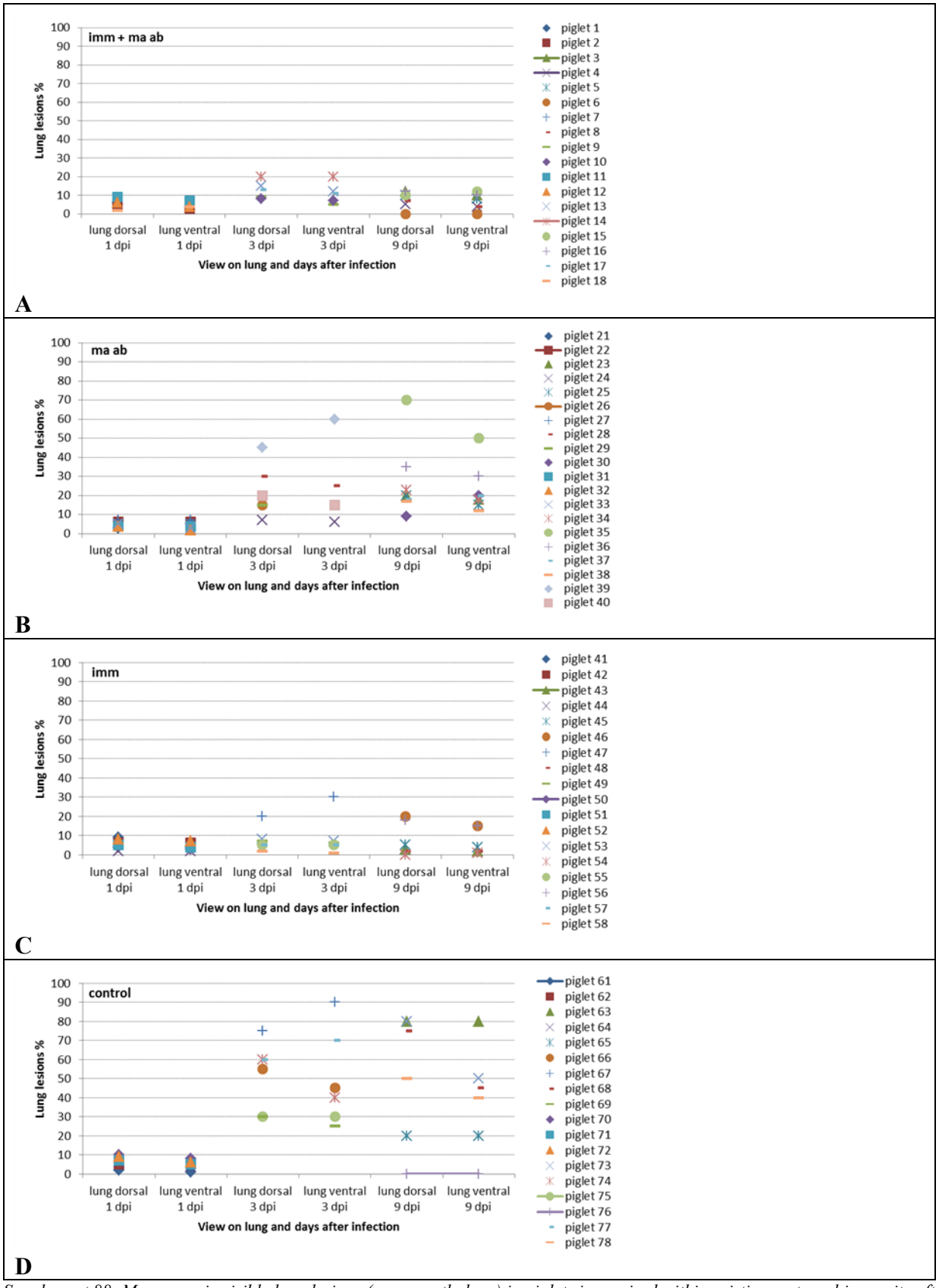
Supplement 85: Individual data of dyspnoea score in piglets immunised within existing maternal immunity after high-dose infection with A/Hamburg/NY1580/2009 H1$_{pdm}$N1 April 2009 virus (imm + ma ab), with maternally-derived antibodies (ma ab), immunised without maternal immunity (imm) and not immunised without maternally-derived immunity (control); because this trial was a high-dose infection approach innate immunity responds to the high viral load immediately installed in the lung after infection by dyspnoea and fever (next page) indicating that under this circumstances short-timed disease cannot prevented by vaccination; looking at the further course of disease in comparison to the control group all other groups were protected with the combination maternally-derived immunity + vaccination performing best; the ending lines stand for piglets that were removed for investigation of lung samples



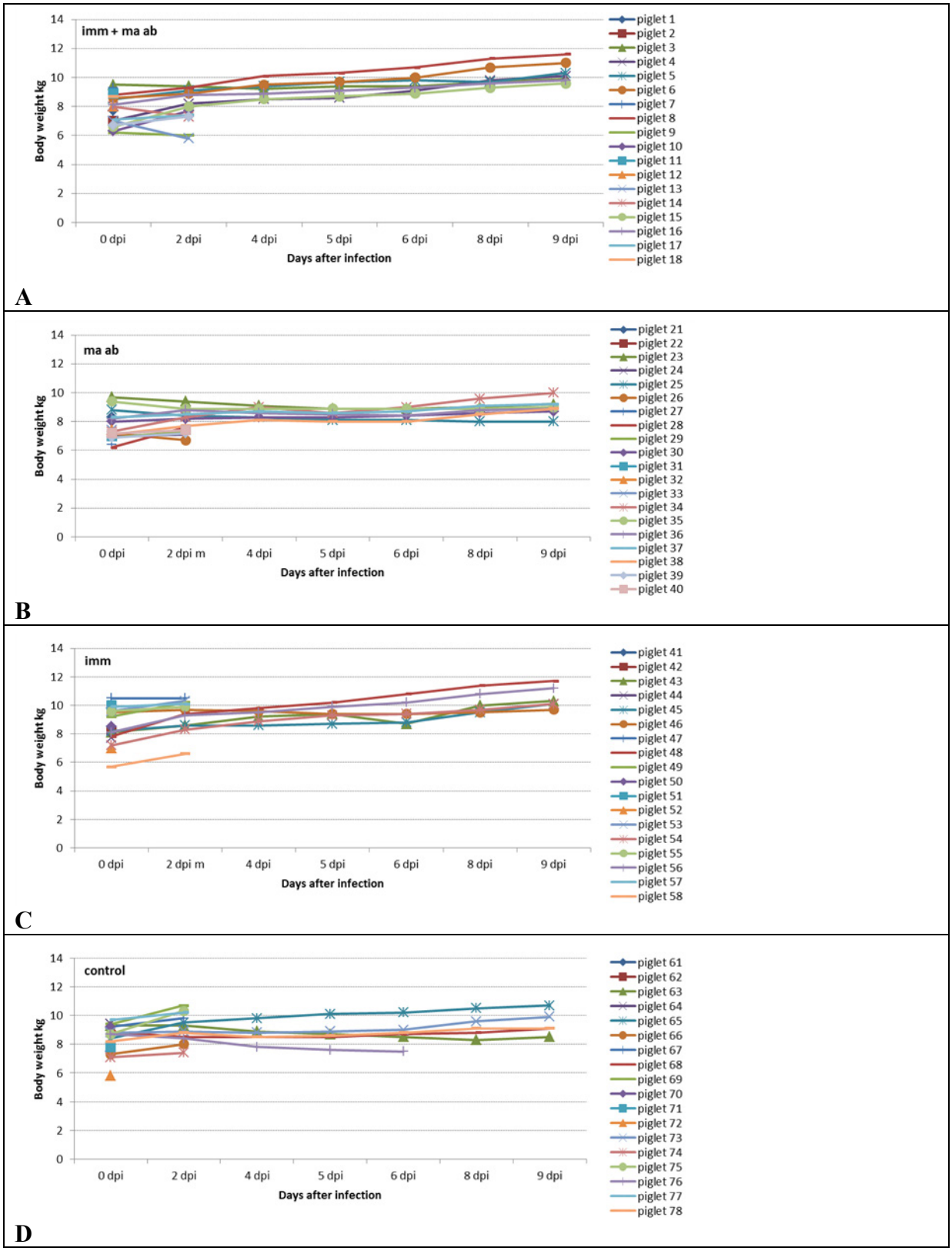
Supplement 86: Individual data of rectal temperatures in piglets immunised within existing maternal immunity after high-dose infection with A/Hamburg/NY1580/2009 H1$_{pdm}$N1 April 2009 virus (imm + ma ab), with maternally-derived antibodies (ma ab), immunised without maternal immunity (imm) and not immunised without maternally-derived immunity (control); because this trial was a high-dose infection approach innate immunity responds to the high viral load immediately installed in the lung after infection by fever (next page) indicating that under these circumstances short-timed disease cannot prevented by vaccination: the immunised group performed best in terms of prevention of the second peak in fever on 3 dpi



Supplement 87: Individual data of viral lung load in piglets immunised within existing maternal immunity after high-dose infection with A/Hamburg/NY1580/2009 H1$_{pdm}$N1 April 2009 virus (imm + ma ab), with maternally-derived antibodies (ma ab), immunised without maternal immunity (imm) and not immunised without maternally-derived immunity (control); due due the high infection dose approach there were no differences in viral lung load on 1 dpi; on 3 dpi the immunised group performed the best; despite no differences in viral lung load between the control group and the groups with maternally-derived immunity the latter were protected clinically from 3 dpi onwards



Supplement 88: Macroscopic visible lung lesions (gross pasthology) in piglets immunised within existing maternal immunity after high-dose infection with NY1580/2009 H1$_{pdm}$N1 April 2009 virus (imm + ma ab), with maternally-derived antibodies (ma ab), immunised without maternal immunity (imm) and not immunised without maternally-derived immunity (control); the data show that there is protection by maternally-derived immunity and vaccination but vaccination is superior in prevention of lung lesions



Supplement 89: Body weights in piglets immunised within existing maternal immunity after high-dose infection with A/Hamburg/NY1580/2009 $H1_{pdm}N1$ April 2009 virus (imm + ma ab), with maternally-derived antibodies (ma ab), immunised without maternal immunity (imm) and not immunised without maternally-derived immunity (control); the data show that there is a short period of stagnation in body weight development in the group with maternally-derived immunity probably due to unlocking the immune blockade; vaccination already in the first weak of their lifes is of benefit to the piglets

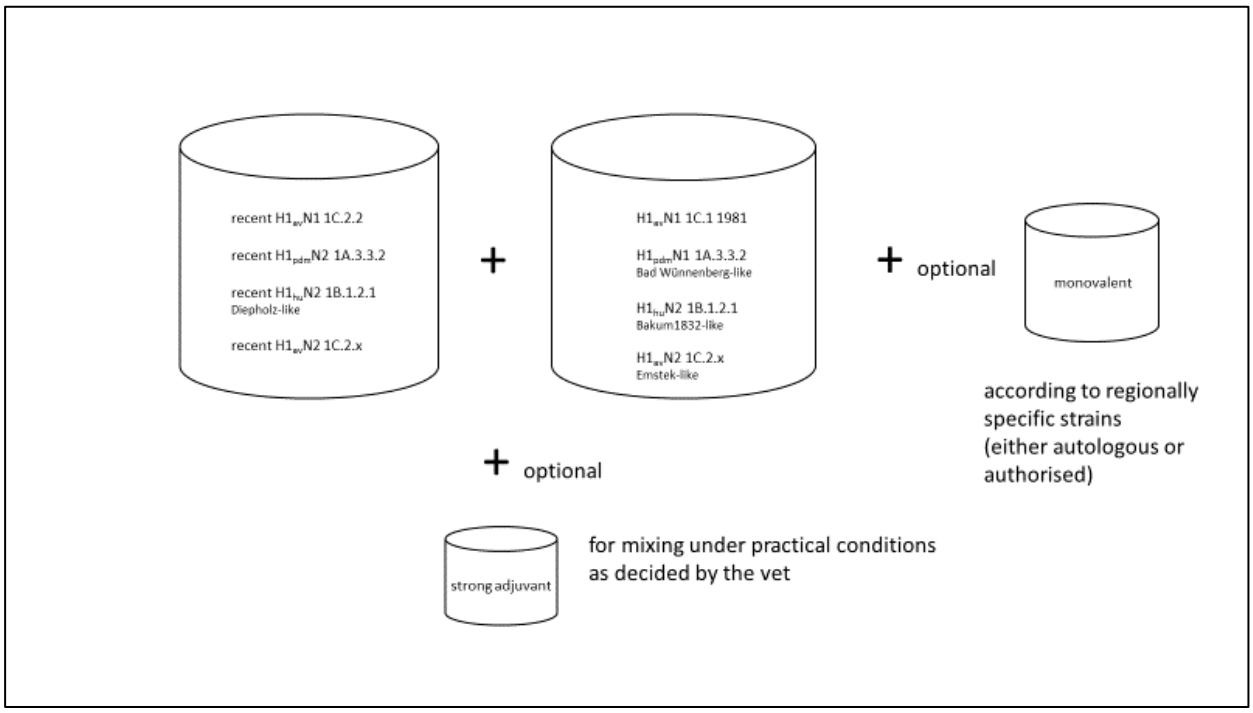
SUPPLEMENT CHAPTER 21 – THOUGHTS ON INFLUENZA AND VACCINATIONS

When I started working with influenza viruses, I always envied those who were able to work with the 1918 influenza virus, which is categorised as an S4 pathogen. In 2009, such a virus came to me in the form of the A(H1N1)pdm09 virus. My colleagues and I were used to the fact that H1N1 viruses do not cause severe symptoms. However, when we carried out the first test on pigs with $H1_{pdm}N1$ 2009 virus, we saw unusually severe symptoms. Influenza viruses are segmented viruses. Unlike C and D viruses, influenza A and B viruses have a division of labour between the two surface proteins in attaching to and detaching from the cell. This has advantages, but also disadvantages. On the one hand, the efficiency of these processes is increased. On the other hand, both proteins have to harmonise with each other. This is not always successful. For example, the combination H3N1 has proven to be incompatible. Although the virus was infectious and was even transmitted between pigs, it was unable to establish stable infection chains. So far, HxN1 has only been successful as an infectious chain in mammals in the H1N1 combination. And here, too, an adaptation phase appears to be necessary. In freshly reassorted viruses, this adaptation is not yet complete and due to the non-optimised division of labour, especially in the process of particle detachment by the neuraminidase, these viruses are more virulent than the viruses that develop from them as a result of adaptation to mammalian cells. There are no genetic markers for this process. This is why viruses of similar virulence are sometimes categorised as S4 pathogens (A(H1N1)pdm1918) and sometimes as S2 pathogens (A(H1N1)pdm2009). It is the stories behind them that are very important for this categorization. The stories were very dramatic for A(H1N1)pdm1918. Yet we don't know much about it. People had other worries at that time. It is difficult to piece together a picture from individual details. It was hoped that the sequence of the 1918 virus would provide a solution. But when this was available, it turned out to be a simple swine influenza virus. With today's knowledge, it must be assumed that it was also a freshly reassorted virus on its way to adaptating, which, in contrast to 2009, unfavourably took its course in the northern hemisphere in autumn 1918. A second disadvantage was the combination of existing immunity to other influenza viruses with a lack of immunity to H1 and N1 in some parts of the human population, which could have triggered an antibody-dependent enhancement of disease. Then there was the post-war situation with many possibilities of infection, the gathering of many people in confined spaces and malnutrition. Experiments on pigs have shown that around 25% of animals are susceptible to severe disease, i.e. in these animals the innate immunity of the lung cells reacts more strongly to the infection than in others. This corresponds to a genetic factor supporting stronger responses of innate immunity that is inherited recessively. It might be similar in humans. However, as shown in this study, deficient cells respond more strongly with an innate immune response. The deficiency could therefore have played an additional role in 1918. We can therefore assume that there will be no serious pandemics with influenza viruses in the near future. What we have to fear more than viruses are people. If the world's population continues to grow and the resulting ecological crisis develops into a major conflict, we can expect infections and pandemics to take on unprecedented proportions.

The years I spent developing swine influenza vaccines were very successful. I led a very small team, but every 7 years a new swine influenza vaccine came on the market. This achievement was never recognised. Comments such as ‘Luck or skill?’, ‘Influenza is easy’, ‘They only developed simple inactivated vaccines’ repeatedly questioned our achievement. What we had really done, however, was to intensively study the basics of swine influenza and the use of vaccines. Thanks to the diagnostic programme I initiated, we had a good overview of the epidemiological situation and a large collection of strains. We were able to select any influenza virus strain for challenge experiments and for the establishment of immune sera and hyperimmune sera. With our own diagnostics, we were able to provide good advice to veterinarians and respond to

changes in the field at any time. We were able to produce large numbers of vaccine laboratory batches in our laboratory. We had a large number of cooperation partners. The vaccine strains of all three swine influenza vaccines were fully sequenced and made available to the scientific community. More than 20 publications have since been released by other groups using our vaccines. In many cases, the sequence information provided was useful. We had a great deal of experience with infection experiments and were able to realistically assess the efficacy of our vaccines. On this basis, we were able to explain to practitioners how to use influenza vaccines to be successful. We were in the process of expanding our diagnostic programme to Europe when other options opened up. The photos and instructions for proper swab collection in pigs that we took in preparation for this programme were then used by others and are still part of CEVA's Influenza Sampling Kit today. By developing the third vaccine, we had completed the first stage of a development process that could have formed the basis for further optimisation of swine influenza vaccinatiom. But the first vaccines were already successful in themselves. Whenever there is economic success, whether in business or politics, management is expanded. More and more people are hired who are supposed to lead, but in fact only restructure, cause meeting inflation, create chaos and keep others from working. That's just the way this world is.

In a next step, I would have developed two non-adjuvanted inactivated influenza virus suspensions that could have been combined with each other as well as with a strong adjuvant at the veterinarian's decision (Supplement 90). This would allow flexibility of use. The integration of different H1 components would have significantly increased the breadth of the immune response. The adjuvant would also have increased the breadth and duration of the immune response, depending on the veterinarian's decision regarding safety for the particular age group. Such vaccines could have been on the market now.



Supplement 90: My proposal for future vaccine development: The combination of two non-adjuvanted suspensions, each containing four inactivated influenza viruses (current and old strains), and the provision of an adjuvant that can be mixed under field conditions should ensure a broad application of influenza vaccination in practice. This approach allows very broad coverage within the H1 group. H3N2 viruses are no longer considered because of their declining importance. However, protection against H3N2 is still possible through the frequent use of HxN2 viruses in vaccines. The application is as follows: first the suspension with the current vaccine strains is applied and then, 2-3 weeks later, the suspension with the older vaccine strains. The third vaccination is again carried out with the suspension containing the current vaccine strains. Pregnant sows and pig herds with health problems receive the suspension without adjuvant, for all others the adjuvant is added. The third vaccination is given after 3 months if vaccines without adjuvant were used, otherwise after 6 months (for vaccines with a strong adjuvant). Depending on regional characteristics, this vaccine could be combined with regional monovalent vaccines to increase effectiveness

After that, I would have turned to the development of haemagglutinin vector vaccines based on modified vaccinia virus Ankara. The use of a vaccine that can also be used as a paraimmunity inducer would be of great advantage and would allow for a wide range of applications. But these have remained only theoretical approaches because that's just the way this world is.

In my opinion, it is not necessary to kill animals and examine lungs for the development of swine influenza vaccines. Simply examining antibodies is sufficient. By using 30 pigs in immunisation trials, I could generate all the information I need. After each application, I would take the temperature every 2 hours within the first 24 hours after vaccine administration, check the injection site and monitor the general condition of the animals. This would give me all the safety information I need. I would take blood samples and determine the antibody levels 10 days after each vaccine application. I would administer the second vaccine dose 2-3 weeks after the first. After that, I would divide the animals into 6 groups and vaccinate 5 pigs in each group a third time at monthly intervals after the second vaccination. I would take blood samples 10 days after each vaccination and determine the antibodies. As long as there is interference between the third vaccination and the second (i.e. there is no effect, i.e. no increase in antibodies from the third vaccination compared to the second), the vaccine virus is neutralised, and this is how long immunity lasts. By using a wide range of influenza virus strains in serological investigations of the sera obtained, the spectrum of coverage provided by the vaccine could be explored. Then I would have all the information I need. This can be applied and tested under practical conditions at any time.

Even in human medicine, there are still misconceptions about the possibilities of influenza vaccinations. The nature of the immune system does not give us much leeway in developing vaccines that provide lasting protection against infection. In principle, the existing vaccines are good. The key is to improve and optimise the use of vaccines. The effectiveness of vaccines (VE), which is regularly calculated for human vaccines, is often misinterpreted. VE is used to determine the success rate of vaccine use. VE reflects the proportion of potentially exposed individuals protected from symptomatic influenza after vaccination in comparison from potentially exposed unvaccinated. Influencing factors are the vaccine (composition of vaccine strains), the timing of vaccination (timing in relation to the influenza wave, vaccination coverage), and the strength of the wave, since the probability of exposure to high viral loads is significantly higher (during stronger circulations, there is a higher probability of high exposure, which can undermine the immune system's response time). Based on the calculations of vaccine effectiveness (VE), it is believed that conclusions can be drawn about the vaccines. However, this is not that simple. VE also reflects the result of its application. It definitely does not allow any conclusions to be drawn about the accuracy of fit of the vaccine strains. Indeed, in most cases vaccine viruses fit well (https://www.rki.de/DE/Themen/Forschung-und-Forschungsdaten/Nationale-Referenzzentren-und-Konsiliarlabore/Influenza/zirkulierende/VirolAnalysen_2024_25.html?nn=16778680). A VE of 43% only indicates that in the examined population, 43% of those vaccinated were protected against clinical influenza. This can also happen with the best vaccine, which fits 100%. When evaluating the results, one must always take into account the high initial replication rate of the influenza viruses and the kinetics of the immune response. Based on both kinetics, one can conclude that in order to achieve good results, the vaccination must be administered as close as possible to the influenza wave in the corresponding year. If you want to interrupt the chain of infection, you have to vaccinate large parts of the population at the same time. You could achieve great success against influenza if you vaccinated all schoolchildren at the end of December/beginning of January. This would have a huge impact on the epidemiology of influenza and could even break the wave in the corresponding influenza season. Surprisingly, mankind always ignores simple solutions and prefers to look for complicated solutions at great expense. Therefore, the potential that could be achieved is not realised. Simple solutions would be feasible, but that’s ...

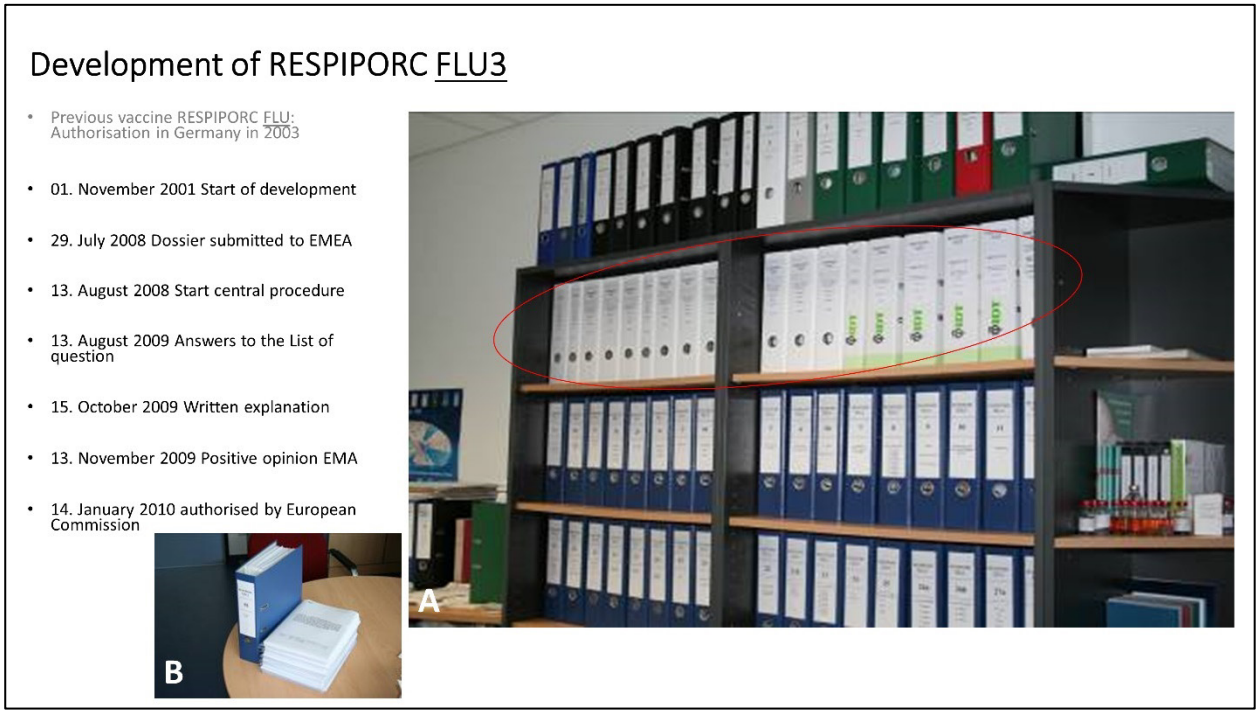
SUPPLEMENT CHAPTER 22 – SCIENTIFIC BACKGROUND OF THE AUTHOR

After completing my diploma thesis (internal medicine)[512] at the veterinary faculty of the university of Leipzig and working at the veterinary clinic of the Institute for Veterinary Medicine in Neubrandenburg for three months, I started my work in the field of virology in December 1990 at the Institute for Microbiology and Animal Epidemic Diseases at the Faculty of Veterinary Medicine in Leipzig under the direction of Prof. Dr. Dr. Heinrich Liebermann. My dissertation was to deal with Borna disease viruses, since the area around Leipzig was known to be an endemic area and a collaboration had been established with Prof. Dr. Hanns Ludwig at the Institute of Virology at the Free University of Berlin. The topic was relatively open and I first had to give it form. A first step was to organise the delivery of diseased animals to the Clinic for Internal Medicine at the faculty for clinical examination. If the pathology section books for the years 1991 to 1994 show a larger amount of material received from animals that died of Borna disease, this is not a sign of increased prevalence of the infection, but a result of my activities at the time. With the materials I collected, we established the first PCRs in Berlin to detect the pathogen[513]. At that time, due to the political changes, large numbers of sheep flocks were culled and I travelled a lot in the endemic areas to take blood samples before these animals were no longer available. Furthermore, I initiated serological studies after vaccination with the live Borna vaccine 'Dessau', which was still in use at the time. Through my contacts at the state investigation offices, I was able to collect data on the occurrence of the disease in the federal states of Saxony, Thuringia and Saxony-Anhalt.

In 1993, I summarised the results of my research in a dissertation and completed my doctorate two and a half years after starting the work[514]. After a year of working as a veterinarian in a cattle practice in Mittelfranken, during which time I continued to deal with Borna virus infections in practice[515], I was a research assistant at the Institute of Virology at the Free University of Berlin from 1994 to 1997. During this time, I conducted molecular biological, serological, virological and animal experiments on Borna virus infection and was able to acquire extensive knowledge in virological methods; I was head of animal husbandry at the institute. During a guest stay at the Scripps Research Institute La Jolla in California in 1994, I carried out sequencing of Borna viruses (sequencing at that time was still done using radioactive probes) in the research group of Juan Carlos de la Torre at the institute of Mike Oldstone[516]. Increasing differences in the interpretation of research results in the Berlin group led me to leave the institute in August 1997. In conclusion, I published a review article; over the years, I had collected all available literature on bornavirus infection and was able to make it available to the English-speaking world with this publication[517].

In the following 20 years, I was involved in the development of vaccines at the Impfstoffwerk Dessau-Tornau in the research department headed by Prof. Dr. Hans-Joachim Selbitz. That was a very successful period, during which, in addition to numerous other projects under my professional direction, three vaccines for the prophylaxis of swine influenza were developed and approved (2003 RESPIPORC® FLU, 2010 RESPIPORC® FLU3, 2017 RESPIPORC® FLUpan H1N1)[86,88,495,518]. The RESPIPORC® FLU3 vaccine successfully passed the company's first centralised approval procedure at the European Medicines Agency (EMEA/EMA) and was approved simultaneously in 29 countries in 2010. Supplement 91 gives an impression of the scale of the investigations. All vaccines were produced in cell cultures and the production was continuously optimised[496]. RESPIPORC® FLU3 contained the novel H1N2 subtype, which had recently emerged in the pig population, and a tolerable adjuvant that enabled the introduction of sow vaccination on a broad basis. We obtained the vaccine strain precursors from cooperation partners or established them from our own diagnostic programme. The vaccines RESPIPORC® FLU3 and RESPIPORC® FLUpan H1N1 are still on the market today and are very successful economically (they are among the top ten products of

the current marketing authorisation holder CEVA; at the previous marketing authorisation holder, IDT Biologika, RESPIPORC® FLU3 was the veterinary product with the highest turnover).



Supplement 91: Development of RESPIPORC FLU3

During 19 years and 4 months I contributed to the development of 3 influenza virus vaccines; the central vaccine RESPIPORC® FLU3 was concepted by me and accompanied by me from development, authorization to marketing; A, view of the registration documentation of this vaccine in my former office (above: white folders, 13 volumes dossier, 5 volumes answers to EMEA list of questions, 1 volume list of outstanding issues; below: 58 folders with data collected during project development; B, Answers to the major questions; timeline: I consider the handover of the first German H1N2 isolate to me by Dir. and Prof. Dr. Jochen Süss at the Coswig motorway exit on 1 November 2001 to be the beginning of vaccine development; on 14 January 2010, the product was authorised by the European Commission; without the experience gained from the previous development of RESPIPORC® FLU, the project would not have been able to be implemented so quickly; when I joined the company Impfstoffwerk Dessau-Tornau GmbH in 1997, there were just over 250 employees; the small structures had the advantage that we were able to work on all areas of vaccine development ourselves, which is no longer the case in larger companies with their specialisations, but also the disadvantage of high workloads. For example, from March to July 2009, I worked every weekend working on the List of Questions; in addition, we had a former university professor as our research director, Prof. Dr. Selbitz, who gave us the freedom to do scientific work, which was essential for the international success of the projects and ultimately made the company a leader in the field of swine influenza prophylaxis, as well as other projects

I have accompanied these product developments with numerous scientific investigations. For example, I initiated a programme for the surveillance of swine influenza in 2003, which I maintained until 2015. The research group I lead (1 scientist, 6 laboratory technicians) offered veterinarians and farmers free diagnostic testing of swab and blood samples. These activities enabled me to participate in numerous research networks such as FLURESEARCHNET and ESNIP and to acquire research funding[519-521]. Numerous publications have been produced in collaboration with the Institute for Virology and Antiviral Therapy at the Friedrich Schiller University Jena (Prof. Dr. Michaela Schmidtke, Prof. Dr. Roland Zell, Prof. Dr. Peter Wutzler)[114,339,344-348,358,497,522]. Further collaborations have been established with Prof. Dr. Georg Herrler and Prof. Dr. Ludwig Haas at the University of Veterinary Medicine Hannover[287,401,523]. Joint projects were also carried out with the working groups of Prof. Dr. Peter M. H. Heegaard and Prof. Dr. Lars E. Larsen at the University of Copenhagen[355,356,524], with Prof. Dr. Kristin Van Reeth, Ghent University, Belgium[519], and with the Institute of Immunology of Prof. Dr. Saalmüller at the University of Veterinary Medicine Vienna[138,139]. In addition to my work on influenza vaccines, I worked on or supervised other research priorities (e.g. BVDV DNA vaccines for cattle, PMV-1+Salmonella combination vaccine for pigeons, investigation of the compatibility of adjuvants with PRRSV, parvovirus vaccine vaccine for pigs with the establishment

of an infection model, combination vaccine PPV/influenza/erysipelas for pigs with comprehensive studies on the duration of immunity in the infection model erysipelas; I had a contract to carry out quality tests for in-process controls and end product testing of veterinary products).

During all these years, I also continued my research on Borna disease. In collaboration with the research group of Prof. Dr. Norbert Nowotny at the Institute of Virology at the University of Veterinary Medicine in Vienna, we were able to show for the first time in 2005 that Borna viruses cluster according to their regional origin[525-529]. The cluster nomenclature I established in 2014[529] is still in use today[530]. After the detection of bornaviruses in shrews in Switzerland, I conducted extensive research on the occurrence of bornaviruses in shrews in Germany[529]. In addition, we were able to detect new hantaviruses (now known as Seewis virus) in wood shrews from the vicinity of Magdeburg, but another research group had just beaten us to it and our manuscript was not accepted. Since 2015, I have been a member of the Borna Virus Study Group of the ICTV (International Committee on Virus Taxonomy)[531]. I had already been involved in the group in 2014 and we had thoroughly revised the taxonomy of Borna viruses and defined new taxa[532], as well as editing the section on Borna viruses for the textbook Clinical Virology[533]. Further investigations dealt with the occurrence of the infection in new world camelids[534]. In 2022, I retrospectively summarised my work on the immunoprophylaxis of Borna disease in a publication[535]. The investigations indicate that vaccinations are successful when vaccine compositions containing the glycoprotein are used.

After the outsourcing of the swine influenza diagnostic programme, and later its discontinuation, scientific work in the company, for which I had successfully worked for almost 20 years, was no longer possible. In addition, the further development of influenza vaccines was deprived of an essential basis. Therefore, I reoriented myself in 2017. After a short period of employment at Micromun in Greifswald, I moved to the Robert Koch Institute in Berlin in June 2017. Since August 2018, I have been head of the National Reference Centre for Influenza Viruses (6 research assistants, 14 technical assistants) and deputy head of Unit 17, Influenza and other respiratory viruses. My focus is now on human respiratory viruses in close collaboration with ECDC and WHO[536-541]. I act as Operational Focal Point Influenza (Virology) Germany. The majority of the laboratory diagnostic methods are accredited according to both DIN EN ISO/IEC 17025 and DIN EN ISO 15189 and are regularly assessed by the Deutsche Akkreditierungsstelle. Furthermore, I am working on the zoonotic potential of swine influenza viruses[542,543]. Working at the Robert Koch Institute presented me with two challenges: the severe B/Yamagata influenza wave of 2017/18[544] and the COVID-19 pandemic of 2020-2023[430,523,545-550]. Due to these challenges, I had to interrupt my work on the completion of my habilitation thesis several times.

Since 1990, I have been involved in teaching in the field of virology. I have given individual lectures in both Leipzig and Berlin and participated in every virology course from 1991-1993 and 1994-1997. I also sat on the annual exams at the Free University of Berlin. Since 2012, I have been giving lectures at the University of Veterinary Medicine Hannover. For many years, I offered the elective course on vaccine development in consultation with Prof. Dr. Ludwig Haas. This took place at the Impfstoffwerk Dessau-Tornau. In addition to learning the basics of vaccine development, students were able to produce laboratory samples of vaccines in the laboratory and then apply them to pigs in the animal husbandry. The course has been consistently rated very highly by students. In 2016, I completed a university didactics training course at the University of Jena, which was also connected with teaching (at the Institute of Virology and Antiviral Therapy). During my time at the Free University of Berlin, I supervised doctoral students. In the working group I led in Dessau-Tornau, two graduates of the Anhalt University of Applied Sciences were able to successfully carry out their diploma theses. In 1998 I became a specialist veterinarian in virology. In 2006 I applied for authorisation to provide further training in this field and was able to successfully train my colleague. I myself have also participated in the examination of colleagues from the veterinary associations of Saxony-Anhalt and Lower Saxony for the qualification as a specialist in virology.

Vaccine developments

2003 RESPIPORC® FLU, inactivated bivalent ($H1_{av}N1$+H3N2) o/w vaccine for pigs

- First cell culture-based swine influenza vaccine
- National authorisation in Germany (licence no. PEI.V. 02317.01.1)
- Co-authorisation as Ingelvac® Flu (License no. PEI.V. 03016.01.1)
- Own contribution to the work: continuation of a project that had already been started: optimisation of production in cell culture, tolerability studies, proof of induction of fever after application of mineral oil, conception, organisation, implementation and evaluation of all efficacy studies, evaluation of field trials, significant collaboration in the preparation of the dossier, answering the Paul Ehrlich Institute's list of questions, project management up to approval, presentations for market launch

2010 RESPIPORC® FLU3, inactivated trivalent ($H1_{hu}N2$+$H1_{av}N1$+H3N2) vaccine for pigs

- First European swine influenza vaccine with subtype H1N2, tolerated adjuvant and improved cell culture production technology
- Central marketing authorisation, dossier submitted to the EMEA on 29 July 2008, authorisation by the European Commission on 14.01.2010, EU/2/09/103/ - EMEA/V/C/153 (co-authorisation as GRIPOVAC® 3, EU/2/09/102 - EMEA/V/C/157)
- Own contribution: Idea, concept and implementation of the concept; selection of vaccine strains and preparation of the production of Master Seed Virus; scientific management of the project until approval; pharmaceutical development; further optimisation of production in cell culture; validation of all virological and serological methods for in-process controls and final product testing including preparation of validation reports; design, organisation, conduct and evaluation of all efficacy studies: Dosistitration, onset of immunity, duration of immunity, efficacy after revaccination; presentation of the vaccine at the presubmission meeting of the EMEA in London; significant contributions to the preparation of the dossier and to answering the list of questions and list of outstanding issues; first successful centralised procedure of the company with approval in 29 countries; presentation of data at national and international conferences

2017 RESPIPORC® FLUpan H1N1, inactivated monovalent ($H1_{pdm}N1$) vaccine for pigs

- Centralised authorisation, dossier submitted to the EMA on 2 October 2015, authorisation by the European Commission on 17 May 2017 (EU/2/17/209/001-002)
- Own contribution: Concept; selection of the vaccine strain and adaptation to cell culture; project management until the EMA's response to the answers to the list of questions; pharmaceutical development; validation of all virological and serological methods for in-process controls and end product testing including preparation of validation reports; design, organisation, conduct and evaluation of all efficacy studies: Dosistitration, onset of immunity, duration of immunity; presentation of the vaccine at the EMA presubmission meeting in London; significant contributions to the preparation of the dossier and to the response to the list of questions and to the EMA's response to the list of questions; then change of job and workplace within the ongoing procedure

ABBREVIATIONS

α	anti
a	afternoon
aa	amino acids
AB	antibody
ADCC	antibody-dependent cell cytotoxity
ADE	antibody-dependent enhancement of disease
AG	antigen
ANP32	acidic nuclear phosphoprotein 32
AU	antibody units
A(H1N1)v	variant H1N1 influenza virus, not identical to seasonal viruses (zoonotic transmission)
BAEE	N-benzoyl-L-arginine ethyl ester (Trypsin hydrolyzes BAEE; one BAEE unit of trypsin is the amount of enzyme causing an increase in absorbance of 0.001 per minute at 25ºC and 253 nm)
BALT	bronchus-associated lymphoid tissue
BTN3A 3	butyrophilin subfamily 3 member A3
Cf	correction factor
CIC	circulating immune complex
COVID-19	Coronavirus disease caused by SARS-CoV-2
CPE	cytopathic effect
cRNA	complementary RNA
DALY	disability-adjusted life years
DI	disease index
dbi	days before infection
dbv1	days before first vaccination
dbimm	days before immunisation
dpi	days after infection (days *post infectionem*)
dpimm	days after immunisation
dpv1	days after first vaccination
dpv2	days after second vaccination
DSG	Dextrose – Sucrose – Glutamate solution
EA	European Asian (referring to the swine H1N1 lineage)
EID_{50}	egg infectious dose 50
EMA	European Medicines Agency (formerly EMEA)
ERD	enhanced respiratory disease
Erys	Erythrocytes control
FADD	Fas-associated protein with death domain
FFU	focus formin units
FLUAV	Influenza A virus
FLUBV	Influenza B virus
FLUCV	Influenza C virus
FLUDV	Influenza D virus
G1/G2 H1N2	genotype 1 of French H1N2 virus/ genotype 2 of French H1N2 virus
GLP	Good Laboratory Praxis
GDR	German Democratic Republic
GMHIU	geometric mean of hemagglutination inhibiting units
GMNIU	geometric mean of neuraminidase inhibiting units
GMNU	geometric mean of neutralizing units
H1, H2. H3	hemaggluinin types 1, 2, 3
$H1_{av}N1$	porcine avian-like H1N1 influenza A virus ($H1_{av}N1_{av}$)
$H1_{cl}N1$	classical swine H1N1 influenza A virus ($H1_{cl}N1cl$)
$H1_{hu}N2$	porcine human-like H1N2 virus ($H1_{hu}N1_{av}$)
$H1_{pdm}N1$	pandemic H1N1 2009 virus ($H1_{pdm}N1_{pdm}$), A(H1N1)pdm09
$H1_{pdm}N2$	porcine pandemic H1N2 virus ($H1_{pdm}N2_{hu}$)
H3N2	porcine human-like H3N2 virus ($H3_{hu}N2_{hu}$)
HA	haemagglutinin
HAT	human airway trypsin-like protease
HE	hematoxylin and eosin staining

HI	hemagglutination inhibition assay
HIU	hemagglutination inhibiting units
HKU1	endemic coronavirus HKU1
HMPV	Human metapneumovirus
Hp	Haemophilus parasuis
HPAIV	highly virulent (pathogenic) avianm influenza viruses
Hpi	hours after infection (hours *post infectionem*)
HRV	Human rhinovirus
HU	hemagglutinating units
HyImSera	hyper immune sera
HxNx	this stands for different possibilities of combinations of HA and NA
IFTMs	Interferon-induced transmembrane proteins
IHC	immunohistochemistry
ICG	internal cassette of genes (means genes other than HA and NA)
ID	infectious dose
IF	immunofluorescent; immunofluorescence assay
IFN	interferon
IFR7	interferon regulatory factor 7
IFTM	interferon-induced transmembrane protein
II	Imbalance in immunity against variable and conserved structures of FLUAV
IL-1 / IL-6	interleukin-1 / interleukin-6
Imm	immunisation
Imm2-1	second immunisation – 1 week (one week before second immunisation
Imm + ma ab	immunisation into maternally immunity (immunization + maternal antibodies)
ImSera	immune sera
i.o.	in order
IOG	Imbalance of the functional interaction of gene segments of newly reasserted viruses
kD	kilo Dalton
l	midday (lunchtime)
LPAIV	low virulent (pathogenic) avian influenza viruses
m	morning
MA-104	kidney cell line from African green monkeys
ma ab	maternally-derived antibodies
MALT	mucosa associated lymphoid tissue
MAPK	mitogen-activated protein kinase
mat imm	maternal immunity, maternally-derived immunity
MDBK	Madin Darby Bovine Kidney cell line
MDCK	Madin Darby Canine Kidney cell line (MDCK-2)
MHC-II	major histocompatibility class II
MLKL	mixed lineage kinase domain-like pseudokinase
mpv	months *post vaccinationem*
moi	multiplicity of infection
mRNA	messenger RNA
M(P)	matrix protein
MUNANA	2'-(4-methylumbelliferyl)-α-d-N-acetylneuraminic acid
Mx	orthomyxovirus resistence gene
MxA	orthomyxovirus resistence gene A
Mx1	orthomyxovirus resistence gene 1
MVA	modified vaccina virus Ankara
n	noon
N1, N2	neuraminidase 1, neuraminidase 2
na	not available
NA	neuraminidase
n.d.	not done
ND_{50}	neutralisation dose 50
NEP	nuclear export protein
NF-κB	Transkritionsfaktor, nuclear factor „kappa-light-chain-enhancer" of activated B cells
n.i.	not investigated
NI	neuraminidase inhibition assay
NIC	National Influenza Centre
NP	nucleoprotein
NRLs	nucleotide-binding domain and leucine-rich-repeat-containing proteins
NS	nonstructural protein
n.s.	not significant ($p>0.05$)
NT	neutralisation test

nt	nucleotides
OAS	2'-5' oligoadenylate synthetase
OC43	Endemic coronavirus OC43
OD	optical density
OL	Oligodendroglia cells (human origin)
p	statistical probability (levels of significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)
ps	passage
PA	polymerase PA (polymerase acidic protein)
PAMPs	pathogen-associated molecular patterns
PAS	Periodic Acid-Schiff staining
PB1	polymerase PB1 (polymerase basic protein 1)
PB2	polymerase PB2 (polymerase basic protein 2)
PFU	plague forming units
PI3K	phosphatidylinositol-3-kinase
Pig ID	pig identification (ear tag)
PIV	Parainfluenzavirus
PKR	protein kinase R
Pm	Pasteurella multocida
pNPP	para-nitrophenylphosphate
PRRs	pattern recognition receptors
PRRSV	Porcine reproductive and respiratory syndrome virus
ps	passage
qPCR	real time polymerase chain reaction
reH1N1	reassortant H1N1 virus
RIG-I	retinoic acid inducible helicase
RIPK3	receptor-interacting serine threonine-protein kinase 3
RKI	Robert Koch-Institut
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RSV	Respiratory syncytial virus
RT-PCR	reverse transcriptase – polymerase chain reaction
SABC	StreptAvidin-Biotin Peroxidase Complex staining
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SPC	Specific product characteristics
SP-D	Surfactant protein D
ssRNA	single stranded RNA
sw	swine
TBS-T	Tris-buffered saline – Tween 20
$TCID_{50}$	tissue culture infectious dose 50 determined in MDBK cells (also $TCID_{50}$ MDBK)
$TCID_{50}$ MDCK	tissue culture infectious dose 50 determined in MDCK cells
TMPRSS	transmembrane serin-like protease
TNF-α	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TLR	Toll-like receptors
tripleUS	triple reassortant USA swine influenza viruses
TTSP	type II transmembrane serine proteases
unvacc	not vaccinated
US	United States of America
v1	first vaccination
v2	second vaccination
VAERD	Vaccine-induced enhancement of respiratory disease
vacc	vaccination (pvacc, after vaccination = *post vaccinationem*)
VE	vaccine effectivenes
vRNP	viral ribonucleicproteins
vRNA	viral ribonucleic acid
vs.	versus
wpi	weeks after infection (weeks *post infectionem*)
wpv	weeks *post vaccinationem*
YLD	numbers of years lost due to disability
YLL	life years lost due to premature death
ZBP1	Z-form nucleic acid binding protein 1 (DNA activator of proteins)
Ø	negative

The list of abbreviations is valid for both volumes of the monograph.