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].