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Analyses of immune competence traits and their association with candidate genes in pigs

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Dedication

"To Remember My Beloved Mother"

Analysen von Merkmalen für die Immunkompetenz und ihre Assoziation mit Kandidatengenen bei Schweinen

Die vorliegende Arbeit wurde zur Untersuchung der Immunkompetenz in einer Duroc× Berliner Miniatur Schwein (DUMI) Rückkreuzungspopulation durchgeführt. Die Komplementaktivität über den klassischen und den alternativen Signalweg wurde mit dem hämolytischen Aktivitätsansatz ermittelt. Antikörperantworten zu *Mycoplasma hyopneumoniae*, Tetanus Toxoid und PRRS Virus wurden mit einem Enzyme-Linked Immunosorbent Assay (ELISA) gemessen. Darüber hinaus wurden die Komplementkomponenten C3c und Haptoglobin, wichtige Parameter der Immunantwort bei Tieren, bestimmt. Diese Parameter wurden im Folgenden als Phänotypen für die Kopplungskartierung zur Detektion von quantitativen trait loci (QTL) behandelt. Eine Genotypisierung von Mikrosatelliten wurde zur Untersuchung der QTL für Immunmerkmale durchgeführt. Es wurden insgesamt 220 Rückkreuzungstiere für 74 Mikrosatelliten auf den 18 Autosomen des Schweins für die QTL-Kartierung genotypisiert. Es konnten 42 signifikante und 24 hoch signifikante QTL für alle Immunmerkmale detektiert werden. Die meisten QTL wurden auf SSC3, SSC16 und SSC18 (neun signifikante F-Werte auf jedem Chromosom) gefunden. Die meisten hoch signifikanten QTL wurden für die Antikörperantwort auf Mykoplasma, Tetanus und PRRS Impfung, C3c und Hp Konzentration gefunden. Für die Merkmale AH50 und CH50 wurden 22 signifikante und 9 hoch signifikanten QTL Regionen mit Hilfe des Programms QTLexpress abgeleitet. Zwei porcine Mannose-binding Gene *MBL1* und *MBL2* wurden in dieser Untersuchung als Kandidatengene untersucht. Eine phylogenetische Studie ergab eine höhere Identität der porcinen *MBL* Gene zu den bovinen Sequenzen als zu den von Primaten oder Nagern. Beide Gene wurden mit Radiation Hybrid Panel und Kopplungskartierung dem SSC14 zugeordnet. Diese waren hoch exprimiert in der Leber. *MBL1* war ebenfalls in Lunge, Hoden und Gehirn exprimiert, eine geringe Expression von *MBL2* wurde in Hoden und Niere gefunden. Single strand polymorphisms (SNP) des porcinen *MBL2* Gens wurden gefunden und in einer porcinen F2 Versuchspopulation parallel zu einem vorher beschriebenen SNP in *MBL1* genotypisiert. *MBL1* Genotypen unterschieden sich in der C3c Serumkonzentration, d.h. *in viv*o Komplementaktivität mit p<0,1. Entsprechend ergab die Kopplungsanalyse einen QTL für das C3c Serumlevel nahe der Position der *MBL* Gene. Diese Untersuchung bestätigt somit, dass die porcinen *MBL* Gene funktionelle und positionelle Kandidatengene für die Komplementaktivität sind.

Analyses of immune competence traits and their association with candidate genes in pigs

The present study was carried out to investigate the immune competences of a backcross DUMI population. Complement activity via classical and alternative pathways were determined using haemolytic activity assay. Antibodies response to *Mycoplasma hyopneumoniae*, Tetanus toxoid and PRRS virus were determined using an Enzyme-Linked Immunosorbent Assay (ELISA). Moreover, complement component C3c and Haptoglobin (Hp), the important parameters of animal immune response were also determined. The parameters were further utilized as phenotypes for the linkage mapping to detect quantitative trait loci (QTL). Microsatellite genotyping was employed to detect the QTL of the immune traits. A total of 220 backcross animals were used for QTL analysis. Seventy-four microsatellites from 18 autosomes of Sus scrofa have been used for QTL mapping. Forty-two significant and twenty-four highly significant QTL could be detected for all immune traits. Most QTL were detected on SSC3, SSC16, and SSC18 (nine significant F-values on each chromosome). No significant F-value was detected on SSC12 and SSC13. Highly significant QTL could be detected for antibody response to Mycoplasma, Tetanus and PRRS vaccination, C3c and Hp concentration. For AH50 and CH50, twenty-two significant and nine highly significant QTL could be detected by using the program QTL express. Mannose-binding lectin (MBL) genes were proposed as a candidate gene in this study. Two porcine genes *MBL1* and *MBL2* were investigated. A phylogenetic study revealed that the porcine *MBL* genes had higher identities to bovine rather than primate and rodent sequences. Both genes were assigned to chromosome 14 by the radiation hybrid panel and linkage mapping. Both *MBL* genes were highly expressed in liver. *MBL1* was also found to be expressed in lung, testis and brain, while low expression of *MBL2* was detected in testis and kidney. New single nucleotide polymorphisms (SNP) of the porcine *MBL2* gene were found and genotyped in an experimental F2 pig population together with a previously reported SNP of *MBL1*. *MBL1* genotypes differed in C3c serum concentration, i.e. *in vivo* complement activity, at $p < 0.1$. Correspondingly, linkage analysis revealed a QTL for C3c serum level close to the position of the *MBL* genes. The study thus promotes the porcine *MBL* genes as functional and positional candidate genes for complement activity.

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List of abbreviations

A	: Adenine
A_{260}	: Absorbance at 260 nm wavelength (UV light)
Ab	: Antibody
Ag	: Antigen
APS	: Ammonium persulfate
ATP	: Adenosine triphosphate
BSA	: Bovine serum albumin
C	: Cytosine
cM	: Centimorgan
DMSO	: Dimethyl sulfoxide
DNA	: Deoxynucleic acid
dNTP	: deoxyribonucleoside triphosphate
	(usually one of dATP, dTTP, dCTP and dGTP)
dpi	: day post-inoculation
DTT	: Dithiothreitol
E. coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetraacetic acid (powder is a disodium salt)
G	: Guanine
IPTG	: Isopropylthio-ß-D-galactoside
kb	: Kilobase
LOD	: Logarithm of odds
mA	: Milliamperes
Mh	: *Mycoplasma hyopneumoniae*
$MgCl_2$	: Magnesium chloride
MW	: Molecular weight
NPL	: Non Parametric LOD Score
OD_{260}	: Optical density at 260 nm wavelength (UV light); = A_{260}
PBS	: Phosphate buffered saline
PRRS	: Porcine Reproductive and Respiratory Syndrome
QTL	: Quantitative trait loci
RACE	: Rapid amplification of cDNA end

RFLP   : Restriction fragment length polymorphism
RNA   : Ribonucleic acid
rpm   : Revolutions per minute
SDS   : Sodium dodecyl sulfate
SMART^{TM} : Switching mechanism at 5'end of RNA transcript
SNP   : Single nucleotide polymorphism
T   : Thymine
T1   : Time point 1
T2   : Time point 2
T3   : Time point 3
T4   : Time point 4
T5   : Time point 5
T6   : Time point 6
TAE   : Tris-acetate buffer
TBE   : Tris- borate buffer
TE   : Tris- EDTA buffer
TT   : Tetanus toxoid
U   : Units
UTR   : Untranslated region
UV   : Ultra-violet light
wk   : weeks

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1   Introduction

Animal health has become an increasingly important issue for livestock producers and consumers. Animal diseases, causing morbidity and mortality, significantly decrease the profitability of animal production. Antibiotics resistance of pathogenic organisms and newly emerged diseases in livestock production such as BSE have led for a call of genetic selection for disease resistance animals. Selective breeding of high disease resistance animals based on their phenotypic value and the presence or absence of some specific resistance genes in their genotypes are the important tools for animal breeders to improve genetics of disease resistance. In order to accomplish the breeding (improvement) goal, the relevant immune response traits from individual animals have to be defined. To date, numerous assays have been established and developed regarding immune response traits.

Molecular genetics approaches including the whole genome scan for quantitative loci (QTL) mapping and candidate gene study have been widely used to investigate genetic variation. Particularly, a combination of theses two approaches has been the most successful method of identifying "disease genes" to date. The QTL approach provides the ability to discover a number of genetic markers at the DNA level. The currents status in pig shows several QTL for various traits including meat production and quality, reproduction as well as disease resistance, that have been mapped on nearly all chromosomes in divergent breed crosses and commercial breeds (Bidanel and Rothschild 2002). The current status (February, 2007) of pig quantitative loci in the 'PigQTLdb' available at the website http://www.animalgenome.org indicating 1,657 QTL were reported from 110 publications represent 281 different pig traits. However, the QTL affecting immune response traits and disease resistance are still far less numerous (Rothschild 2003). The candidate gene approach is useful for quickly determining the association of a genetic variant with a disorder and for identifying genes of modest effect (Kwon and Goate 2000).

In this study, several experiments were conducted. Haemolytic complement activities were performed to determine the complement activities via classical and alternative pathways. Several enzyme-linked immunosorbent assays (ELISA) were performed to determine the antibody responses to different vaccination treatments in experimental

animals, including some important immunological parameters (e.g. haptoglobin, C3c). Moreover, molecular genetics methods regarding linkage mapping and candidate gene approaches were also employed in order to achieve the following aims:

1. Evaluation of the porcine immune competencies including total complement activities of classical and alternative pathways, complement component C3c, acute phase protein, and also antibodies of individual pigs in a backcross DUMI resource population.

2. Detection of quantitative trait loci (QTL) using genome scan over all autosomes in a backcross DUMI resource population regarding immunological traits.

3. Identification of single nucleotide polymorphism (SNP) in the porcine *MBL* candidate genes and their association on immunological traits in an F2 DUMI resource population.

2   Literature review

2.1   The immune system

The immune system is an organization of cells and molecules with special roles in defending against infection. It comprises of two functional types of responses, innate or natural and specific or adaptive responses (Figure 1). Its major functions are to differentiate self from non-self and to maintain host defences against foreign substances and pathogens. The innate responses use phagocytic cells including neutrophils, monocytes and macrophages, that release inflammatory mediators (basophils, mast cells and eosinophils) and natural killer cells. The molecular components of innate response include complement, acute-phase proteins and cytokines. The adaptive or specific immune response involves the proliferation of antigen specific B and T cells (Delves and Roitt 2000, Medzhitov and Janeway 1997). Its composition is a complex series of cells and molecular from various tissues that interact to protect the body against invading microorganisms (de Souza 2006). In multicellular animals, immune systems contain different kinds of cells such as tissues or organs and their molecular products that encompass and protect the whole organism against potentially harmful pathogens such as bacteria, viruses and parasites that inhibit the external environment (Cooper 2000). It is considered that the immune system must be capable of doing three actions including recognition of a diverse array of pathogens, killing these pathogens once they are recognized and sparing tissues of the host (Beutler 2004).
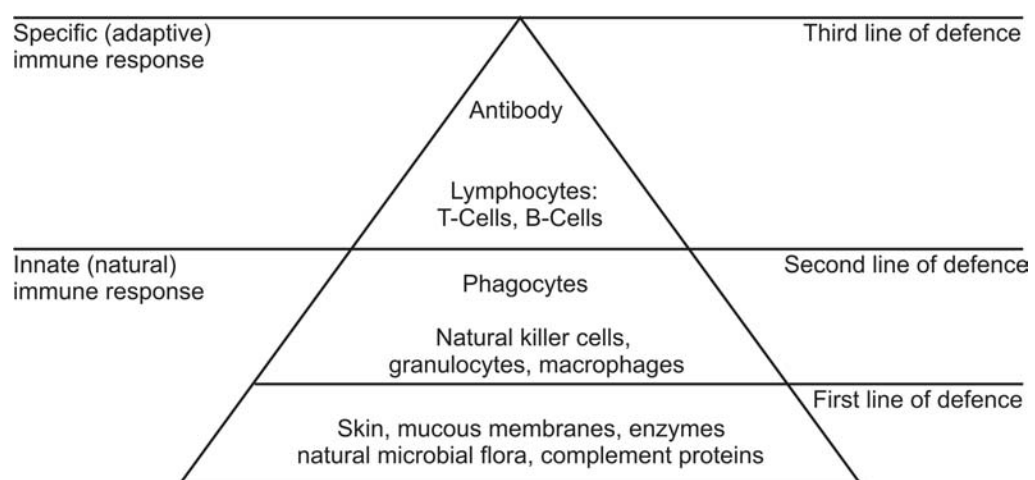
Figure 1:   Scheme of the immune response

2.1.1   Innate immune response

The innate immune system can be expressed on the cell surface, in intracellular compartments or secreted into the bloodstream and tissue fluids using a variety of pattern recognition receptors (PRRs) (Medzhitov and Janeway 1997). PRRs functions include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signalling pathways and induction of apoptosis (Janeway and Medzhitov 2002). Two mechanisms are involved in the innate response in order to distinguish self cells from foreign organisms. The first mechanism involves an array of cell-bound and soluble molecules that have evolved to pathogen-associated molecular pattern (PAMPs) recognition, such as MBL. The second mechanism of self-nonself discrimination involves protecting self cells from the destructive effects of innate immunity such as the alternative pathway of complement activation on the surface of self cells (Parish 2005). The innate immune system evolved long before adaptive immune system in many respects (Beutler 2004).

2.1.2   Acquired immune response

The acquired immune response is a specific response against a particular pathogen or antigen, Lymphocytes are the primary effector cells, a memory response is generated and increases with each exposure to the antigen. The adaptive immune response is distinguished from innate immune mechanisms by a higher degree of specific reactivity for the including agent and recall memory (Doenhoff 2000). Its function is mediated by specific antibody or humoral immunity and a specific cellular immune response or cell-mediated immunity (CMI) (Corbeil 1991). These specificities are governed by antigen handling and recognition molecules on each of the three cell types such as, major histocompatibility antigens (MHC) on antigen-presenting cells (APC), T-cell receptors (TCR) on T-cell and immunoglobulin (Ig) molecules on B cells. One of the acquired immune system enhancements is also represented by successful vaccination against an infectious disease. Vaccines against bacterial and viral infections have employed attenuated live or inactivated whole organisms (Bahr 1999).

2.1.3   Complement system

The complement system is part of the innate immune system and plays an important role in both natural host defence against invading pathogens and the induction of acquired immunity (Roos et al. 2006). The complement system in mammals consists of a complex group of more than 35 soluble proteins and receptors that play an important role in innate and acquired host defence mechanisms against infection and participate in various immunoregulatory processes. The functions mediated by complement activation products include phagocytosis, cytolysis, inflammation, solubilization of immune complexes, apoptotic cells clearance and promotion of humoral immune responses. Activation of complement through any of the three pathways lead to activation of C3, the central protein of the complement system (Holland and Lambris 2005) and the formation of C5 convertase and enzyme complex that activates the terminal pathway and leads to the end product of complement activation, so called 'membrane attack complex' (MAC) (Sodetz and Plumb 2005). Most parts of the complement system are synthesized from liver and are taken into the circulation there (Carroll 2004).

2.1.3.1   Classical pathway

The classical pathway of complement is a major system of innate immunity and triggered through activation of several multimolecular proteases: C1, the MBL associated serine protease 2 (MBL-MASP-2) complex and the ficolin-MASP-2 complexes. These convert an initial recognition signal into proteolytic activity, thereby initiating the 'classical' and 'lectin' routes of complement activation. Both routes then lead to the formation of C3 convertase and the generation of protein fragments triggering diverse biological activities, such as opsonization, endocytosis and inflammation (Arlaud and Colomb 2005). The classical pathway was the first studied and found activated by either antibody released after humoral response or by natural antibody (Carroll 2004). It is triggered by activation of the C1-complex, either by C1q binding to antibodies complexes with antigens, or by binding C1q to the surface of the pathogen. The C1 complex is inhibited by C1-inhibitor. The C1-complex now binds to and splits C2 and C4 into C2a and C4b. C4b and C2a bind to form C3-convertase (C4b2a complex). The production of C3-convertase signals is the end of the classical

pathway, but cleavage of C3 by this enzyme brings us to the start of the alternative pathway.

2.1.3.2 Lectin pathway

The lectin pathway closely resembles the classical complement pathway. The major molecules of this pathway consist of mannose MBL, the homologous molecule to C1q and the MBL-associated serine proteases (MASPs) including MASP-1 and MASP-2, which are homologous to C1r and C1s (Nakao et al. 2001, Sato et al. 1994, Thiel et al. 1997) and form a complex molecule in the presence of the Ca^{2+} ions. This is similar to the C1q/C1r/C1s molecules of the classical pathway. The lectin pathway activation is initiated by binding of the complex between MBL and its associated serine protease MASP-1 and -2 to the mannose groups on bacterial cell surfaces, that leads to the activation of this protease (Fujita 2002, Walport 2001). By MASP2 activation, the complement component C4 and C2 are then cleaved to form the C3 convertase (C4aC2b), which is similar to the classical pathway activation (Fujita 2002). This C4aC2b molecule binds to C3b leading to the C5 convertase generation for the terminal pathway. Three members of this pathway have been identified including MBL, ficolin H and ficolin L (Carroll 2004).

2.1.3.3 Alternative pathway

The alternative pathway, the oldest and most important activation pathway of the complement system, assists in maintaining the integrity of an organism by inactivating invading organisms, pathogens and modified tissue cells (Zipfel 1999). It can be activated non-specifically without the necessity of antigen-antibody complexes (Corbeil 1991). The alternative pathway is activated by a variety of microorganisms including viruses, bacteria, fungi and protozoa. Although the initiation of the activation is essentially antibody-independent, aggregated antibodies have been shown to enhance the activation process. The alternative pathway is kept at a low level of steady-state activation as a result of the hydrolysis of the thioester group of native C3, which leads to the formation of hydrolysed C3 (Holland and Lambris 2005). This pathway is triggered on the surface of a pathogen. Then, C3 is split into C3a and C3b in the alternative pathway. Some of the C3b is bound to the pathogen where it will bind to

factor B; this complex will then be cleaved by factor D into Ba and the alternative pathway C3-convertase, Bb.

2.1.3.4   Terminal pathway

The terminal or lytic pathway of the complement system consists of C5-C9 components which are involved in the formation of the MAC. The MAC causes cell lysis in the pathogen (Holland and Lambris 2005). The activation of the terminal pathway begins with the activation of C5 convertase enzyme (C4b2a3b and C3b3bBb), then breakdown the C5 component to release C5a and C5b. The C5b molecule binds to C6, C7, C8 and C9 respectively, to form MAC that facilitates the killing of microorganisms by changing the permeability of their membranes causing the osmotic lysis of microorganism cells (Sodetz and Plumb 2005).

2.1.3.5   Complement activation

Once the complement system is activated, a chain of reactions involving proteolysis and assembly occurs, resulting in destruction of the pathogen membranes. Complement is activated by three different pathways, classical, alternative and lectin. All pathways share the common step of activating the central component C3, but their recognition mechanisms are different (Carroll 2004). Various pathogenic microorganisms, including bacteria and viruses, as well as many infected cells, efficiently activate the classical pathway after their recognition by antibodies. The activation is triggered upon interaction of the serum C1 complex with antigen-antibody complexes or immune aggregates containing immunoglobulin G (IgG) or IgM (Arlaud and Colomb 2005). The lectin pathway is triggered by C1-like complex proteases in which the recognition function is mediated by either MBL, a member of the collectin family (Turner 1996) or the L- and H-ficolins (Matsushita et al. 2002). The activation of the pathway starts in the fluid phase including plasma or serum by a change of C3 which leads to the enzyme complex formation that cleaves further C3 molecules and sets in motion an amplification reaction leading to complement activation and deposition of a large number of C3b molecules on the cell surface (Zipfel 1999).

A) Classical

C1q binds to:
- antibody-antigen complexes
- certain invasion pathogens

B) Lectin

MBL binds to:
- oligosaccharides on
  certain invasion pathogens

C) Alternative

spontaneous
breakdown
of C3 in serum

$C1_{inactive} \longrightarrow C1_{active}$

MBL $\longleftarrow$ complex$_{active}$ $\longleftarrow$ MBL complex$_{inactive}$

$C3b+C3a$

C4 $\longrightarrow$ C4b+C4a

C3bB $\longleftarrow$ Surface-bound C3b

C2 $\longrightarrow$

C4b2

Factor D $\longrightarrow$

Factor B

C3bBb+Ba

$\longleftarrow$ Properdin (P)

C4b2a
Classical/MBL
C3 convertase

C3bBbP
Alternative
C3 convertase

D) Terminal

C3 convertase

C5 convertase (C4b2a3b & $C3b_2Bb$)

C5 $\longrightarrow$ C5b+5a

$\longleftarrow$ C6, C7, C8

C5b678

$\longleftarrow$ C9

$C5b678(9)_n$
(membrane attack complex, MAC)

Membrane disruption

Figure 2:   Activation of the complement system via A) classical, B) alternative and
                C) lectin pathways.

2.1.4   Antibody response

Antibodies are produced by B cells in response to antigens such as bacteria, viruses and protozoa. The development of the antibody response is dependent on the type of antigen and whether the immune system has previously encountered the antigen (Wingren 2001). Antibodies belong to glycoprotein families known as Ig. They circulate in the plasma and lymph, are present in mucosal and lymphoid tissues and can be found on the surfaces of B lymphocytes, where they function as receptors for antigens. From lymphocytes, antibodies are secreted in response to foreign antigenic stimulation, which consist of the principal component of the adaptive humoral immune response. Antibody molecules consist of four polypeptide chains including two identical light (L) chains and 2 identical heavy (H) chains as demonstrated in figure 3. The chains are linked by disulfide bonds and are arranged such that the H and L chains form pairs. The domain

function created by the juxtaposition of the V_H and V_L regions is to recognize and bind antigens, whereas the function of the C_H domain(s) is to mediate biological effector functions (Lucas 1999).

There are five classes of immunoglobulin: IgM, IgD, IgG, IgA and IgE, which can be divided into subclasses. For example, the IgG class consists of four subclasses (IgG1, 2, 3 and 4) and the IgA class consists of two subclasses (IgA1 and IgA2). Biological properties of immunoglobulin vary within a class, such as IgA1 is able to utilize the alternative complement pathway, whereas IgA2 is not. Similarly, IgG1 and IgG3 fix complement (classical pathway) efficiently while IgG4 does not. These differences could be important in immune responses where a particular antibody specificity is restricted to a single IgG subclass (Lucas 1999). The genes encoding antibody molecules are organized such that the same VH region is able to associate with different CH regions. A particular VH region may at one time be expressed in the context of IgM and at another time in the context of IgG. Thus, antibodies with the same V regions specificity may associate with different CH regions, a process which links specificity with different effector capabilities and provides functional flexibility (Lucas 1999).
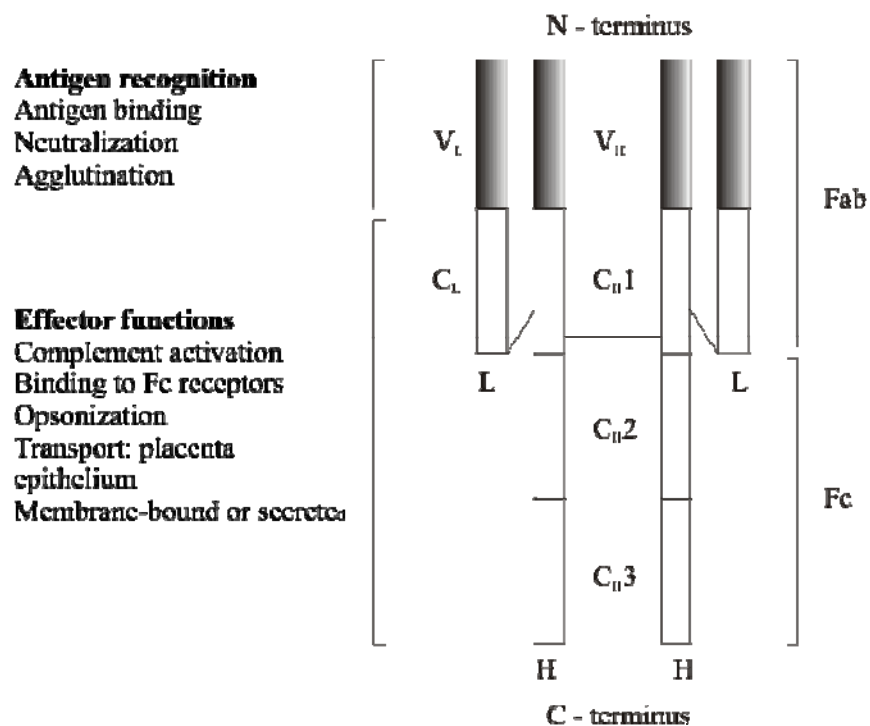
Figure 3:   Schematic diagram illustrating immunoglobulin G structures modified
                 from Lucas (1999)

2.1.4.1   Antigen-antibody complex

Antigen-antibody complexes are formed when the immune system raises antibody against antigenic determinants of host or foreign substances that recognize and bind to the unwanted molecules. Normally, the immune complexes that are formed are cleared by the cellular immune system, but when an excess of antibody or antigen is present, the immune complexes are either detected in tissues or involved in the pathogenesis of tissue lesions in a variety of diseases (Eggleton 2006). Antigen-antibody binding involves non-covalent interactions between atoms of the antigen and atoms of the combining site. Antibody-combining site character and the extent of the bonding interactions with the antigen varies, and also depends upon the type of antigenic determinant (epitope) which is being recognized. Antibodies can recognize small chemical groups or haptens, short peptides, polysaccharides and the surfaces of native proteins (Cisar et al. 1975).

2.1.4.2   Antibody response to *Mycoplasma hyopneumoniae*

M. hyopneumoniae is recognized as the causative agent of porcine enzootic pneumonia and plays an important role in the porcine respiratory disease complex (PRDC) (Thacker et al. 1999). Up to 80 % of pigs around the world are affected by this chronic disease which leads to a high morbidity, low mortality rates, thus being a significant pathogen to pig industry (Fano et al. 2005). *M. hyopneumoniae* causes widespread and persistent disease in pigs, and in combination with other respiratory pathogens e.g. porcine reproductive and respiratory syndrome (PRRS) or swine influenza virus, can cause more serious pneumonia than single pathogen infection (Kim et al. 2006, Thacker et al. 2001). The mechanism of *M. hyopneumoniae* is to attach to the cilia of the respiratory epithelium of tracheal epithelial cells, resulting in damage to epithelial cells and the mucociliary apparatus. *M. hyopneumoniae* infection is characterized by a chronic, mild, dry, non-productive cough beginning 10 to 14 days after experimental infection. Fever, lethargy, or anorexia is rarely observed in pigs infected only with *M. hyopneumoniae*. Lung lesions observed in pigs infected with *M. hyopneumoniae* are similar to those observed in pigs with swine influenza virus (SIV), with dark purplish areas of lung consolidation occurring primarily in the cranioventral areas of the lung (Debey et al. 1992, Thacker et al. 2001). Clinical manifestation of mycoplasmal

pneumonia is common in young pigs but generally not seen in adults. Previous infection at the young age protecting from re-infection is probably the reason of this pattern (Kobisch et al. 1993). Antibodies to *M. hyopneumoniae* were first detected in sera of pigs inoculated intranasally with *M. hyopneumoniae* at 2 to 4 wk after inoculation and 7 to 8 wk after pigs were contact-exposed to the same Mycoplasma (Kazama et al. 1989). Active immunization, using mainly whole cells of M. hyopneumoniae inactivated bacteria, has been recommended in order to protect animals from mycoplasmal pneumonia, has been shown experimentally to induce protection against *M. hyopneumoniae* (Okada et al. 1999). Various methods have been employed in order to monitor mycoplasmal pneumonia and its antibodies in pig, such as the complement fixation (CF) test, the indirect hemaglutination (IHA) test and ELISA (Kazama et al. 1989). It is possible to monitor the development of infection by measuring the immune response in animals maintained under commercial conditions using *M. hyopneumoniae* specific ELISA. The ELISA tests have a higher sensitivity at the individual level compared to other methods (e.g. IHA) (Armstrong et al. 1983, Sheldrake et al. 1990, Sørensen et al. 1992).

2.1.4.3   Antibody response to Tetanus toxoid (TT)

Tetanus is caused by the bacterium *Clostridium tetani* which produces toxins that affect the central nervous system. The organism, which can form spores, lives in the large intestines and faeces of many mammals, including pigs. It must enter through a dirty abrasion or a cut. In the sucking pig, the most common source is castration. Several *In vitro* serological assays for measuring the level of Tetanus antitoxin have been developed, such as, passive and indirect hemaglutination (Peel 1980), the toxin binding inhibition (ToBI) test (Hendriksen et al. 1989) and several versions of ELISA (Esparza and Kissel 1992, Gentili et al. 1985, Gupta and Siber 1994, Simonsen et al. 1987b). Stiffler-Rosenberg and Fe (1977) described a TT antibody ELISA procedure in which 1-ml volumes in test tubes are used, three serum dilutions, two overnight incubation steps and alkaline-phosphatase as the enzyme. In an effort to make the test more economical, (Layton 1980) developed a Tetanus ELISA assay in which smaller volumes in microtiter plates and the less expensive enzyme horseradish peroxidase are used. One serum dilution (1:10) with one overnight incubation step was used. (Bullock and Walls

1977) found that short incubation periods (total, 5 h) were satisfactory in their study of the variables in the *Toxoplasma* antibody ELISA test. They concluded that measurement of a single dilution of test serum was inadequate for quantitative tests according to their reactivity curves which showed an extremely steep slope at the endpoint. The ELISA test is, however, commonly used to determine TT antibody titres and several modifications have been developed to overcome the unreliability it may show (Aybay et al. 2003).

2.1.4.4   Antibody response to PRRS

PRRS is a widespread and (becoming) one of the most important viral diseases in pig industry (Dee et al. 1997, Greiner et al. 2000). Its emergence across the world has a critical economic impact on the production. PRRS is caused by a virus, which eventually lyses the cell whose are contents released systemically into the body resulting in fever, anorexia, vomiting and coughing in young pigs and non-pregnant sows (Benfield et al. 1992, Bierk et al. 2001). In general, clinical signs caused by PRRS involve reproductive disorders in pregnant animals which are manifested as late-term abortions or premature farrowing, and/or respiratory disease in pigs of all ages. Furthermore, it also affects litters by having higher proportions of stillbirths, piglets born weak and increased pre-weaning mortality (Bilodeau et al. 1994). PRRS causes a long acute infection in pigs which is defined as the continued presence of a pathogen in the host beyond the acute phase of infection. Its persistence has been detected up to 157 day post-inoculation (dpi) in weaned pigs (Wills et al. 1997), but much shorter (42 to 86 dpi) in sows (Bierk et al. 2001). However, it has been reported that PRRS virus persistence in female pig was not detected during the period of 120 to 180 dpi (Batista et al. 2002).

Pigs develop both antibody and cell-mediated immune responses following PRRS virus infection. However, the mechanisms and specific roles of each type in the development of protective immunity and clearance of the virus are not yet known (Batista et al. 2004, Chang et al. 2002). The IgM antibodies are detected approximately 5 to 7 dpi. They decrease rapidly to undetectable levels after 2 to 3 wk (Joo et al. 1997). Additionally, the IgG antibodies are detected at 7 to 10 dpi and reach the peak at 2 to 4 wk post-

inoculation (wpi). These remain constant for months until they decrease to low levels by 300 dpi (Nelson et al. 1994).

For diagnosis of PRRS, serological tests are used. An immunoperoxidase monolayer assay (IPMA) is widely performed in Europe, while the indirect fluorescent antibody (IFA) test is more prefered in Canada and the United States (Cho et al. 1996). An ELISA has been developed for the rapid detection of antibodies to the PRRS virus in pig sera. By comparing to the IPMA, the only test available up till recently for serodiagnosis of the disease, the ELISA test proved to be more sensitive particularly in early detection of antibodies. It gave a sensitivity of 100% and specificity of 66% relative to IPMA using 165 field sera and 9 experimentally obtained sera (Albina et al. 1992). Moreover, a reliable ELISA would be used because the advantages in term of semi-automation and cost effective for large-scale measurement (Cho et al. 1996). Serum antibody detection by the HerdChek® PRRS virus antibody test kit is used for herd screening to prevent the spread of disease to uninfected animals (Botner 1997, Cho et al. 1996).

2.1.5   Acute-phase proteins

Acute phase proteins (APP) are liver-derived plasma proteins whose concentrations can change rapidly in response to abnormal events that disturb physiologic homeostasis, including infection, tissue injury and trauma. Changes in APP concentrations, fever, leukocytosis, vascular permeability and an increase of metabolic responses are components of innate immunity mediated by cytokines, such as IL-1, IL-6 and TNF-α (Suffredini et al. 1999). The major porcine APP are C-reactive protein (CRP), haptoglobin (Hp) and serum amyloid A (SAA). The concentrations of these APP increase rapidly in response to infection. Acute phase proteins are sensitive indicators of illness even of sub-clinical diseases and immunological stress. In pig, Hp is one of the most important acute phase proteins (Francisco et al. 1996, Hall et al. 1992).

2.1.5.1   Complement component 3 (C3)

The importance of C3 as the central coordinator between innate and acquired immunity is to eliminate pathogens (Sahu and Lambris 2001). Its functional and genetic regulation is widely investigated. C3 is mainly synthesized in liver and regulated by cytokines or

interleukins (IL) such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), IL-1 and IL-6 (Mitchell et al. 1996), transforming growth factor-β1 (TGF- β1) and TGF-β2 (Høgåsen et al. 1995).The C3 protein is normally secreted in plasma at about 1.1-1.5 µg/ml which is considered as an acute-phase protein with many times of normal concentration during inflammation or infection. C3 is an essential protein for binding covalent bonds to a wide range of biological surfaces such as pathogenic cell surfaces due to its thioester bond site in the α-chain molecule (Law and Dodds 1997). Native plasma C3 consists of a two chain (α and β) structure which is an inactivated molecule that does not bind to any antigen target. It is activated by C3 convertase from all three pathways in the complement system by cleaving the α-chain, releasing C3a and generating C3b molecules (Sahu and Lambris 2001). Native C3 is not a functional molecule and all of the ligand-binding sites on C3 are hidden until the molecule is activated (Holland and Lambris 2005). The C3b molecule plays a key role in regulating immune response in host defence due to the existence of a thioester bond which is essential to form a covalent bond with the cell surface of microorganisms as described above. C3b is produced by proteolytic cleavage of the C3-α chain by the classical (C4b2b) or alternative pathway convertase (C3bBb) with release of the amino terminal C3a peptide. Initial inactivation of C3b prevents its participation in C3 or C5 convertase formation and is mediated by factor I, which, in the presence of factor H, catalyzes the proteolysis of two peptide bonds in the α' polypeptide chain of C3b. The resulting products are C3b, which consists of two α-polypeptides covalently bonded to the β-chain and C3f, which is released from the remainder of the molecule. Surface-bound and fluid-phase iC3b can be further degraded by limited tryptic digestion or by elastase digestion to produce the C3c and C3d fragments (Davis et al. 1984).

2.1.5.2 Haptoglobin (Hp)

Numerous functions of Hp have been proposed including the prevention of iron loss by the formation of very stable complexes with free haemoglobin in the blood, binding haemoglobin, bacteriostatic effect, stimulation of angiogenesis, role in lipid metabolism/development of fatty liver, immunomodulatory effect and inhibition of neutrophil respiratory burst activity. The increase of Hp also reflects the respiratory

infections caused by different serotypes, such as *Actinobacillus pleuropneumoniae*, *Mycoplasma hyorhinis*, or PRRS virus (Petersen et al. 2004).

Hp is one of the major APP in pigs which has been used for identification in both clinical and sub-clinical disease, objectively monitoring antibiotic therapy and assess the health of animals (Alava et al. 1997, Eckersall et al. 1996). It has been used to evaluate systemic inflammation after cardiopulmonary bypass and acute phase reaction to antimicrobial agents after vascular implantation and in response to anti-inflammatory drugs due to its highly sensitive, nonspecific reactants present at low concentrations in normal sera. Hp serum concentration increases rapidly and significantly during inflammation or infection and precede the development of specific antibody in 4 to 5 days later (Chen et al. 2003). Pigs aged 10 to 14, 15 to 19 and 20 to 25 wk in conventional herds had higher Hp concentrations than high health SPF (SPF-x) pigs of the same age. There was no significant difference between SPF-x pigs of different ages. Conventional pigs aged 15 to 19 and 20 to 25 wk had higher Hp concentrations than conventional pigs aged 10 -14 wk (Petersen et al. 2002).

Hiss et al. (2003) developed the enzyme immunoassay (EIA) to determine the Hp in body fluids including meat juice, serum, plasma and saliva. According to their result, the in-house developed ELISA indicated reliably quantification of Hp in body fluids.

2.2   Immune competence determination

Immune competence of the pigs can be monitored by measuring the immune response induced by infection or detect the pathogen itself. Most common diagnostic assays are based on detection of antibodies specific to the pathogen. Normally, serum is used as a sample, although thoracic fluid, colostrums and muscle fluids are also used. The existing techniques for these purposes are based on the collection of blood samples in herds at regular intervals with subsequent serological analyses of the sera (Mortensen et al. 2001).

2.2.1   Haemolytic complement activity assay

In recent years, the relation between the amount of complement used and the proportion of red cell lysed reflecting haemolytic complement activity is represented in a sigmoidal curve. This curve indicated a high sensitivity to small changes of the complement

amount at the central region. For the haemolytic complement activity precision, usually 50% or the central part of the curve was chosen to define the activity. The 50% haemolytic unit of complement designated as CH_{50}, is defined as the quantity of complement required for 50% lysis (haemolysis) which is an arbitrary unit depending on many factors including red cell concentration, cell fragility, the quality of antibody used for sensitization, the nature of that antibody, the ionic strength of the reaction system, the concentration of Ca^{++}, Mg^{++}, pH, reaction time and temperature (Mayer 1961). Moreover, storage temperatures also influence the activity. The results of the haemolytic complement activity are influenced by storage conditions of sera as found in chickens (Demey et al. 1993). In cattle and sheep, a little loss (13% loss) of activity was found in samples which had been stored under -70 °C after 4 months. On the other hand, samples which were stored at higher temperature (-20/-10 °C) had significant loss (67 and 80% in cattle and sheep, respectively) of the activity within a month (Pandey et al. 1993). But, it was found that the haemolytic complement activity was not affected by the coagulation temperature of serum sample (Baatrup et al. 1992).

A simple, automated microassay for the serum complement-dependent haemolytic activity has been described (Liu and Young 1988). In contrast to the traditional titration haemolysis assay established by Mayer (1961), the new method depends on a single experimental step using a fixed volume of serum sample and sheep erythrocytes. This assay is based on the change in light scattering properties of erythrocytes upon haemolysis. It relies on the spectrophotometric reading of microtiter well samples at 700 nm using a microplate reader. The measured absorbance correlates proportionally with the extent of haemolysis. A good correlation between the results obtained using this technique and those obtained by the traditional CH_{50} titration method is observed. This simple procedure can be applied to the rapid, semi-quantitative diagnostic screening of complement activities of a large number of serum specimens.

2.2.2   Immunoassay

ELISA was found to be potentially a very attractive and practical serodiagnostic test for mycoplasmal pneumonia in porcine. A study conducted by Armstrong et al. (1983) indicated an extremely sensitivity of these method for detecting porcine antibodies to *M. hyopneumoniae*, and also let itself to automation that would be economical for testing

when compared to indirect hemaglutination (IHA) and complement fixation (CF) methods. For the detection of Ab to PRRS virus in porcine, an immunoperoxidase monolayer assay (IPMA) and an indirect immunofluorescent (IFA) were previously used in Europe and North America, respectively (Cho et al. 1997a). Recently, researchers have conducted the comparative study to compare in-house and commercial tests for the serological diagnosis of porcine PRRS using IPMA and ELISA. The results also indicated a high sensitivity of ELISA similar to IPMA method (Drew 1995). At present, numbers of immunoassay formats are available to measure almost any substance ranging from small molecules to complex cellular antigens, and the ELISA have become the most widely used for immunological assay (Andreotti et al. 2003).
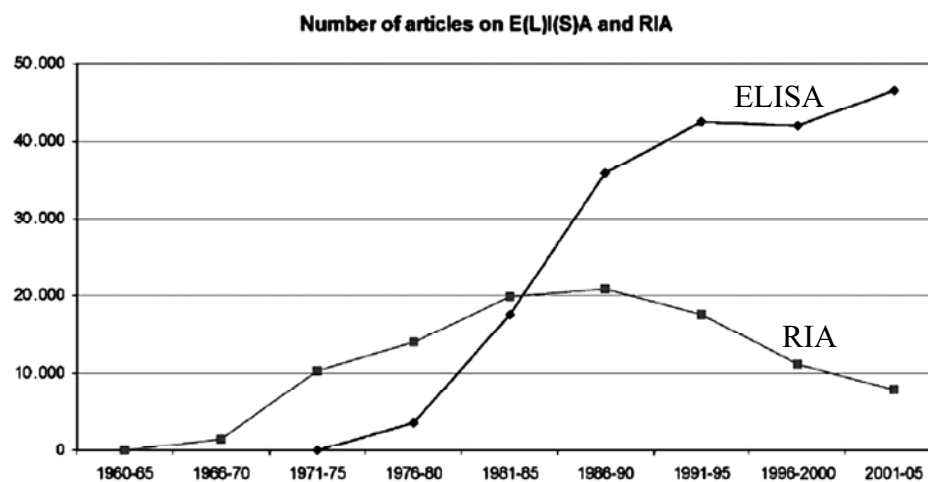
Figure 4:   Estimates of the number of articles published involving EIA/ELISA and RIA modified from Lequin (2005).

2.3   Quantitative traits loci (QTL) analysis

The effect of a QTL on the phenotype is usually expressed as the percentage of the total phenotypic variance that can be explained by variation in DNA at the locus. A massive number of QTL have been identified which influence economic important traits e.g. growth, carcass- and meat quality on almost all chromosomes in pig (Hu et al. 2005). The detection of QTL may be carried out by using a genome scan of either an F2 or backcross between two inbred strains that differ for the phenotype of interest. Due to the reason that all members of an inbred strain have identical genomes, there should be no genetic difference between the F1 progeny. Therefore, the familial effects in the

analysis of F2 or backcross individuals should not be found. Quantitative trait loci mapping methods are statistical methods for identifying loci associated with a quantitative phenotype. The goal of QTL mapping is to determine the loci that are responsible for variation in complex, quantitative traits. The immune competence is a quantitative trait. It expresses the ability of an individual to protect itself against any pathogen by using innate and humoral immunity. QTL mapping procedures can be distinguished into three steps as follow (Mott 2006);

1. QTL detection, where the objective is to find and map a QTL to within about 10-30 cM, usually accomplished by linkage analysis of family data;

2. QTL fine-mapping, where the QTL is localized to within 1-2 cM, small enough that a search for candidate genes within the region is feasible, usually by association analysis in either population or family data; and

3. Cloning, in which the molecular basis of the trait variation is determined.

Functional genomics has been applied to the genetic dissection of immune response in different ways (de Koning et al. 2005). The QTL detection underlying experimental crosses between lines that differ in their innate and specific immune response is also one of many methods used. Using these methods, the experimental populations have to be custom bred and challenged to study genetic differences in immune response and map genetic loci underlying these differences in most infectious disease study (de Koning et al. 2005). Antibody response was one of the first immune competence traits to be examined by QTL analysis (Edfors-Lilja et al. 1998).

Many QTL underlying the immune response variation could be detected on various chromosomal regions in many studies in mouse, chicken and human (Hall et al. 2002, Siwek et al. 2003a, b , Zhou and Lamont 2003a, Zhou et al. 2003). By screening these chromosomal regions, evidences for significant association of candidate genes were found e.g. genes related to interferon which plays an important role for primary and secondary antibody response to different antigens as shown in chicken (Zhou et al. 2001). These results in chicken confirmed the genetic association between immune response and disease resistance, thus promoting the improvement of poultry immune competence using marker assisted selection or 'MAS' programs (Yonash et al. 2001). The transforming growth factor beta 2 (*TGFB2*) gene was already suggested as candidate gene to be applied in MAS to improve antibody production (Zhou and Lamont 2003a). Similar results were found in pig, QTL close to the mast/stem cell

growth factor receptor (*KIT*) gene was detected and candidate gene analysis showed significant effects of this gene on the immune response-related traits (Wattrang et al. 2005). In this study the QTL analysis is used to detect the loci and candidate genes regarding the innate and adaptive (humoral) immune response traits in pig. By QTL detection, the linkage between loci is calculated to localize the chromosome region including the candidate genes.

2.4   Mannose-binding lectin (MBL)

MBL also called mannose-binding protein (MBP) is a calcium-dependent carbohydrate-recognizing protein (C-type lectin protein). It is a member of the collectins family that includes both collagen and globular regions. Its structure is similar to C1q in the complement system. Two types of MBL, namely MBL-A and MBL-C, were characterized in many species. In rodents, both types of MBL have been reported (Drickamer et al. 1986, Hansen et al. 2000, Mizuno et al. 1981), as well as in rhesus monkey (Mogues et al. 1996). Only one MBL was identified in human, chimpanzees (Mogues et al. 1996), chickens (Laursen and Nielsen 2000) and cattle (Kawai et al. 1997). It has been, however, reported that bovine MBL-A was isolated and characterized recently (Lillie et al. 2006). In rabbit, although two forms of MBL (from serum and liver) were characterized by Kawai et al. (1998), their results indicated that those are identical and encoded by one gene (Carroll 2004, Håkansson and Reid 2000). Binding of MBL leads to opsonization through complement activation and C3 deposition (Holmskov et al. 2003). In addition, serum mannose-binding lectin is able to trigger the classical complement system, via serine proteases (MASP-1 and MASP-2), resulting in lysis of the invading microorganism (Matsushita and Fujita 1992).

2.4.1   MBL molecular characterization

Collectin molecules comprise of four different regions including an N-terminal region containing a 20-residue signal peptide, a collagenous region containing 19 Gly-Xaa-Yaa triplets, an helical coiled coil so called 'neck region' and a C-terminal calcium-dependent carbohydrate-binding lectin domain (also called carbohydrate-recognition domain; CRD) (Håkansson and Reid 2000, Holmskov et al. 2003, Presanis et al. 2003). The CRD is followed by an a-helical neck domain, a collagen-like domain and an N-

terminal cysteine-rich domain. Three neck domains will form a triple coiled-coil structure and the collagen-like domain will assemble into a triple helix, leading to the formation of trimeric subunits. Trimeric subunits are assembled subsequently via cysteine residues in the N-terminal domain into higher oligomeric forms (Laursen and Nielsen 2000). MBL was first isolated from rabbit serum in the late 1970s (Kawasaki et al. 1978). The overall similarity in the organization of MBL as a collagen containing protein with 16 head domains with C1q, the first component of the classical complement pathway, led to the assumption that MBL could substitute for C1q and activate the classical pathway of the complement system (Ikeda et al. 1987). The collagenous regions of the collectins vary considerably in length in mammal collectins, especially for MBL comprising 19 Gly-Xaa-Yaa triplets. These are about 2-3 fold less than other collectins (e.g. 59 triplets in surfactant protein-D; SP-D). N-terminal to the collagenous region is a stretch of 7–28 residues of indeterminate structure, but, importantly, displaying 1–3 cysteine residues involved in the covalent interactions between the 3 polypeptide chains of the subunit and also responsible for covalent joining of several subunits into an oligomeric structure of up to 6 subunits (Holmskov et al. 2003). MBL forms multimeric (octadecamers) of this 6 trimeric subunits resembling a bouquet of flowers as in surfactant protein-A (SP-A) (van de Wetering et al. 2004). Characterization of cDNA that encoded MBL-A in rat revealed homologies to surfactant apoprotein A in that both molecules had C-terminal lectin–like domains and collagen tails that form trimers and then multimers of trimers up to hexamers of trimers (Drickamer et al. 1986).
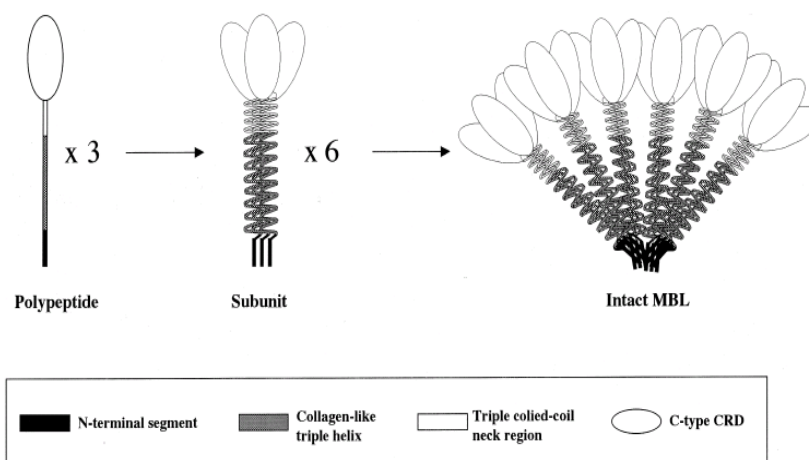
Figure 5:   Schematic representation of the domain organisation and structure of mannose-binding lectin modified from Laursen and Nielsen (2000)

Table 1:   Porcine MBLs characterization

Protein	Gene (Accession No.)		
-	-	First porcine MBLs were isolated and characterized	Storgaard et al. (1996)
-	*MBL2* (AF205528)	SNP (C to T at 328 bp) found to be segregating Somatic cell hybrid panel assigned location at SSC14q26-q29 Linkage mapping assigned location between S0007 and SW210 on SSC14	Marklund et al. (2000)
MBL-C	*MBL2* (NM_214125)	Porcine liver MBL cDNA of 723 bp ORF of 241 amino acid was obtained Shares overall identity to rat MBL-A and –C proteins of 50.2 and 56.7%, respectively	Agah et al. (2001)
MBL-A	*MBL1* (AY771222)	A 750 bp segment encoding MBL cDNA, with 249 amino acid Evidence indicated *MBL2* previously reported by Marklund is *MBL1*	Lillie et al. (2006)

2.4.2   MBL activation and its biological activity

MBL is considered as an important factor in the lectin pathway in innate immunity (Kilpatrick 2002). It is the only member of the collectin family of proteins to activate the complement system (Presanis et al. 2003). Its association to MASP according to the structural and genetic similarities between C1q and MBL and C1r/C1s and MASP can initiate the classical complement pathway. However, the biological relevance of MASP in the lectin pathway has not been strongly confirmed yet (Matsushita and Fujita 1992). In addition, MBL may also directly opsonize microorganisms for phagocytosis (Kuhlman et al. 1989). MBL synthesis is regulated and influenced by inflammatory status and other stimuli, and has been reported as an acute phase protein (Holmskov et al. 2003). MBL binds to immobilized mannan in a $Ca2^{+}$-dependent manner, and also

binds to a variety of phospholipids. Furthermore, MBL can also bind to a wide variety of microorganisms, including yeasts, bacteria, and viruses (Kilpatrick 2002).

2.4.3   MBL genetics

Two distinct, but related genes named *MBL1* and *MBL2*, were found encode MBL-A and –C, respectively in mammals. The genes encoding human MBL (Sastry et al. 1989), rodent MBL-A and -C (Sastry et al. 1991), rhesus monkey MBL-A and -C (Mogues et al. 1996), chicken MBL (Laursen and Nielsen 2000), bovine MBL-A (Lillie et al. 2006) and -C (Kawai et al. 1997) and pig MBL-C (Agah et al. 2001) have been sequenced and characterized. In addition, porcine MBL-A cDNA was (also) characterized and proposed as the porcine *MBL1* gene which is homologous to the rodent *MBL1* gene and *MBL1P1* pseudogene of humans and chimpanzees (Lillie et al. 2006).

2.4.3.1   Structure and sequence of the MBL gene

The human MBL is encoded by four exons of *MBL2* gene (Taylor et al. 1989), the first exon encodes a signal peptide, the N-terminal cysteine rich segment and part of the collagen-like region. The rest of the collagen-like region is encoded by exon 2. The neck is encoded by exon 3, while exon 4 encodes the CRD. For the *MBL1* gene in human, a pseudogene was reported (as *MBL1P1*) according to the posttranslational modification, it encodes a truncated 51-amino acid protein that is homologous to the MBL-A in rodents and primates (Guo et al. 1998). In rodents, rat MBL-A and MBL-C are encoded by 4 and 6 exons, respectively, while mouse MBL-A and MBL-C has 5 and 6 exons, respectively. The extra exons in the rodent genes encode 5'-untranslated regions of the mRNA (Laursen and Nielsen 2000).

2.4.3.2   MBL gene polymorphisms

In human, three SNPs identified in exon 1 causing amino acid Arg → Cys substitution at codon 52 (Madsen et al. 1994), Gly → Asp substitution at codon 54 (Lipscombe et al. 1993) and Gly → Glu substitution at codon 57 (Sumiya et al. 1991) were detected and have been widely known showing association with many innate immunological factors in human (Holmskov et al. 2003). The variation in MBL levels can be attributed to these

three structural mutations of the MBL gene which are likely to result in defective polymerization, interacting with several polymorphisms in the MBL promoter region which influence the level of expression (Presanis et al. 2003).

2.4.3.3   Positional and functional mapping of MBL gene

In human, *MBL2* gene was assigned to chromosome 10q11.2-q21 by a combination of Southern analysis of somatic cell hybrids and *In situ* hybridization (Sastry et al. 1989, Schuffenecker et al. 1991). The RFLP result indicated that the *MBL2* gene locus is close to the *MEN2A* locus. In rodents, White et al. (1994) mapped the gene for murine *MBL1* genes to chromosome 14 (MMU14) and 19 (MMU19), respectively. The murine *MBL1* gene is located on a region homologous to human chromosome 10 (HSA10). However, it has been found that murine *MBL2* gene location on MMU19 shows homology to HSA10, but does not include the MBL locus. Marklund et al. (2000) reported the chromosomal localization study of porcine *MBL1* which is thought at that time to be porcine *MBL2* (Lillie et al. 2006) by using a pig/rodent somatic cell hybrid panel (SCHP). The result showed that porcine *MBL2* is located on pig chromosome 14q26–q29. This was also confirmed by linkage mapping, showing that porcine *MBL2* is most likely located between *S0007* and *SW210* on SSC14.

3 Material and methods

3.1 Materials

3.1.1 Animals

For this study, F2 and backcross animals of a resource population based on the cross of Duroc and Berlin miniature pig (DUMI resource population) were used for determining the immunological competences and their association on porcine genetics using candidate gene approaches.

3.1.1.1 F2 DUMI resource population

The resource population used in molecular genetics study was generated based on the reciprocal cross of Duroc and Berlin miniature pig (Hardge et al. 1999). Five sows of Berlin miniature pigs were crossed with a Duroc boar and 4 Duroc sows were crossed with a Berlin miniature boar to produce F1 animals (parental generation). Fourteen F1 animals generated the F2 animals (n = 417) of Bonn-DUMI resource population (figure 6). F2 animals were reared and performance tested at the research farm of Frankenforst, Institute of Animal Science, University of Bonn. Theses animals were used for complement activity and other immune responsiveness evaluation. The piglets were vaccinated with a Mycoplasma vaccine at 6 wk of age, with an Aujeszky's vaccine at 14 wk of age and with a PRRS vaccine at 20 wk of age. Blood samples were taken from each piglet immediately prior to immunisation (day 0) and on day 4 and 10 after vaccination with the exception that after PRRS vaccination blood was only taken at day 0 and 10 (figure 7). Blood was cooled immediately. Serum and plasma were harvested within 2 hours then stored at -80°C for further analysis. A pool of sera from several pigs was used as a reference serum.
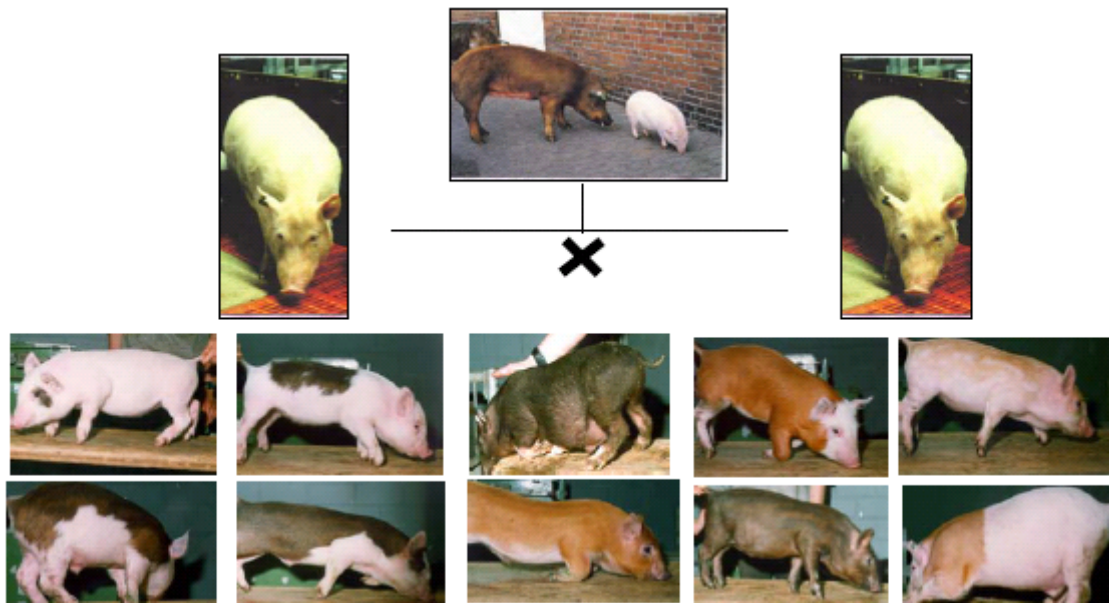
Figure 6:   F2 DUMI resource population

3.1.1.2   Backcross DUMI resource population

The experimental animal population used for immunological traits measurement and QTL analysis in this study was a DUMI backcross population, comprising three generations. The first generation (BC1) derived from twelve F2 DUMI pig sows (see 3.1.1.1) were crossed back with 6 Duroc boars. The second generation (BC2) was derived from the cross of 7 DUMI-BC1 sows and 1 Duroc boar. The third generation (BC3) was derived from the cross of 6 DUMI-BC2 sows and 3 Duroc boars. All backcross animals were reared and performance tested at the research farm of Frankenforst, Institute of Animal Science, University of Bonn and were used for complement activity and other immune competences evaluation. The piglets were vaccinated with a mycoplasma vaccine at 6 wk of age, with a TT vaccine at 9 wk of age and with a PRRS vaccine at 15 wk of age. Blood samples were taken from each piglet immediately prior to immunisation (day 0) and on day 10 and day 20 after mycoplasma vaccination (Time point 1, 2, 3), day 10 and day 20 after TT vaccination (Time point 4 and 5). The last blood sampling was taken at day 10 after PRRS vaccination (Time point 6) as demonstrated in figure 7. Blood was cooled immediately. Serum and plasma were harvested within two hours and stored at -80°C for further analysis. A pool of sera of several pigs was used as a reference serum.
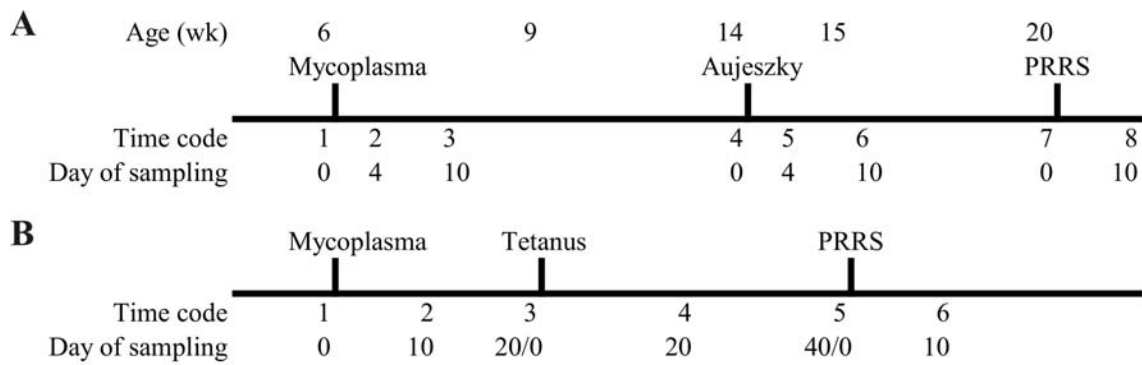
Figure 7:   Schematic display of vaccination program and time point of blood sampling from A) F2 and B) Backcross DUMI

3.1.2   Materials for laboratory analysis

3.1.2.1   Chemicals, biological materials, kits and other materials

Acris antibodies and abcam® (Hiddenhausen):	Sheep polyclonal Anti Human C3c Antibody, and Lambda DNA *Hind*III
Biomol (Hamburg):	Phenol
Biozym Diagnostik (Hessisch-Oldendorf):	Sequagel XR sequencing gel (National Diagnostics)
Clontech (Heidelberg):	SMART™ RACE cDNA Amplification Kit
Corning Incorperated (USA):	Costar® 3590 96 Well EIA/RIA Plate, and EIA Plate 9108
DakoCytomation (Denmark):	Polyclonal Rabbit Anti-Human C3c Complement Horseradish Peroxidase and Dako *Mycoplasma hyopneumoniae* ELISA kit
Gibco/BRL, Life Technologies (Karlsruhe):	Trizol™ reagent and SuperScript™ II reverse Transcriptase
IDEXX GmbH (Wörrstadt):	HerdChek® PRRS virus antibody test kit
MWG Biotech (Ebersberg):	Oligonucleotide primers
Promega (Mannheim):	DTT, pGEM®-T vector, Pfu DNA polymerase and 10x reaction buffer, Restriction endonucleases *Hind*I, 10 x BSA and 10x buffer, Rnase free-Dnase, Rnasin Ribo-nuclease inhibitor, RQ1 Rnase-free Dnase and 10 x buffer, T4 DNA ligase and 2x rapid ligation buffer

Qiagen (Hilden):	RNeasy Mini Kit (RNA purification kit), QIAquick PCR purification kit
Roth (Karlsruhe):	Acetic acid, Agar-Agar, Ampicillin, Ammonium peroxodisulphate (APS), Boric acid, Bromophenol blue, Chlorofrom, Dimethyl sulfoxide (DMSO), dNTP, EDTA, Ethanol, Ethidiumbromide, Flatted bottom 96-well microplates, Formaldehyde, Formamide, Glycerin, Hydrochloric acid, Hydrogen peroxide (30%), N,N´-dimethylformamide, Nitric acid, Peptone, Proteinase K, SDS, Silver nitrate, Sodium carbonate, Sodium chloride, Sodium hydroxide, TEMED, Tris,
Sigma (Germany):	Oligonucleotide primers
Sigma-Aldrich (Taufkirchen):	Agarose, Anti-Pig IgG (whole molecule) Peroxidase conjugate, Anti-sheep red blood cell stroma (Hemolysin), Blue dextran, Calcium chloride, Diethyl barbituric acid, EGTA, Gelatin, Foetal calf serum, Isopropanol, Magnium chloride, o-Phenylendiamine dihydrochloride, Phosphate-citrate buffer with Sodium perborate capsules, Sodium barbituric acid, Streptavidin-peroxidse conjugate, *Taq* polymerase, TRI REAGENT^{TM} and Tween 20.
WDT (Garbsen):	EquimaTe® Tetanus toxoid vaccine

3.1.2.2   Buffers and reagents

All solutions used in this investigation were prepared with deionised Millipore water and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

Alsever's solution	Dextrose	20.5	g
	tri-Sodium citrate dihydrate	8.0	g
	Sodium chloride	4.2	g
	Citric acid monohydrate	0.6	g
	Water added to	1000.0	ml

APS solution:	Ammoniumpersulfat	5.0	g
	Water added to	50.0	ml
Coating buffer	Sodium hydrogen carbonate	1.6	g
	di-sodium hydrogen carbonate	42.0	g
	Water added to	1000.0	ml
Dextran blue buffer:	Dextran blue (50mg/ml)	1.0	ml
	EDTA (0.5 M)	50.0	μl
	Formamide	5.0	ml
Digestion buffer:	Sodium chloride	2.5	ml
	Tris	2.5	ml
	EDTA	0.1	ml
	Water added to	50.0	ml
EGTA-Mg	EGTA	38.4	g
	Magnesium chloride	100	ml
	Water added to	1000.0	ml
	pH 7.5		
10 × FA buffer:	MOPS	41.8	g
	Sodium acetate	4.1	g
	EDTA (0.5M)	20.0	ml
	Water added to	1000.0	ml
	pH 7.0		
FA-gel (1.2 %)	Agarose	1.2	g
	10 × FA buffer	1.0	μl
	Ethidium bromide	1.0	μl
	Formaldehyde (37%)	20.0	μl
	Water added to	100.0	ml

GVBSS:	5× Veronal buffer saline (VBS)	200.0	ml
	2% Gelatine	25.0	ml
	Magnesium chloride	2.0	ml
	Calcium chloride	0.6	ml
	Water added to	1000.0	ml

GVBS-Mg-EGTA:	5× VBS	200.0	ml
	EGTA-Mg	160.0	ml
	2% Gelatine	50.0	ml
	Water added to	100.0	ml

| IPTG solution: | IPTG | 1.2 | g |
| | Water added to | 10.0 | ml |

LB-agar plate:	Sodium chloride (Roth)	8.0	g
	Peptone (Roth)	8.0	g
	Yeast extract (Roth)	4.0	g
	Agar-Agar (Roth)	12.0	g
	Sodium hydroxide, 1N (Roth)	480.0	μl
	Water added to	800.0	ml

LB-broth:	Sodium chloride (Roth)	8.0	g
	Peptone (Roth)	8.0	g
	Yeast extract (Roth)	4.0	g
	Sodium hydroxide, 1N (Roth)	480.0	μl
	Water added to	800.0	ml

PBS:	Sodium chloride	8.0	g
	di-sodium hydrogen phosphate	1.5	g
	Potassium dihydrogen phosphate	2.0	g
	Potassium chloride	0.2	g
	Water added to	1000.0	ml

Polyacrylamidgel:	Sequagel XR	20.0	ml
	Sequagel buffer	5.0	ml
	APS (10%)	250.0	µl
	Dimethylsulfoxid	200.0	µl
SDS solution (10%):	Sodium dodecylsulfat	10.0	g
	Water added to	100.0	ml
TAE (50×) buffer	Tris	242.0 mg	mg
	Acetic acid	57.1 ml	ml
	EDTA (0.5 M)	100.0 ml	ml
	Water added to	1000.0 ml	ml
	pH 8		
TBE (10×) buffer:	Tris	108.0 g	g
	Boric acid	55.0 g	g
	EDTA (0.5 M)	40.0 ml	ml
	Water added to	1000.0 ml	ml
TE buffer:	Tris (1 M)	10.0 ml	ml
	EDTA, (0.5 M)	2.0 ml	ml
	Water added to	1000.0 ml	ml
5× Veronal Buffer (VBS)	Diethyl barbituric acid	2.9	g
	Sodium barbiturate	1.9	g
	Sodium chloride	42.5	g
	Water added to	1000.0	ml
X-gal:	X-gal	50.0	mg
	N,N´-dimethylformamide	1.0	ml

3.1.2.3 Software used

BLAST program	http://www.ncbi.nlm.nih.gov/blast/
CRIMAP	Version 2.4 (Green et al. 1990)
DNA to protein translate tool	http://us.expasy.org/tools/dna.html
Image Analysis (version 4.10)	LI-COR Biotechnology, USA
MapChart2.2	http://www.biometris.nl/uk/Software/MapChart/
MEGA3	http://www.megasoftware.net/mega.html
Multiple Sequence Alignment	http://saturn.med.nyu.edu/searching/promultali.html
	http://prodes.toulouse.inra.fr/multalin/multalin.html
OneDScan	Scanalytics Inc., Billerica, MA
PedCheck	http://watson.hgen.pitt.edu/register/docs/pedcheck.html
Pig Genome	http://linkage.rockefeller.edu/soft/list.html
	http://www.marc.usda.gov/genome/genome.html
	http://www.genome.kvl.dk/piggenome/misc.html
	http://www.genome.iastate.edu/pig
	http://nitro.biosci.arizona.edu/zbook/book.html
QTL-express	http://qtl.cap.ed.ac.uk/ (Seaton et al. 2002)
Restriction enzyme analysis	http://www.firstmarket.com/cutter/cut2.html
	http://tools.neb.com/NEBcutter/index.php3
RH-Panel	http://imprh.toulouse.inra.fr/
Primer design	Primer Express -ABI prism
	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
SAS version 8.02	SAS Institute Inc., Cary, NC
Soft Max Pro Software	Molecular Devices, USA

3.1.2.4 Equipments

Automated sequencer (LI-COR 4200)	MWG Biotech, Ebersberg, Germany
Centrifuge	Hermle, Wehingen, Germany
Carbon dioxide incubator	Heraeus, Hanau, Germany
CEQ™ 8000 Genetic Analysis System	Beckman Coulter, Krefeld, Germany
Electrophoresis (for agarose gels)	BioRad, München, Germany

Thermomax ELISA reader   Molecular Devices, USA

PCR thermocycler (PTC100)   MJ Research, USA & BioRad, Germany

Power Supply PAC 3000   BioRad, München. Germany

Spectrophotometer UV/visible light   GE Amersham Biosciences, Germany

UV Transilluminator (Uvi-tec)   Uni Equip, Martinsried, Germany

3.2   Immunological methods

3.2.1   Haemolytic complement activity

The activity of complement system was determined in both alternative and classical pathways in this study. Its activity is measured based on the change in light-scattering properties of lysed sheep red blood cells as previously described (Liu and Young 1988).

3.2.1.1   Classical pathway activity

The method used to measure total complement activity was modified from Liu and Young (1988) and Demey et al (1993). It is based on the measurement of the change in light-scattering properties of erythrocytes upon lysis. The test conditions were evaluated and standardised for the assay of pig complement. Sheep red blood cells (SRBCs) were collected from healthy animals into modified Alsever's solution and stored at 4 °C. A commercially prepared antibody, haemolysin (Sigma), was used to sensitize SRBCs. Sensitised SRBCs were used as a 2 % cell-suspension. Serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) of test sera (50 µl) in GVBS buffer (50 µl) were made in replicates of 2 in flat-bottomed 96 wells microtiter plates. To each serum dilution, 50 µl of sensitised SRBCs were added and the plates were incubated for 90 min at 37 °C. The results were read at 650 nm in a microplate reader (ThermoMax ELISA reader and Soft Max Pro software, Molecular Devices, USA). A pool of sera of several pigs was used as a reference. The reading results were transformed according to the method of von Krogh (1916) and the haemolytic titre was expressed as the titre that lysed 50 % of the erythrocytes (CH_{50}, U/ml).

3.2.1.2   Alternative pathway activity

Twenty-five microlitres of diluted sera (1:2, 1:3, 1:4 and 1:6) with GVBS-Mg-EGTA buffer and 25 µl of 0.5 % (v/v) rabbit erythrocytes were incubated at 37 °C for 90 min in duplicate wells of flatted bottom 96-well microplates (Roth). A 150 µl GVBSS-Mg-EGTA buffer was added in each well and the absorbance values at 650 nm were read by a microplate reader (ThermoMax, Molecular Devices). A pool of sera of several pigs was used as a reference. Data were transformed by the method of von Krogh equation according to Mayer (1961). The activity was expressed in term of serum amount which causes 50% haemolysis of rabbit erythrocytes (AH_{50}, U/ml).

3.2.2   Immunoassays

3.2.2.1   Antibody response to mycoplasma vaccination

The antibody response to M. hyopneumoniae vaccination was determined by a monoclonal blocking ELISA (Feld et al. 1992) using the M. hyopneumoniae ELISA kit (DakoCytomation, Hamburg, Germany). A peroxidase-conjugated monoclonal antibody to M. hyopneumoniae 74 kDa protein epitope competes with antibodies in the serum samples to bind with a M. hyopneumoniae antigen, which was previously coated in the microplate. The porcine serum samples were diluted 1:10 and added in the wells pre-coated with the antigen. After incubation for 90 min at room temperature, a specific peroxidase conjugated monoclonal antibody to M. hyopneumoniae was added into the wells to compete with an antibody in the samples. The conjugate and the samples were incubated together for 15 min and the wells were washed. A chromogenic o-phenylendiamine substrate (OPD) was added and the colour reaction was developed in dark for 10 min and stopped by adding sulphuric acid. The optical density (OD) at 490 nm was read using a microplate reader (ThermoMax, Molecular Devices). The results were interpreted as percentage of the buffer control wells (Buffer + conjugate + chromogen), and samples which had an OD of less than 50 % of the buffer control were regarded as positive sample.

3.2.2.2   Antibody response to tetanus toxoid vaccination

Microtiter plates were coated with 100 µl of antigen (0.2 µg/µl coating buffer) by incubation for 2 h at 37 °C. The plates were washed 4 times with 200 µl of PBS-Tween 20 buffer. The plates were blocked with 1 % (v/v) foetal calf serum (FCS) in PBS-Tween 20 at 37 °C for 60 min, and then washed as described above. The test sera were diluted 1:100 with PBST containing 1 % (v/v) FCS and 100 µl of diluted sera were added in duplicate to the antigen coated wells. The plates were incubated at room temperature for 60 min and washed 3 times with PBS-Tween 20. One hundred µl of horseradish peroxidase conjugated rabbit antibody against pig IgG (Sigma-Aldrich, Munich, Germany) diluted to 1:10,000 in PBS-Tween 20 was added to the plates and incubated at 37 °C for 60 min then washed as described above. Then o-phenylendiamine dihydrochloride, OPD solution was added in 100 µl volume to the wells. The plates were incubated at room temperature for 30 min and the reaction was stopped with 50 µl of 2 M sulphuric acid (H_2SO_4). The OD values were determined with the ThermoMax ELISA reader at a wavelength of 490 nm. The result of antibody to TT vaccination was expressed as S/P:

$$S/P = \frac{(\text{mean of sample OD}_{490}) - (\text{mean of blank OD}_{490})}{(\text{mean of positive control OD}_{490}) - (\text{mean of blank OD}_{490})}$$

3.2.2.3   Antibody response to PRRS vaccination

Serum samples from pigs at 10 days after PRRS vaccination were measured for antibodies against PRRS virus by enzyme immunoassay using a HerdChek[®] PRRS virus antibody test kit (IDEXX's, Ludwigsburg, Germany). Microtiterplate wells were coated with PRRS virus and normal host cell (NHC) antigen in alternating wells. The NHC antigens coated on the plate are used to assess whether antibody against tissues culture component present in vaccine are contributing to test results. The serum samples were diluted (1:40) and added to duplicate wells coated with PRRS and NHC antigen. The positive and negative control sera were included in every plate of the assay. After incubation of 30 min at room temperature, plates were washed and an anti-porcine horseradish peroxidase conjugate was added to each well. Plates were incubated for 30 min at room temperature and were washed with washing buffer to remove unbound

anti-porcine conjugate. The substrate (hydrogen peroxide) and chromogen (3, 3′, 5, 5′ tetramethylbenzidine) were added into each well and incubated for 15 min at room temperature. The enzymatic colour reaction was stopped with hydrofluoric acid. The optical density was determined with a microplate reader (ThermoMax, Molecular Devices) at a wavelength of 650 nm. The antibodies to PRRS are expressed as S/P:

$$S/P = \frac{(\text{mean of sample OD}_{650}/ \text{PRRS well}) - (\text{mean of sample OD}_{650}/ \text{NHC})}{(\text{mean of PC OD}_{650}/ \text{PRRS well}) - (\text{mean of PC OD}_{650}/ \text{NHC})}$$

3.2.2.4 Complement component 3 (C3c) concentration

Serum C3c concentration of individual porcine serum samples prior and after vaccinations were measured by ELISA. Microtiter plates (EIA plate 3590, Corning Costar®, New York, USA) were coated with anti-human C3c (sheep polyclonal to human C3c, abcam®, UK) in a volume of 100 µl (diluted 1/100 in coating buffer, pH 9.6) per well at 37 °C for 2 h. The plates were washed 4 times with 200 µl of PBS-Tween 20 buffer. The plates were blocked with 1 % (v/v) FCS in PBST at room temperature for 60 min, and then washed as described above. The serum samples were diluted 1:40 with PBS-Tween 20 containing 1 % (v/v) FCS and 100 µl of diluted sera were added in duplicate to the coated microtiter plate wells. The plates were incubated at 37 °C for 60 min and washed 4 times with PBS-Tween 20. One hundred µl of horseradish peroxidase conjugated polyclonal rabbit anti-human C3c (Dako Cytomation, Germany) diluted to 1:500 in PBST was added to the plates and incubated at 37 °C for 60 min then washed for four times. Then, o-phenylendiamine dihydrochloride, OPD solution was added in 100 µl volume to the wells. The reaction was stopped by 100 µl of 2 M sulphuric acid (H2SO4) after 30 min. The OD was determined using a microplate reader (ThermoMax, Molecular Devices) at a wavelength of 490 nm. The results of complement component C3c concentration were expressed as S/P:

$$S/P = \frac{(\text{mean of sample OD}_{490}) - (\text{mean of blank OD}_{490})}{(\text{mean of positive control OD}_{490}) - (\text{mean of blank OD}_{490})}$$

3.2.2.5   Haptoglobin concentration

Porcine Hp concentrations were measured by enzyme immunoassay as described by Hiss et al. (2003). Microtiter plates were coated with anti-rabbit-Fc fragment antibodies generated in sheep by incubating 150 ng of this sheep IgG in a volume of 100 μl 50 mM NaHCO3 (pH 9.6) per well at 4 °C for 20 h. After decanting, a second coating with 300 μl 2.5% casein in 0.05 M NaCl (pH 7.4) was done to saturate potentially remaining binding sites of the plastic surface at room temperature for 1.5 h. The plates were then decanted and washed five times with washing buffer. They were filled with assay buffer to avoid desiccation and were stored at 4 °C up to several weeks without appreciable loss of sensitivity. The plates were decanted and 50 μl of biotinylated porcine Hp, diluted 1/1000 in assay buffer containing 1 mg haemoglobin per ml were added in duplicate. Purified porcine Hp was used as standard at the same volume in HEM buffer. Serum samples were first diluted 1/10,000 in HEM buffer and 50 μl were then pipetted per well. After adding 50 μl rabbit anti-Hp serum the plates were incubated for 1 h. After three washes using a microtiter plate washer (EL404, Bio-Tec Instruments, Winooski, VT, USA), 100 μl of a streptavidin-peroxidase conjugate solution (200 ng/ml assay buffer; Sigma-Aldrich, Germany) were added per well. After 30 min and five further washes, the wells were filled with 150 μl of a freshly prepared substrate solution containing 0.05 M citric acid, 0.055 M Na2HPO4, 0.05% urea hydrogen peroxide, 2 % ProClin 150®, and 2 % of a tetramethylbenzidine solution (12.5 mg/ml DMSO). The reaction was stopped after 30 min with 1 M of moxalic acid and dye development was determined photometrically at 450 nm in a microtiter plate reader (ELX800, BioTec Instruments). The Hp concentration in unknown samples was calculated from the standard curve using the four-parameter method.

3.3   QTL analysis

3.3.1   Selection of markers and genotyping methods

In this study, 74 microsatellite markers were selected covering all 18 autosomes of the pig. The markers were selected according to previous at the Institute of Animal Science, University of Bonn, Germany (Liu 2005, Oltmanns 2003, Ün 2002). These markers were mainly selected from the USDA/MARC map. Names and genome position in Centimorgan (cM) units of markers used in this (http://www.marc.usda.gov/genome/

swine/swine.html) are demonstrated in figure 8. Note that the names of the markers used and the position are indicated on the right and left side of the bar, respectively.
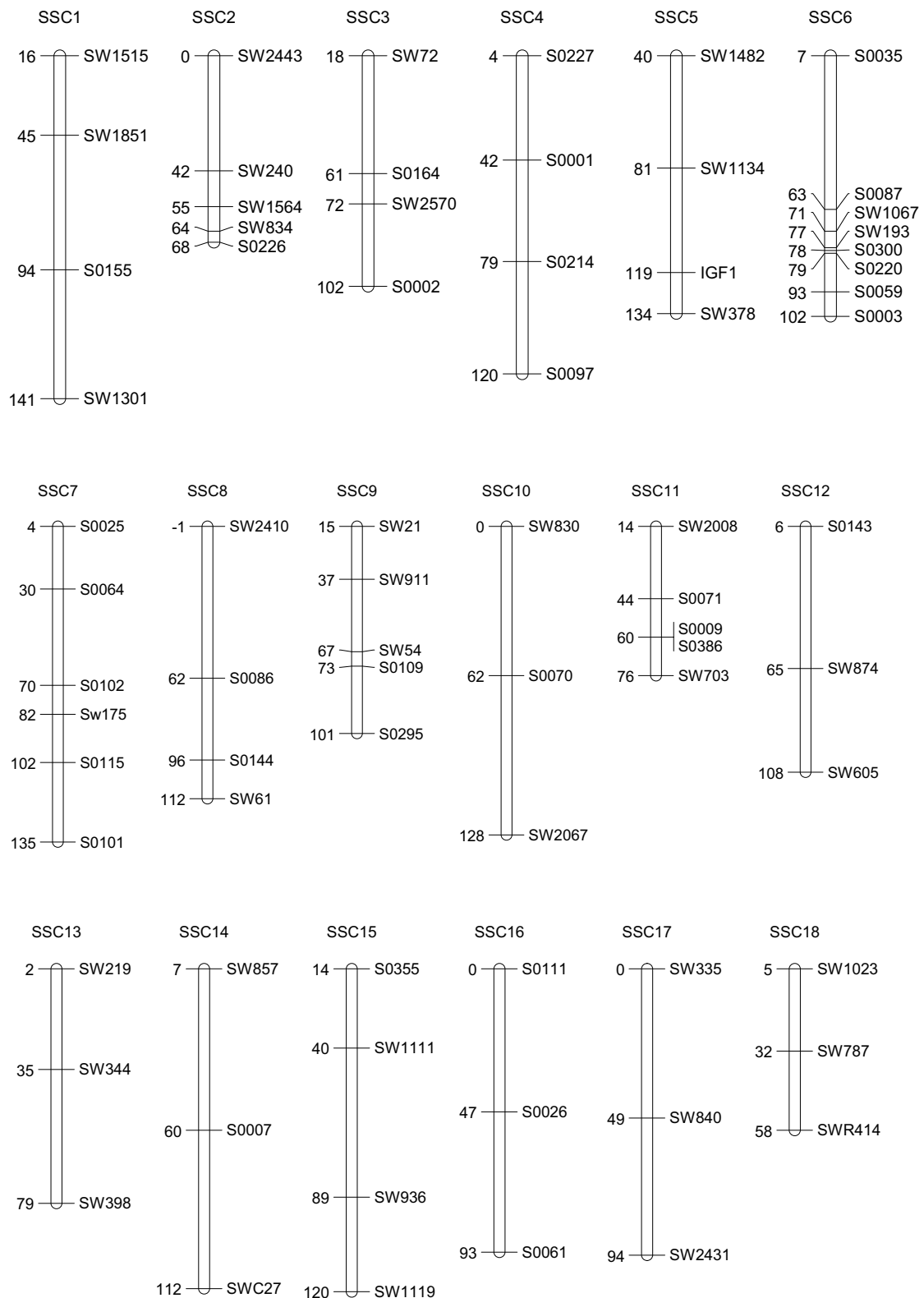
Figure 8:   Positions and names of microsatellite markers used in this study

Tissue samples from tail of the backcross animals were collected at the research farm of Frankenforst, Institute of Animal Science, University of Bonn. Genomic DNA was isolated following the protocol of phenol-chloroform extraction, as described in chapter 3.4.1. Genotyping, electrophoresis, and allele determination were done by laboratory technicians at the Institute of Animal Science. Different PCR protocols were used. Mainly, multiplex PCR were performed containing 2 to 4 primer pairs. Primers used for multiplex reaction showed at least more than 30 bp difference between the bands with similar annealing temperatures. Single and multiplex PCR were carried out in 12.5 µl reaction volume included 50 ng of genomic DNA, 0.2 µM of each primer, 50 µM of dNTP, 0.5 units of Supratherm DNA Polymerase (GeneCraft, Lüdinghausen, Germany) in 1xPCR buffer containing 1.5 mM $MgCl_2$. The PCR amplifications were performed using standard and touchdown protocols as described previously by Liu (2005), Oltmanns (2003) and Ün (2002).

PCR products were run in a LI-COR 4200 Automated Sequencer. Polyacrylamide gels were mixed by adding 20 ml Sequagel XR, 5 ml Sequagel buffer, 250 µl DMSO and 200 µl APS (10%). Glass plates were prepared by cleaning with ethanol (75%), application of 60 µl haftsilane on the area where the comb was placed and two plates were fixed together with two spacers (0.04 mm) on the left and right side. The gel solution was filled between the plates, the comb inserted and the gel was allowed to polymerise for one hour. The PCR products were diluted 1:10 with dextran blue buffer and loaded on the 6% polyacrylamide gel. PCR products with a standard size were loaded as markers on both ends of the gel. Electrophoresis was performed in 1×TBE-buffer at 50 °C, 50 W, 40 mA and 1500 V. Each gel was loaded 2 to 5 times. The gel image data were analysed by using Image Analysis program, version 4.10 (LI-COR Biotechnology). The allele sizes were determined with the software 1D Scan. The fragment lengths were calibrated for each sample in relation to the size of the standard PCR products (75bp, 100bp, 105bp, 120bp, 145bp, 175bp, 200bp, 204bp, 230bp, 255bp, 300bp and 325bp). The fragment sizes were identified with an interval of 2bp and assigned to an Excel spreadsheet with the report function.

3.3.2 Statistical analysis of QTL

3.3.2.1 Characterization of markers

To characterize the markers used in this study, two different measurements were performed. The heterozygosity was used to identify the allelic diversity or the informativeness of genetic markers. The informativeness of a genetic marker increases as the heterozygosity increases. The informativeness of genetic markers in outbred species can also be measured by the polymorphic information content, the PIC (Botstein et al. 1980). The heterozygosity of a genetic marker is estimated by:

$$H = 1 - \sum_{k=0}^{k} p_i^2$$

where:

      p_i: the frequency of the ith allele

      k: the number of alleles.

      (Nei 1978, Otto and Goldstein 1992)

The PIC is estimated by:

$$PIC = 1 - \sum_{i=1}^{k} p_i^2 - \sum_{i=1}^{k} \sum_{j=l+1}^{k} p_i^2 p_j^2$$

where:

      p_i: the frequency of the ith allele

      k: the number of alleles.

      (Botstein et al. 1980, Otto and Goldstein 1992)

Regarding a codominant genetic marker, the PIC was developed for ascertaining the allele transmitted by an affected heterozygous parent carrying a dominant disease allele (Otto and Goldstein 1992). The PIC estimates the probability that the codominant marker genotype of an offspring can be used to deduct which of the two marker alleles were transmitted by a parent carrying a dominant disease allele. The term polymorphic information content is alternatively and frequently used for heterozygosity and possibly other measures of marker informativeness.

3.3.2.2   Construction of genetic maps

The data obtained from the fragment sizes analysis were firstly checked for any genotyping errors using Pedcheck, version 1.1 (O'Connell and Weeks 1998). The following multipoint analyses were carried out for female, male, and sex-average maps using the CRIMAP software, version 2.4 (Green et al. 1990). A two-point linkage analysis of recombination between two markers was used. Furthermore, the 'fixed' function was used for known order of markers, including 'flipsn', 'all' and 'build' functions. For the resulting map, CRIMAP calculated the expected recombination between markers. The recombination units were converted into map distances using Kosambi's mapping function (Kosambi 1944). The generated sex-average recombination units can be converted to Kosambi centimorgan by:

$$M = 1/4 \ln (1+2R/1-2R),$$

where:

M   = map distance in Morgan

R   = recombination

3.3.2.3   Identification of QTL

Using a regression approach, we calculated QTL with effects on immune traits measured in this investigation. A QTL interval mapping analysis was performed using the web-based program QTL express available at http://qtl.cap.ed.ac.uk/ (Seaton et al. 2002).

The QTL-express program including backcross/F2 dataset was used following an additive and dominant model with permutated chromosome-wide permutations at a total of 10,000 iterations. The chromosome-wide analysis was done by measuring QTL for all traits at the same time. As fixed effects, we used cross type mean (CTM) and sex. No interactions between the fixed effects and the traits were used in the QTL analysis. It is meaningful to include the litter number as fixed effect, but all animals used in this analyses were derived only from the first litter each sow.

Based on a robust two-step procedure for QTL mapping in the QTL express program, marker genotypes were used to estimate the identity-by-descent (IBD) probabilities at 1-cM intervals through the chromosomes. These probabilities are used to calculate

additive and dominance coefficients for a putative QTL at each position and the trait values are regressed onto these coefficients to calculate *F*-ratios testing the existence of a QTL at given position. Linear models are fitted to phenotypic data using a general linear model. For the genetic component in the linear model, a single or a two QTL model is fitted (Green et al. 1990). The regression analysis of the backcross population calculates transmission probabilities using a simple algorithm. The estimable allele substitution effect is defined by QQ-Qq, which contains both an additive and a dominance part. If the effect of the recurrent QTL genotype is larger than the effect of the heterozygous genotype, the value is positive. The QTL regression model for single analysis was:

$$y_{ijk} = \mu + s_j + f_k + \beta\,cov_{ijk} + c_{ai}a + c_{di}d + \varepsilon_{ijk},$$

where:

y_{ijk} = phenotype of the ith offspring

μ = overall mean

s_j = jth fixed sex effect, j = 1, 2

f_k = kth fixed contemporary group effect

β = regression coefficient on the covariate

cov_{ijk} = covariate (CTM and sex)

c_{ai} = additive coefficient of the ith individual at a putative QTL in the genome

c_{di} = dominant coefficient of the ith individual at a putative QTL in the genome

a = additive effects of a putative QTL

d = dominant effects of a putative QTL

ε_{ijk} = residual error

3.3.2.4   Significant threshold

To reduce results showing false positive significance, significant thresholds were determined for all QTL analysis. Errors of type I (false positive) and errors of type II

(false negative) are possible in any statistical analysis, whereas errors of type I play an important role in a QTL analysis.

A total of 10,000 permutations were performed for each chromosome * trait combination. The chromosome-wide 1% and 5% significance thresholds were calculated by QTL express. The 1% and 5% experiment-wide significant threshold were calculated by transformation with Bonferroni correction for 18 autosomes of the haploid porcine genome. As there were no markers genotyped on the X-chromosome, transformation was done only for an experiment-wide, not for a genome-wide significant threshold level. The significant thresholds at the 5 and 1% level were determined empirically by permutation test for individual chromosomes (Churchill and Doerge 1994). This empiric method uses distribution of data from genotypes and phenotypes. The experiment-wide significance level was calculated by the following term:

$$Pg = \frac{1 - (1 - Pc)}{r}$$

where:

     r   = length of a specific chromosome/ sum of length of all chromosomes

     Pc   = chromosome-wide significance threshold

Different from the method and the chosen significance threshold used in this study, some publications use the "drop-off" method by Lander and Kruglyak (1995). By this method in a F2 population, an F-value of 4.3 is necessary for reaching a 1% significant threshold within a genome-wide experiment and a significant QTL. Analysis resulting in a suggestive QTL and a significance threshold of 5% should have F-values of not less than 3.0 (Lander and Kruglyak 1995). This "drop-off" method tends to give underestimated confidence intervals.

3.4   Molecular genetic methods

The basic molecular genetic methods including DNA isolation, polymerase chain reaction (PCR), and sequencing are described in this chapter. Moreover, semiquantitative RT-PCR used for gene expression studied was also explained. In order

to characterize the porcine MBL genes, chromosomal assignment, linkage mapping, gene polymorphism detection and genotyping were done. These experiments were performed in the F2 DUMI population.

3.4.1 DNA isolation

Genomic DNA was isolated from tissue samples. Tissue samples of about 0.1 g were cut into small pieces (2-3) and put it into 2 ml tube, and then 700 µl of digestion buffer, 70 µl of 10% SDS, and 18 µl of proteinase K to digest protein were added and well mixed it well. Samples were incubated overnight at 37 °C, 90 rpm. Seven hundred microlitres of phenol-chloroform were added and then the tube was shaken until an emulsion was formed and centrifuged at 10,000 rpm for 10 min. The upper part was transferred into a 2 ml tube, 700 µl of chloroform were added and shook gently. Samples were centrifuged at 10,000 rpm for 10 min. The upper part was carefully collected into 1.7 ml tube, 700 µl of isopropanol and 70 µl of sodium acetate were added into the tube and the samples were shaken gently until precipitation of DNA and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was discarded, then 200 µl of 70% ethanol were added, the pellet liberated from the tube surface then centrifuged at 10,000 rpm for 5 min. The aqueous phase was discarded and the pellet left to dry. Five-hundred microlitres of 1x TE were added in each tube and the DNA samples were left overnight at room temperature. The DNA samples were diluted to a concentration of 50 ng/µl.

3.4.2 Polymerase chain reaction (PCR)

Standard PCR, in 20 µl reaction volume included 100 ng of genomic DNA, 0.2 µM of each primer, 50 µM of dNTP, 0.5 units of Biotherm or Supratherm DNA polymerase (GeneCraft, Lüdinghausen, Germany) in 1xPCR buffer containing 1.5 mM $MgCl_2$. Thermocycling was performed as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 59 °C for 30 sec, 72 °C for 1 min, and final extension at 72 °C for 10 min. Additionally, touchdown PCR was also conducted to amplify *MBL1* using the following conditions: initial denaturation at 95 °C for 5 min, followed by 8 cycles at 94 °C for 30 sec, 66-62 °C for 30 sec, 72 °C for 1 min (annealing temperature was 0.5 °C decreased each cycle), then followed by 35 cycles of

94 °C for 30 sec, 62 °C for 30 sec, 72 °C for 1 min, and final extension at 72 °C for 5 min. Gene-specific primers used are described in table 2. All PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

Table 2:   Gene-specific primers (5´-3´) used for porcine MBL genes

Primer set	Sequence	Annealing temp. (°C)	Product size
MBL1-a	CCCCAATATTTCCTGGAGGT TCCTCCTTCTGTGTGTGGTG	59	222bp
MBL2-a	GGGAGAAAAGGGAGAACCAG CACACAGAGCCTTCACTCCA	59	278bp
MBL1-b	AAGGGAGAACCAGGTATAGG TGAACCCTGGCCCTGTTG	62-66	702bp
MBL2-b	CTTCGCTCAGGGAAAACAAG GTCATTCCACTTGCCATCCT	59	319bp

3.4.3   Sequencing

PCR products of porcine *MBL1* and *MBL2* genes were generated using standard protocols as described above. DNA was purified from the agarose gel using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Samples were excised from gels, recorded the weight, and put in the spin column. Three sample volumes of QG buffer (Qiagen) were added to the samples and incubated at 50 °C for 5 min until the gel was completely melted. One sample volume of 100% 2-propanol (Roth, Karlsruhe, Germany) was added and mixed by inverting. The spin column was placed into the collection tube, followed by centrifugation for 1 min at 18,000 rpm at 19-20 °C. After the flow-through liquid was discarded, this cleaning up procedure was repeated two times and the buffer was replaced by 500 µl QG and 750 µl PE buffer (Qiagen), respectively. The spin column containing the sample was moved to a new collection tube. Fifty microlitres of dd.H2O were added into the column and incubated at room temperature for 5 min. Centrifugation for 1 min at 18,000 rpm at 19-20 °C was done to collect the dissolved sample in the tube. Dehydration was performed and the sample was diluted by adding 10 µl of dd.H2O for sequencing PCR. Five microlitres of purified

sample was used for cycle sequencing, with specific primers (table 2), and the Quick Start Kit for Dye Terminator Cycle Sequencing or DTCS (BeckmanCoulter, Krefeld, Germany) including DNA polymerase, pyrophosphatase, buffer, dNTPs and dye terminators. Once, sequencing the PCR was performed, three molar NaOAc, 100 mM EDTA and glycogen were added to stop the reaction. To each sample, 60 µl of 98% ethanol (Roth) was added and mixed well by vortexing and then centrifuged for 15 min at 18,000 rpm at 4 °C. All liquid was removed and replaced with 200 µl 70% ethanol without mixing and centrifuged again for 15 min at 18,000 rpm at 4 °C. The ethanol was then removed and the sample was air dried for 10 min. The sample was then resuspended in 40 µl of sample loading solution (SLS) (Beckman Coulter). Cleaned up samples were transferred manually to a CEQ sample plate and overlaid with mineral oil. Samples were sequenced using CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

3.4.4   Expression study of porcine MBL genes

In order to survey expression of the porcine *MBL* genes in different tissues, RT-PCR was employed. Total RNA was isolated from muscle, heart, spleen, tonsil, lymph node, lung, liver, kidney, testis and brain from adult animals of Duroc and Berlin Miniature pig crossbreds using Tri-Reagent (Sigma, Taufkirchen, Germany) following the manufacturer's instruction. All RNA samples were treated with deoxyribonuclease I (DNase I, Promega, Mannheim, Germany) in the presence of RNase inhibitor (Promega) for 1 h at 37 °C to remove the residual DNA. DNA free RNA products were obtained after purification with RNeasy Mini kit (Qiagen, Hilden, Germany). The RNA was visualized on 1.5 % formaldehyde containing agarose gel to check the integrity, the concentration was measured by Ultraspect-photospectrometer (Amersham Bioscience). First-strand cDNA was synthesized from 1 µg of total RNA using oligo (dT)12N and random primers in the presence of the superscript reverse transcriptase enzyme (Invitrogen, Karlsruhe, Germany). Standard protocols as described above using MBL1-a, and MBL2-a primer sets for *MBL1* and *MBL2,* respectively (table 2) were done to detect *MBL1* and *MBL2* transcripts. In order to control for possible variation of the initial RNA input, the expression of the *18S* gene was used as an internal control.

3.4.5 Single nucleotide polymorphism (SNP) detection

A part of the porcine *MBL2* gene was amplified from 15 pigs of the F2 DUMI resource population by using standard PCR with the MBL2-b primer set. Primers (MBL2-b; table 2) were designed based on the conserved region between exon 4 of the human and porcine *MBL2* genes (GenBank accession no. NP_000233 and NM_2141259). The target products were sequenced using CEQ™ 8000 Genetic Analysis System (Beckman Coulter). Individual sequences were aligned and compared using the web-based program "multalin" (http://ribosome.toulouse.inra.fr/multalin/multalin.html) for identification of sequence variation. In order to detect a SNP in porcine *MBL2* gene, multiple sequence alignment was employed. The identified SNPs were used for genotyping analysis.

3.4.6 Genetic and physical mapping of porcine MBL genes

Radiation hybrid mapping of *MBL* genes was performed using the INRA-Minnesota 7000 rads radiation hybrid panel (IMpRH) (Yerle et al. 1998) which consisted of 118 hamster-porcine hybrid cell lines (Hawken et al. 1999). Primers were developed for both porcine *MBL* genes (GenBank accession no. AF208528 and NM_214125), and the entire RH panel was scored by PCR using the INRA protocol available at http://www.toulouse.inra.fr/lgc/lgc.htm. Data analysis was performed using software available at IMpRH database (http://imprh.toulouse.inra.fr) for chromosome assignment. Genetic mapping by two-point linkage analysis using CRIMAP 2.4 (Green et al. 1990) was also done.

3.4.7 Genotyping of porcine MBL genes

PCR restriction fragment length polymorphism (PCR-RFLP) using *Hinf*I and *Ade*I restriction enzymes for *MBL1* and *MBL2* polymorphic sites were performed. For the *MBL1* gene, animals were genotyped at a previously identified C to T substitution within intron 1 at position 328 of the sequence (GenBank accession no. AF208528), reported by Marklund et al (2000). For the *MBL2* gene, animals were genotyped at the G to A transition at position 645 of sequence NM 214125 as found in this study. The fragments covering polymorphic sites of both genes were amplified using specific

primer sets (MBL1-b, MBL2-b; table 2) as given above with touchdown and standard conditions for *MBL1* and *MBL2*, respectively. Digestion of the products was carried out in 10 µl of 10x restriction buffer and incubated at 37 °C overnight to ensure complete digestion. Digested PCR products were visualized on 2.5 % agarose gels to identify the fragment pattern. Data were checked for any genotyping errors by using the program Pedcheck version 1.1 (O'Connell and Weeks 1998).

3.4.8   Phylogenetic analyses of MBL genes

In order to identify the relationships of MBL in different animal species, we used deduced amino acid sequences from previously identified genes reported in GenBank to reconstruct the phylogenetic tree. The tree was constructed by using the neighbour-joining method (NJ). The reliability of internal branches was assessed by using 1000 bootstrap replicates, and sites with pairwise deletion in this analysis. NJ searches were conducted by using the computer program MEGA3 (Kumar et al. 2004).

3.4.9   Linkage QTL study for porcine MBL genes

The association analysis was complemented by linkage QTL analysis, which enables to provide evidence for effects of the MBL genes in absence of linkage disequilibrium between the analyzed silent SNPs and other potentially existing causal mutations. Linkage QTL analysis was carried out by least square regression using the program QTL Express (Seaton et al. 2002). This model assumes a biallelic QTL fixed for alternative alleles in each parental line. For each F_2 animal, genotypes of five markers including *MBL1* and *MBL2* on chromosome 14 (SSC14) were used to estimate the probability of having none, one or two alleles of the putative QTL of the respective founder line (grand-parent generation) in 1 cM intervals. The probabilities are used to calculate additive and dominance coefficients for a putative QTL at each position and the trait values are then regressed onto these coefficients. For QTL analysis, the phenotypes CH_{50}, AH_{50} and C3c, adjusted for systematic effects, were used. These effects also included the residuals of repeated measures analyses using the model detailed above but without the fixed effect of genotype of *MBL1* and *MBL2*. The chromosome-wide 5% significance threshold was determined empirically by permutation (10000 iterations) and transformed to genome-wide 5% significance

threshold by subsequent Bonferroni correction for the number of autosomes (Churchill and Doerge 1994).

3.5   Statistical analyses

3.5.1   Immunological traits analyses

Estimations were performed with the general linear model (GLM) procedure of the SAS software package (SAS System for Windows, Release 9.01). Data were analyzed using the following model;

$$Y_{ijkl} = \mu + \text{sire}_i + \text{dam}_j + \text{sex}_k + \text{time}_l + \varepsilon_{ijkl}$$

where:

$Y_{ijklmno}$	=	immunological traits (CH_{50}, AH_{50}, C3c level, Hp level, mycoplasma, tetanus, and PRRS antibody level)
μ	=	overall mean
sire_i	=	the fixed effect of sire (i=1-8)
dam_j	=	the fixed effect of dam (j=1-22)
sex_k	=	the fixed effect of sex (k=1-2)
time_l	=	the fixed effect of time point prior/after vaccinations (l=1-6)
ε_{ijkl}	=	the residual error

3.5.2   MBL genotype association study

Analysis of variance was performed with the procedure 'mixed' and 'repeated' statement of the SAS software package to investigate effects of *MBL1* and *MBL2* genotypes on AH_{50}, CH_{50} and C3c concentration. The model was fitted in order to identify other significant environmental and genetic effects apart from the *MBL1* and *MBL2* genotypes and its interaction by stepwise elimination of non-significant effects. Those factors found to significantly affect the phenotypes were included in the model to assess association of the *MBL* markers with the immunological trait. The animal effect was the subject specified in the repeated statement. An autoregressive (AR) covariance structure was included in model. The least square means between *MBL* genotype classes

on each time point were compared. The repeated measures (first order autoregressive R-matrix) mixed model for haemolytic complement activity traits and acute phase proteins is:

$$Y_{ijklmno} = \mu + sire_i + dam_j + parity_k + treatment_l + genotype_m + time_n +$$
$$sex_o + animal_{ijklmno} + (genotype*time)_{mn} + \varepsilon_{ijklmno}$$

where:

$Y_{ijklmno}$	=	immunological traits (CH_{50}, ACH_{50}, and C3c level)
μ	=	overall mean
$sire_i$	=	the fixed effect of sire (i=1-3)
dam_j	=	the fixed effect of dam (j=1-11)
$parity_k$	=	the fixed effect of parity (k=1-5)
$treatment_l$	=	the fixed effect of treatment (vaccinated and non-vaccinated)
$genotype_m$	=	the fixed effect of genotype (m=1-3)
$time_n$	=	the fixed effect of time point of measurement prior/after vaccinations (n=1-8)
sex_o	=	the fixed effect of sex (o=1-2)
$animal_{ijklmno}$	=	the random effect of animal
$(genotype*time)_{mn}$	=	the interaction between *MBL2* genotype and time point
$\varepsilon_{ijklmno}$	=	the residual error

4   Results

4.1   Immunological analyses

In this chapter, the immunological traits including haemolytic complement activity of the classical and alternative pathway, complement component C3c, haptoglobin concentration and antibody response to vaccination (mycoplasma, tetanus, and PRRS) are described. QTL of immunological traits observed in the backcross DUMI population are reported. Furthermore, genetic analyses of porcine MBL genes including gene evolution, gene expression, chromosomal assignment, single nucleotide polymorphism detection, and the association between gene variation and phenotypic traits are also described.

4.1.1   Classical complement activity assay

The means of total haemolytic complement activity of the classical pathway of all pigs were 28.44 ± 16.15 U/ml before vaccination and 35.27 ± 18.13 U/ml after three immunizations. The lowest concentration of the complement activity in the classical pathway was measured at T3 with 27.78 ± 14.70 U/ml. The haemolytic complement activity of the classical pathway was elevated during each immunisation. The results indicate that the haemolytic complement activity of the classical pathway was increased over time point of measurement as described in table 3.

Table 3:   Haemolytic complement activity of the classical pathway (CH_{50}) at different time points of measurement prior and after vaccinations

Vaccination	Time	Animals	Mean ± SD	Minimum	Maximum
Mycoplasma	T1	194	28.44 ± 16.15	0.73	78.23
	T2	195	32.14 ± 18.27	3.04	84.57
Tetanus	T3	199	27.78 ± 14.70	5.61	82.88
	T4	188	30.92 ± 14.05	4.59	71.61
PRRS	T5	165	33.13 ± 17.13	2.20	84.39
	T6	163	35.27 ± 18.13	7.96	101.95

The distribution of haemolytic complement activity of the classical pathway can be approximated as a normal distribution at each time point of measurement. The histograms of all observations and the normal curve at each time point of measurement are shown in figure 9.
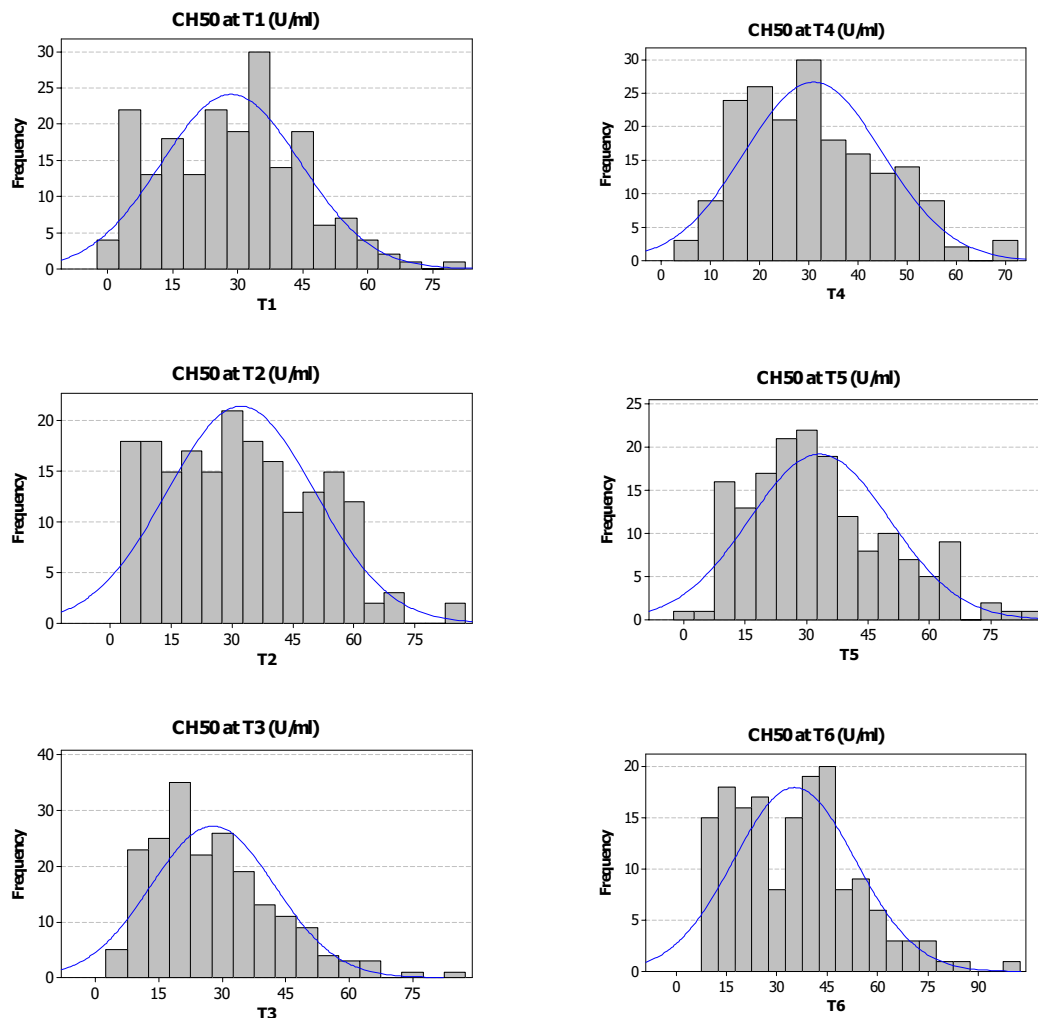
Figure 9:   Distribution of the haemolytic complement activity in the classical pathway (CH_{50}) at each time point of measurement prior and after vaccinations.

The analysis of variance for haemolytic complement activity of the classical pathway prior and after vaccination as described in table 4 indicated that the combined effects in the model accounted for 65 to 74% of phenotypic variation in traits at different time points. The effect of sire contributed significantly to the variation observed in the classical pathway at T1 and T2, while the effect of the dam contributed significantly to the variation observed at T1 to T4. Gender did not affect the traits.

Table 4:   Analysis of variance of CH_{50} values at different time points of
                measurement prior and after vaccinations

Vaccination	Time	R^2	Model	Sire	Dam	Gender
Mycoplasma	T1	0.74	***	*	***	ns
	T2	0.73	***	**	***	ns
Tetanus	T3	0.71	***	ns	***	ns
	T4	0.67	***	ns	**	ns
PRRS	T5	0.74	***	ns	**	ns
	T6	0.65	***	ns	ns	ns

*p<0.05, ** p<0.01, *** p<0.001, ns = not significant

4.1.2 Alternative complement activity assay

The means of total haemolytic complement activity of the alternative pathway of all
pigs were 27.95 ± 12.01 U/ml before vaccination and 56.09 ± 28.68 U/ml after three
immunizations. The haemolytic complement activity of the alternative pathway was
elevated during each immunisation. The results indicated that the haemolytic
complement activity of the classical pathway was increased over time points of
measurement as described in table 5.

Table 5:   Average of haemolytic complement activity of the alternative pathway
                (AH_{50}) at different time points of measurement prior and after
                vaccinations

Vaccination	Time	Animals	Mean ± SD	Minimum	Maximum
Mycoplasma	T1	102	27.95 ± 12.01	1.53	56.82
	T2	115	35.50 ± 16.90	1.75	100.03
Tetanus	T3	96	32.47 ± 19.37	1.29	91.63
	T4	87	44.92 ± 24.65	8.54	154.23
PRRS	T5	90	50.69 ± 24.02	3.74	131.05
	T6	83	56.09 ± 28.68	7.01	194.05

The distribution of the haemolytic complement activity of the alternative pathway can
be approximated as a normal distribution at each time point of measurement. The

histograms of all observations and the normal curves of each time point of measurement are shown in figure 10.
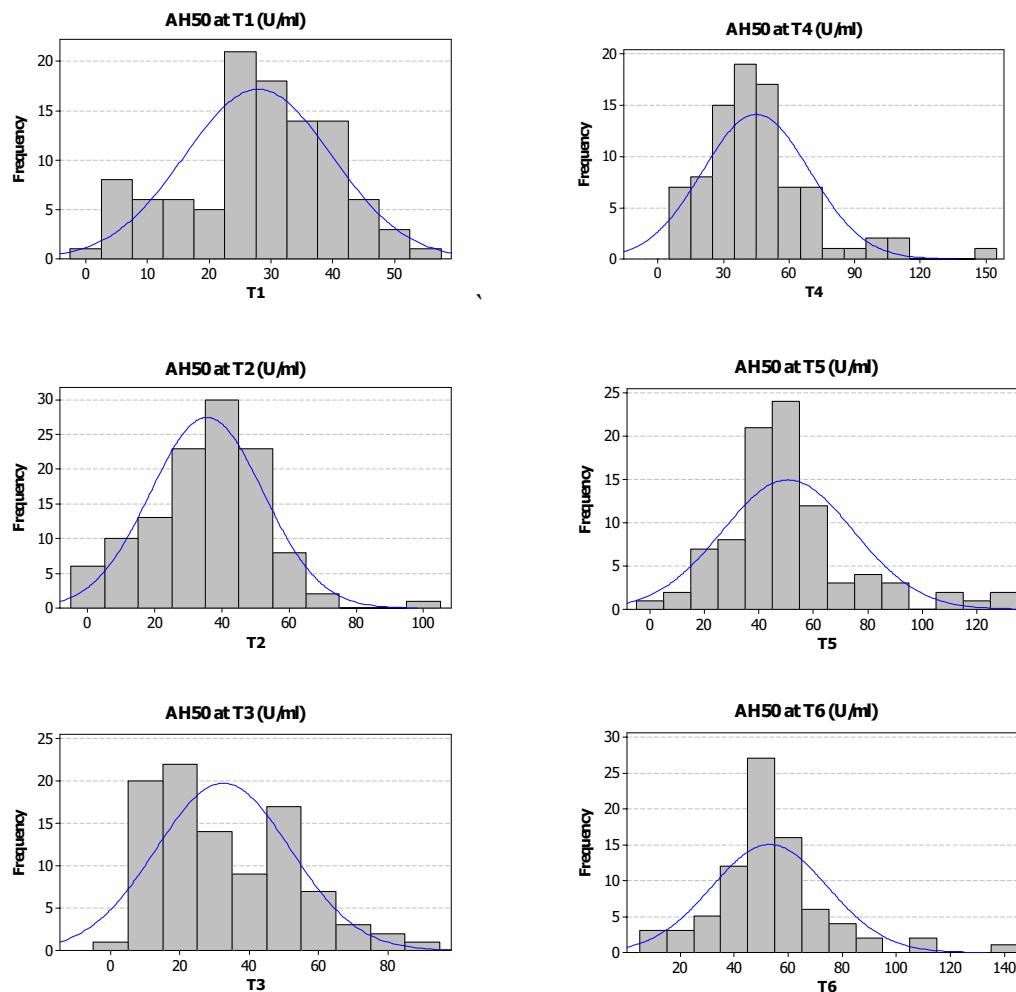
Figure 10:   Distribution of the haemolytic complement activity in the alternative pathway (AH_{50}) at each time point of measurement prior and after vaccinations.

The analysis of variance for the haemolytic complement activity in the alternative pathway prior and after vaccination as described in table 6 indicated that the combined effect in the model accounted for 26 to 77 % of phenotypic variation in traits at different time points. The effect of sire contributed significantly to the variation observed in the alternative pathway at T2, T3 and T6, while the effect of dam contributed significantly to the variation at T2-T6. The results clearly indicate that gender did not affect the traits.

Table 6:   Analysis of variance of the haemolytic complement activity in the alternative pathway (AH_{50}) at different time points of measurement prior and after vaccinations

Vaccination	Time	R^2	Model	Sire	Dam	Gender
Mycoplasma	T1	0.67	***	ns	ns	ns
	T2	0.73	***	***	***	ns
Tetanus	T3	0.77	***	***	***	ns
	T4	0.43	***	-	**	ns
PRRS	T5	0.26	*	ns	***	ns
	T6	0.32	**	***	*	ns

*p<0.05, ** p<0.01, *** p<0.001, ns = not significant

4.1.3   Antibody response to mycoplasma vaccination

The means of antibody response to mycoplasma vaccination which were determined by a monoclonal blocking ELISA using M. hyopneumoniae ELISA kit of all pigs were 37.40 ± 23.76 % before vaccination, 19.86 ± 24.47 % and 14.59 ± 20.76 % at 10 and 20 days (T2 and T3) after the immunizations, respectively. The results as described in table 7 indicated that the antibody response to mycoplasma vaccination was increased over time point of measurement. Samples with a value less than 50% were considered positive, and therefore, an increase in the antibody level would be seen as a decrease in the test results.

Table 7:   Average of antibody response to mycoplasma vaccination at different time points of measurement

Vaccination	Time	Animals	Mean ± SD	Minimum	Maximum
Mycoplasma	T1	130	37.40 ± 23.76	2.02	96.26
	T2	127	19.86 ± 24.47	2.44	95.13
	T3	126	14.59 ± 20.76	1.64	100.26

The analysis of variance for antibody to mycoplasma prior and after vaccination as described in table 8 indicated that the combined effect in the model accounted for 56 to 74% of phenotypic variation in traits at different time points. The effect of sire

contributed significantly only at T1, while the dam contributed significantly to the variation observed in antibody to mycoplasma at all three time points. Gender did not affect the traits.

Table 8:   Analysis of variance of antibody response to mycoplasma vaccination at different time points of measurement

Vaccination	Time	R^2	Model	Sire	Dam	Gender
	T1	0.61	***	**	***	ns
Mycoplasma	T2	0.74	***	ns	***	ns
	T3	0.56	***	ns	***	ns

** p<0.01, *** p<0.001, ns = not significant

4.1.4   Antibody response to tetanus toxoid vaccination

The means of antibody response to TT vaccination which were determined by a competitive ELISA and expressed as an S/P ratio of all pigs were 0.09 ± 0.07 before vaccination, 0.65 ± 0.21 and 0.92 ± 0.10 at 20 and 40 days (T2 and T3) after the vaccinations, respectively. The results as described in table 9 indicated that the antibody response to TT vaccination was increased over the time points of measurement.

Table 9:   Average of antibody response to tetanus toxoid at different time points of measurement

Vaccination	Time	Animals	Mean ± SD	Minimum	Maximum
	T3	186	0.09 ± 0.07	0.02	0.78
TT	T4	187	0.65 ± 0.21	0.07	1.02
	T5	162	0.92 ± 0.10	0.64	1.06

The analysis of variance for antibodies to TT prior and after vaccination as described in table 10, indicated that the combined effects in the model accounted for 22 to 65% of phenotypic variation in traits at different time points. The effect of the sire contributed significantly at T4 and T5, while the dam contributed significantly to the variation observed in antibody to TT at all three time points. Gender did not affect the traits.

Table 10:   Analysis of variance of antibody to tetanus toxoid at different time points
                 of measurement

Vaccination	Time	R^2	Model	Sire	Dam	Gender
	T3	0.22	**	ns	**	ns
TT	T4	0.48	***	*	***	ns
	T5	0.65	***	***	***	ns

*p<0.05, ** p<0.01, *** p<0.001, ns = not significant

4.1.5 Antibody response to PRRS vaccination

The means of antibody response to PRRS vaccination are given in table 11. Antibody
levels were determined by a competitive ELISA and expressed as an S/P ratio.

Table 11:   Average of antibody response to PRRS vaccination at time point 5 of
                 measurement

Vaccination	Time	Animals	Mean ± SD	Minimum	Maximum
PRRS	T5	153	0.84 ± 0.79	-0.17	3.12

The analysis of variance for antibodies to PRRS after vaccination as described in table
12 indicated that the combined effect in the model accounted for 55% of phenotypic
variation in trait. The effect of the dam contributed significantly to the variation
observed in antibody to PRRS at the time point of measurement (T6). The sire and
gender did not affect the trait.

Table 12:   Analysis of variance of antibody to PRRS vaccination time point 5 of
                 measurement

Vaccination	Time	R^2	Model	sire	dam	gender
PRRS	T6	0.55	***	ns	***	ns

*** p<0.001, ns = not significant

The distribution of antibodies response to vaccinations can not be approximated as a
normal distribution at each time point of measurement. The histogram of all

observations and the normal curve of each time point of measurement are shown in figure 11.
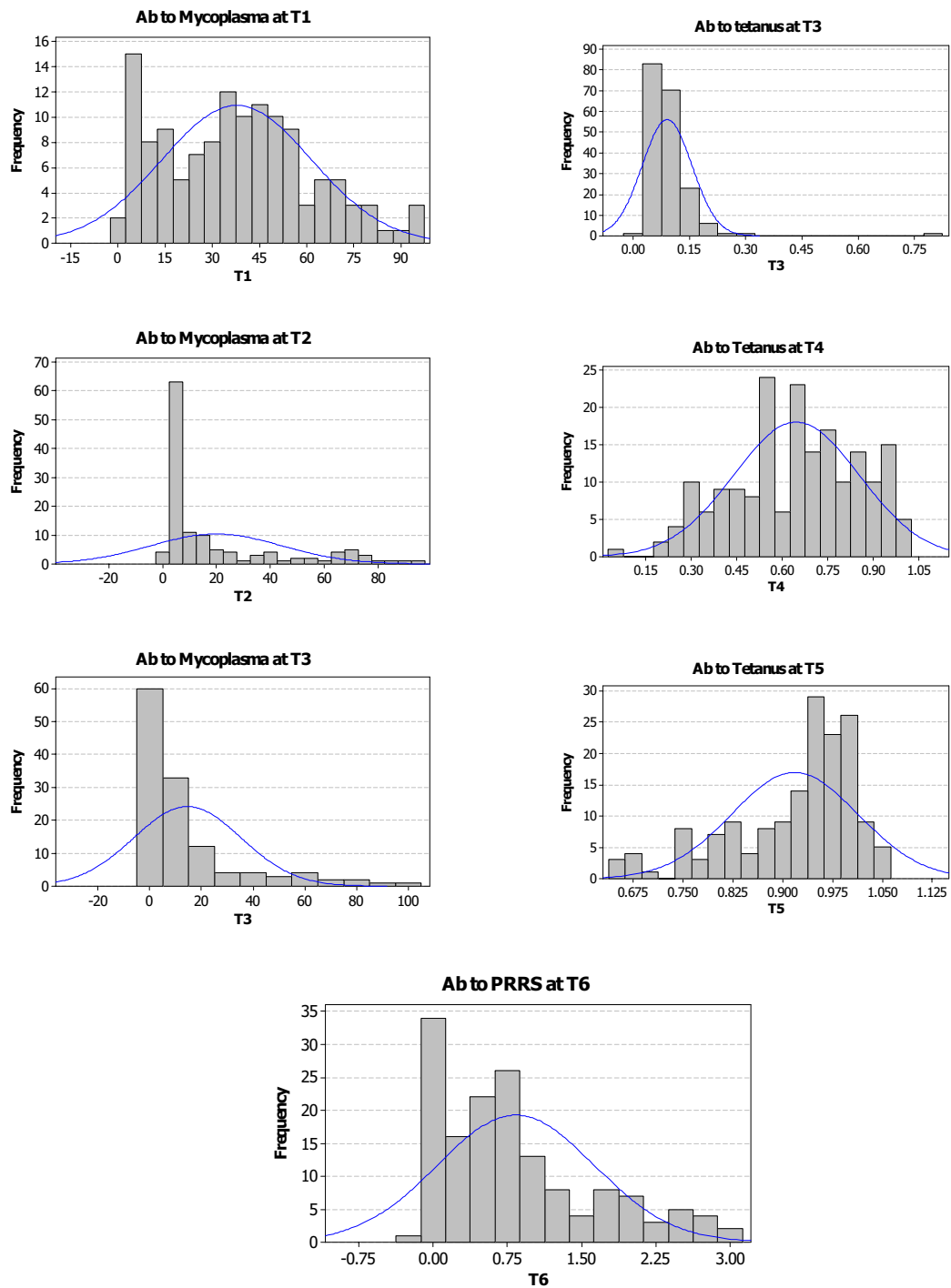
Figure 11: Distribution of antibody responses to mycoplasma at T1 to T3, TT at T3 to t5 and PRRS at T6 of time points of measurement

4.1.6   Complement C3c concentration

The means of complement C3c concentration which were determined by a competitive ELISA and expressed as an S/P ratio of all pigs were found lowest at time point T3 (0.78 ± 0.25), and highest at T2 (0.87 ± 0.23). The results as described in table 13 indicated that the complement C3c concentration is increased after each vaccination.

Table 13:   Average of the complement component C3c concentration at different time points of measurement prior and after vaccinations

Vaccination	Time	Animals	Mean ± SD	Minimum	Maximum
Mycoplasma	T1	125	0.84 ± 0.25	0.18	1.33
	T2	127	0.87 ± 0.23	0.13	1.47
TT	T3	124	0.78 ± 0.25	0.15	1.64
	T4	136	0.82 ± 0.21	0.11	1.95
PRRS	T5	128	0.79 ± 0.24	0.05	1.16
	T6	133	0.82 ± 0.25	0.04	1.15

The analysis of variance for complement C3c concentration prior and after vaccination as described in table 14 indicated that the combined effects in the model accounted for 52 to 78% of phenotypic variation in traits at different time points. The effect of the sire did not contribute during mycoplasma, but TT and PRRS vaccination. The dam contributed significantly to the variation observed during mycoplasma vaccination at both time points and before PRRS vaccination (T5). Gender did not affect the traits.

Table 14:   Analysis of variance of complements component C3c concentration at different time points of measurement prior and after vaccinations

Vaccination	Time	R^2	Model	Sire	Dam	Gender
Mycoplasma	T1	0.70	***	ns	**	ns
	T2	0.78	***	ns	***	ns
TT	T3	0.52	***	*	ns	ns
	T4	0.57	***	*	ns	ns
PRRS	T5	0.64	***	***	*	ns
	T6	0.65	***	***	ns	ns

The distribution of complement C3c concentration can be approximated as a normal distribution at each time point of measurement. The histograms of all observations and the normal curve of each time point of measurement are shown in figure 12.
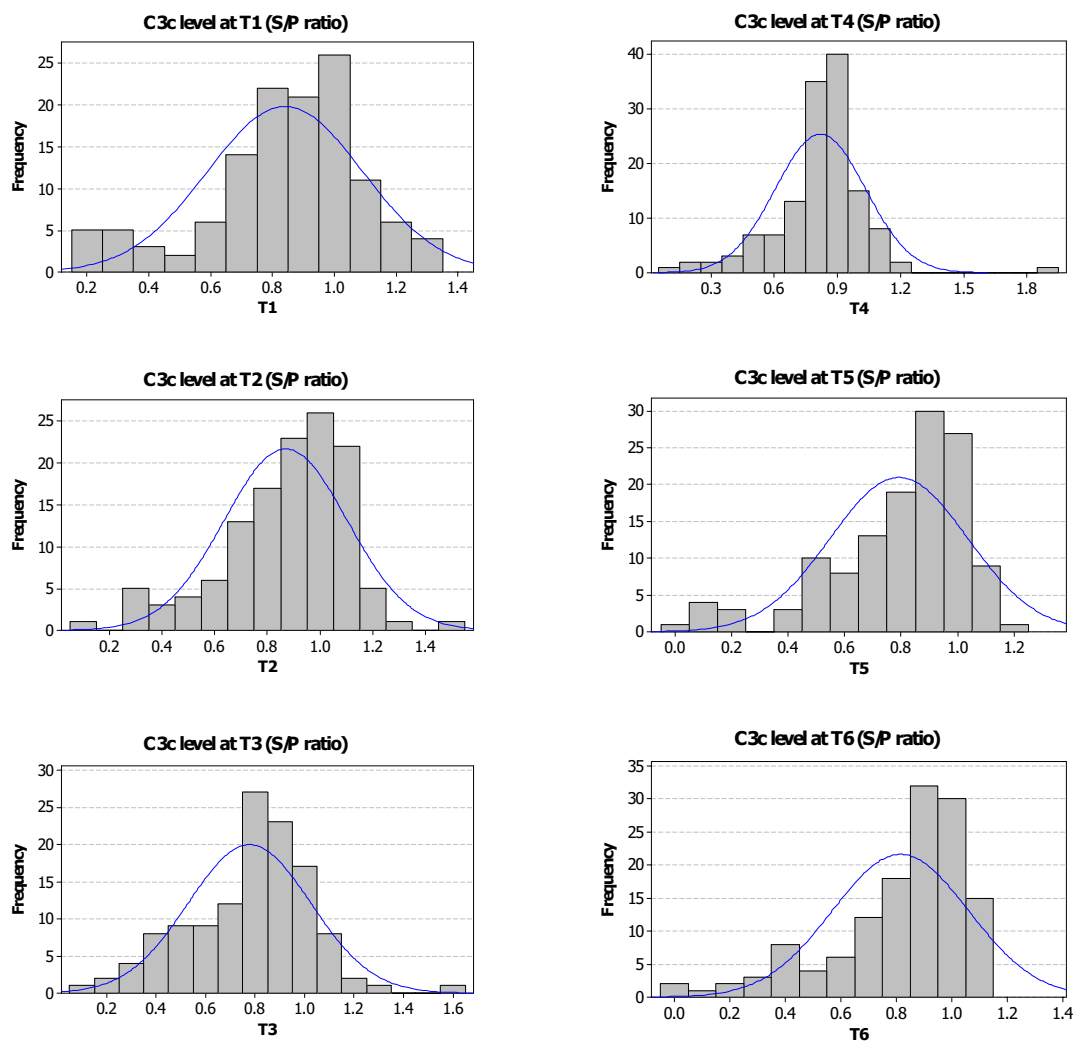
Figure 12:   Distribution of complement component C3c level at each time points of measurement prior and after vaccinations

4.1.7   Haptoglobin concentration

Hp concentrations in the experimental pigs were measured according to the method described by Hiss et al. (2003). The means of Hp concentration which were determined by a competitive ELISA and expressed as mg/ml of all pigs were found lowest at T3 (1.23 ± 0.92 mg/ml) and highest at T6 (2.18 ± 2.07 mg/ml). The results as described in table 15 indicated that the Hp concentration is decreased after the first measurement (T1) until T3, before TT vaccination. The increase of Hp concentration was obtained

after T3 and reached the highest point of measurement at T6 or 10 days after PRRS vaccination.

Table 15:   Average of Hp concentration at different time points of measurement prior and after vaccinations

Vaccination	Time	Animals	Mean ± SD	Minimum	Maximum
Mycoplasma	T1	184	1.76 ± 1.03	0.03	6.30
	T2	166	1.55 ± 1.01	0.12	8.30
TT	T3	184	1.23 ± 0.92	0.01	4.32
	T4	188	1.68 ± 1.05	0.07	6.60
PRRS	T5	170	1.93 ± 1.08	0.04	5.80
	T6	183	2.18 ± 1.07	0.25	6.60

The analysis of variance for Hp concentration prior and after vaccination as described in table 16 indicated that the combined effect in the model accounted for 18 to 49 % of phenotypic variation in traits at different time points The effect of the sire contributed significantly only at T3, while the dam contributed significantly to the variation observed in the Hp concentration at all six time points, except T2 or 10 days after mycoplasma vaccination. Gender did not affect the traits.

Table 16:   Analysis of variance of haptoglobin (Hp) concentration at different time points of measurement prior and after vaccinations

Vaccination	Time	R^2	Model	sire	dam	gender
Mycoplasma	T1	0.30	***	ns	***	ns
	T2	0.18	ns	ns	ns	ns
TT	T3	0.49	***	*	***	ns
	T4	0.44	***	ns	***	ns
PRRS	T5	0.40	***	ns	***	ns
	T6	0.38	***	ns	***	ns

The distribution of Hp concentration can be approximated as a normal distribution at each time point of measurement. The histograms of all observations and the normal curve of each time point of measurement are shown in figure 13.
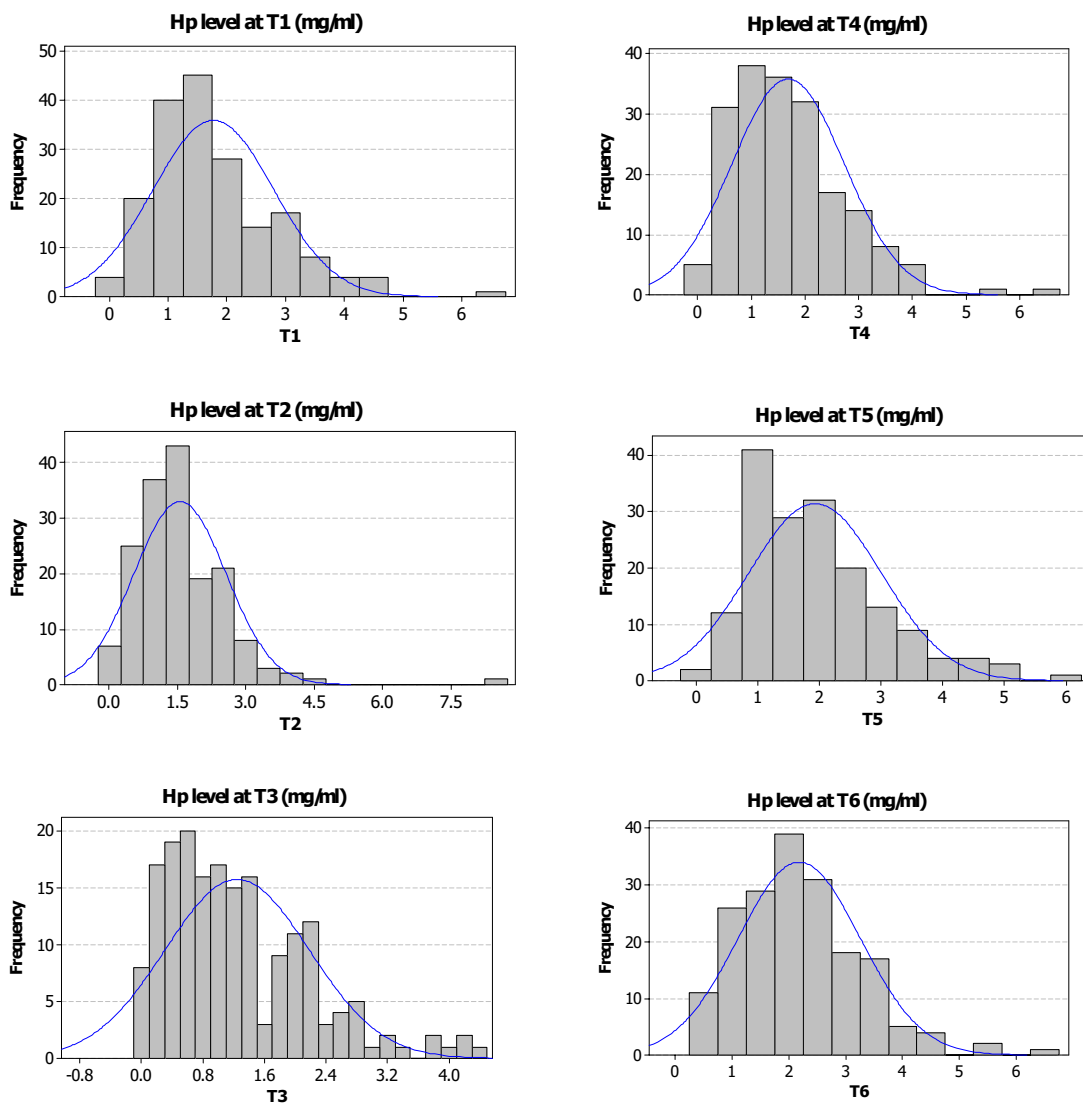

Figure 13: Distribution of Haptoglobin concentration at each time point of measurement prior and after vaccinations

4.2 Linkage analysis

4.2.1 Characterization of markers

By genotyping, the seventy-four markers (an average of 4.11 markers per chromosome) were investigated. The microsatellite markers had between two to seven alleles with an

average of 4.19 alleles per marker. A total of 220 backcross animals were used for QTL analysis in this study. Note that twenty-four animals were derived from F2 (parents of backcross), while thirteen and seventeen animals were also derived from F1 and F0 (parents and grandparents of F2), respectively.

The average heterozygosity and information content was 0.57 and 0.52, respectively. The lowest information content was 0.16 at the locus of the marker *S0155* on SSC1. In total, twenty (27% of all used) markers showed information content less than 0.5.

4.2.2   Mapping of markers

Seventy-four microsatellites from 18 autosomes of *Sus scrofa* have been used for QTL mapping for immune traits in the DUMI backcross population. The sex average, female, and male maps were 2,052 cM, 2,058 cM, and 2,154 cM (Kosambi cM) in length, respectively. The orders of markers were almost in accordance with the published USDA-MARC map, except the marker S0220 on SSC6. This microsatellite marker S0220 was not available on that database. As there was not enough information, the map of SSC17 used for backcross was taken from mapping result in F2. The map of the DUMI F2 population was about 1,983 cM. The map from backcross was approximately with the factor 1.03 larger than the map in F2. It can be concluded that the map used in this study is in good agreement with the map in F2 population. The shortest sex average map was calculated for SSC13 with 32 cM total length, the longest map on SSC15 with 233 cM in length. The markers and their genetic distances used in the QTL analysis and the genetic map as established for the DUMI resource population (sex average, Kosambi cM) for all autosomes (SSC) are shown in table 17.

Table 17: Markers used in the QTL analysis and genetic map as established for the backcross population (sex average, Kosambi cM)

SSC	Coverage [1] [cM]	Markers and genetic distances [cM]
SSC1	16.4 – 140.5 [144.0]	*SW1515* [46.3] *SW1851* [73.7] *S0155* [95.6] *SW1301*
SSC2	0.0 – 74.82 [132.1]	*SW2443* [57.3] *SW240* [20.5] *SW1564* [20.5] *SW834* [5.8] *S0226* [2]
SSC3	17.8 – 102.2 [129.3]	*SW72* [29.8] *S0164* [24.3] *SW2570* [38.6] *S0002*
SSC4	4.1 – 120.0 [130.1]	*S0227* [54.8] *S0001* [17.9] *S0214* [78.7] *S0097*
SSC5	8.4 – 102.9 [114.4]	*SW1482* [31.5] *SW1134* [49.0] *IGF1* [11.5] *SW378*
SSC6	7.3 – 102.0 [165.7][3]	*S0035* [20.4] *S0087* [12.0] *SW1067* [7.5] *SW193* [3.7] *S0300* [8.0] *S0220* [2] [30.9] *S0059* [17.2] *S0003*
SSC7	3.7 – 134.9 [156.6]	*S0025* [28.4] *S0064* [29.4] *S0102* [20.4] *SW175* [37.3] *S0115* [38.7] *S0101*
SSC8	0.0 – 112.3 [127.7]	*SW2410* [67.9] *S0086* [39.4] *S0144* [13.3] *SW61*
SSC9	11.1 – 96.5 [138.5]	*SW21* [29.3] *SW911* [39.5] *SW54* [21.4] *S0109* [68.3] *S0295*
SSC10	0.0 – 124.1 [124.1]	*SW830* [100.0] *S0070* [0.8] *SW2067*
SSC11	14.1 – 76.2 [84.9]	*SW2008* [22.5] *S0071* [12.6] *S0009* [12.6] *S0386* [0.3] *SW703*
SSC12	6.6 – 108.3 [113.1]	*S0143* [58.5] *SW874* [32.7] *SW605*
SSC13	1.6 – 79.3 [126.2]	*S0219* [31.9] *SW344* [0.1] *SW398*
SSC14	7.4 – 111.5 [111.5]	*SW857* [48.9] *S0007* [27.9] *SWC27*
SSC15	1.3 – 107.4 [111.8]	*S0355* [100.0] *SW1111* [91.0] *SW936* [41.9] *SW1119*
SSC16	0.0 – 92.6 [93.2]	*S0111* [37.6] *S0026* [41.8] *S0061*
SSC17[+]	0.0 – 94.0 [97.0]	*SW335* [33.8] *SW840* [33.3] *SW2431*
SSC18	5.0 – 57.6 [57.6]	*SW1023* [30.8] *SW787* [100.0] *SWR414*

[1] relative position of flanking markers set used in this study from public map (USDA-MARC v2);

[2] *S0226* not covered by USDA-MARC v2, but *SW14*, which is closely linked to *S0226* (PigMaP v1.5);

[3] *S0035* at 0.0 cM and *S0003* at 144.5 cM in the International Workshop I SSC6 integrated map with a total length of 166.0 cM;

[+] data observed in F2 population

4.2.3   Significance thresholds

The average F-values for each significant threshold of all traits derived from the QTL express program showing F-values ≥ 2.0 separated by chromosomes and experiment-wide significance levels after transformation are shown in table 18.

Table 18:   Significant threshold and experiment-wide significance levels after transformation

SSC	Numbers of F-values ≥ 2.0	Average of minimum F-value for reaching the following chromosome-wide significance level	
		5%	1%
1	9	4.65	6.54
2	5	4.23	6.13
3	20	4.48	6.35
4	17	4.6	6.44
5	9	4.03	5.88
6	13	4.73	6.67
7	19	4.78	6.62
8	16	4.44	6.22
9	2	3.85	5.58
10	16	3.99	5.66
11	13	3.87	5.66
12	5	4.29	6.14
13	3	3.88	5.64
14	12	4.38	6.31
15	8	4.36	6.18
16	11	3.8	5.59
17	19	3.1	4.73
18	17	4.14	5.87

4.2.4   Significant QTL

Forty-two significant and twenty-four highly significant QTL could be detected for all immune traits. Most QTL were detected on SSC3, SSC16, and SSC18 (nine significant

F-values on each chromosome). No significant F-value was detected on SSC12 and SSC13. Most highly significant QTL could be detected for antibody response to mycoplasma, TT and PRRS vaccination, C3c and Hp concentration. For AH_{50} and CH_{50}, 22 significant and 9 highly significant QTL could be detected. An overview of the detected QTL separated by chromosome and by trait is shown in table 19.

Table 19:   Summarise of the detected QTL in the backcross DUMI population

SSC	Number of detected QTL					
	All traits		Complement activity		Immunoassay	
	*	**	*	**	*	**
1	2	0	2	0	0	0
2	1	0	1	0	0	0
3	7	2	2	0	5	2
4	2	2	2	2	0	0
5	2	1	2	1	1	0
6	1	0	0	0	1	0
7	6	1	5	1	1	0
8	0	1	0	0	0	1
9	1	0	1	0	0	0
10	5	0	2	0	3	0
11	2	2	0	0	2	2
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	1	1	1	0	0	1
15	1	0	0	0	1	0
16	2	7	1	5	1	2
17	4	2	1	0	3	2
18	4	5	2	0	2	5
Total	42	24	22	9	20	15

* p≤0.05 ** p≤0.01 at chromosome-wide level

4.2.4.1   QTL for haemolytic complement activity traits

QTL analysis was performed for two different traits within the haemolytic complement activity, the classical and the alternative pathway activities (CH_{50a} and AH_{50}). For both traits, effects were measured at different time points as mentioned in previous chapters. The CH_{50} activity was measured at six different time points, for four time points QTL could be detected. For CH_{50} at T1, significant F-values were detected on SSC3, SSC10 and SSC14. On SSC17, a highly significant F-value for the measurement at T4 and one significant F-value at T6 were detected at the locus of the marker *SW335* at 0 cM. For the measurement at T5, highly significant and significant F-values were detected on SSC16 and SSC5, respectively. Evidence for QTL significant at the 5% chromosome-wide level for measurements of the CH_{50} obtained in the analysis of backcross DUMI population including estimated significance levels (F-value), position, and gene effects are shown in table 20.

Table 20:   Location and estimated effects of putative QTLs affecting the haemolytic complement activity in the classical pathway at different time points

Trait	SSC	position (cM)	F-value	Additive effect	S.E.	Dominant effect	S.E	Variance (%)[1]
CH_{50}								
T1	3	78	5.58*	422.34	173.41	-191.53	200.8	23.36
T1	10	100	5.65*	411.11	135.26	-253.46	174.94	23.35
T1	14	120	4.21*	696.21	256.99	-1124.72	490.13	23.5
T2	-	-	-	-	-	-	-	
T3	-	-	-	-	-	-	-	
T4	17	0	6.48**	-1210.7	388.16	903.67	424.4	25.00
T5	5	89	3.14*	-222.11	192.46	575.53	249.85	30.22
T5	16	79	9.34**	-69.9	312.45	1447.43	446.55	29.42
T6	7	66	4.41*	84.29	178.19	-441.68	188.99	30.23
T6	17	0	4.15*	-1216.86	469.89	955.37	513.76	34.13

* $p \leq 0.05$ ** $p \leq 0.01$ at chromosome-wide level,

[1]the fraction of phenotypic variance in the backcross explained by each QTL

Similar to CH_{50}, the AH_{50} measurements at six different time points were also used for QTL detection. Significant F-values on SSC2, 3, 4, 9 and 10 were detected for only one measurement. QTL for the complement activity in the alternative pathway at T3 and T4 was found on SSC1 at 74 cM. In addition, the linked regions for similar measurements were also found on SSC18 at 100 to 130 cM. On SSC4, four significant F-values were found for the measurements at T1, T3, T5 and T6 located nearby the marker *SW2570* at approximately 50 cM. This clear picture of an interesting locus on one chromosome could not be found for SSC7, where significant F-values were detected for different measurements (T3, T5 and T6) covering different regions of the chromosome. Significance for almost all measurements was detected on SSC16 except for T5 and almost all QTL were located in the region of the marker *S0026*. The overview of all QTL detected for the different measurement for AH_{50} levels is shown in table 21.

Table 21:   Location and estimated effects of putative QTLs affecting the haemolytic complement activity in the alternative pathway at different time points

Trait	SSC	Position (cM)	F-value	Additive effect	S.E.	Dominant effect	S.E.	Variance (%)[1]
AH_{50}								
T1	4	50	8.09**	56.22	244.63	528.34	284.59	31.91
T1	5	66	7.13**	674.32	220.78	-1091.82	289.82	32.04
T1	7	105	5.65*	1.49	189.34	-498.81	204.7	32.25
T1	9	29	2.12*	-827.24	651.2	1145.43	681.86	32.76
T1	16	38	6.4*	-235.4	167.41	-269.88	227.13	32.14
T2	7	56	4.81*	110.17	187.7	-524.8	203.71	33.02
T2	16	26	9.89**	-80.84	202.65	-686.37	266.36	32.31
T3	1	74	4.94*	750.51	239.56	-695.15	328.75	32.95
T3	4	54	6.04*	200.57	235.58	266.2	273.26	32.79
T3	16	18	8.43**	-12.57	224.67	-715.02	285.84	32.46
T3	18	130	5.78*	-901.37	561.52	182.82	608.55	32.83
T4	1	74	5.04*	-746.69	235.24	769.13	322.83	32.35
T4	7	0	5.5*	411.15	155.41	-578.51	175.08	32.29
T4	10	0	4.33*	224.96	301.7	703.4	446.77	32.46

Table 21 (continued)

Trait	SSC	Position (cM)	F-value	Additive effect	S.E.	Dominant effect	S.E.	Variance (%)[1]
AH$_{50}$								
T4	16	21	7.93**	32.94	214.13	-727.18	275.96	31.95
T4	18	106	5.3*	-676.81	780.88	-450.55	855.36	32.32
T5	4	54	6.08*	29.05	236.97	456.51	274.87	32.98
T5	7	58	5.8*	-20.32	185.64	-470.52	202.12	33.02
T6	2	58	4.25*	651.71	224.59	-695.91	273.05	33.81
T6	3	30	5.2*	-193.1	185.52	561.51	221.16	33.67
T6	4	62	8.31**	-238.07	230.69	791.73	269.91	33.22
T6	5	56	4.87*	638.15	264	-1102.38	353.68	33.72
T6	7	49	7.9**	277.69	193.52	-761.41	208.4	33.28
T6	16	79	4.04*	-182.04	359.4	-844.06	513.64	33.84

** p≤0.05 ** p≤0.01 at chromosome-wide level,

[1]the fraction of phenotypic variance in the backcross explained by each QTL

The F-values of QTL of the haemolytic complement activity in the alternative pathway for the most interesting chromosomes including SSC1, SSC4, SSC7 and SSC16 are shown in figures 14, 15, 16 and 17, respectively.
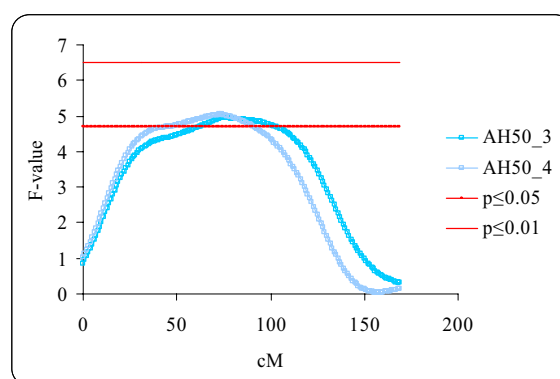
Figure 14:   Relative position of QTL for AH$_{50}$ at T3 and T4 on SSC1

Figure 15:   Relative position of QTL for AH$_{50}$ at T1, T3, T5, and T6 on SSC4

Figure 16:   Relative position of QTL for AH$_{50}$ at T1, T2, T4, T5, and T6 on SSC7

Figure 17:   Relative position of QTL for AH$_{50}$ at T1, T2, T3, T4, and T6 on SSC16

4.2.4.2   QTL for complement component C3c and Hp levels

For C3c, different QTL could be detected in the backcross population. Only one significant F-value was detected on SSC16 and SSC17, whereas three interesting results on SSC11 for the measurements at T1, T3 and T6 were observed, indicating the region

between the markers *S0071* and *S0386* as important for search of possible candidate genes for this trait. On SSC3, there were significant F-values found mainly at 30 cM next to the marker *S0164* for measurements excluding T5. The most interesting QTL on complement component C3c was found on SSC18 similar for all measurements, being located at 130 cM with the peak at the marker *SWR414*. All significant and highly significant F-values including the additive and dominant effects are shown in table 22.

Table 22:   Location   and   estimated   effects   of   putative   QTLs   affecting   the complement component C3c at different time points

trait	SSC	Position (cM)	F-value	Additive effect	S.E.	Dominant effect	S.E.	Variance (%)[1]
C3c								
T1	3	0	5.91*	-200.1	212.94	828.34	285.42	33.17
T1	11	23	4.41*	225.2	214.02	-607.48	254.12	33.39
T1	18	130	5.29*	-339.48	568.89	1112.3	616.54	33.26
T2	3	31	8.28**	-405.8	180.85	804.89	215.58	32.37
T2	18	130	6.35**	448.5	558.31	377.14	605.07	32.64
T3	3	30	6.38*	-71.6	180.07	487.9	214.67	32.68
T3	11	35	6.24**	591.06	231.13	-966.62	277.75	32.7
T3	18	130	7.55**	1038.3	556.19	-228.93	602.77	32.52
T4	3	29	4.98*	10.44	180.89	361.1	215.6	32.35
T4	17	0	3.45*	-1210.7	388.16	903.67	424.4	32.57
T4	18	130	6.94**	145.67	548.7	721.7	594.66	32.08
T5	16	0	3.57*	-12.75	236.14	418.74	280.6	32.83
T5	18	130	5.17*	104.82	557.55	656.82	604.25	32.60
T6	3	29	4.84*	3.36	182.99	368.24	218.12	32.73
T6	11	42	5.48**	369.79	224.41	-822.67	274.15	32.64
T6	18	130	6.55**	62.24	555.68	793.02	602.23	32.49

** p≤0.05 ** p≤0.01 at chromosome-wide level,

[1]the fraction of phenotypic variance in the backcross explained by each QTL

For C3c measurements, SSC3, SSC11 and SSC18 could be detected as the most interesting chromosomes. Within these chromosomes, significant F-values for all six

measurements where detected at the locus of the marker *SWR414*, at 130 cM. The
following figures 18, 19 and 20 show the QTL effects for the different C3c
measurements on SSC3, SSC11 and SSC18, respectively.
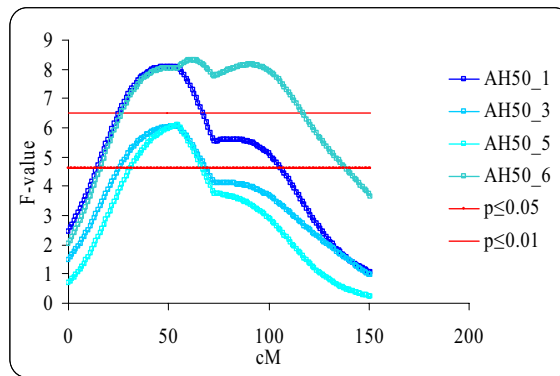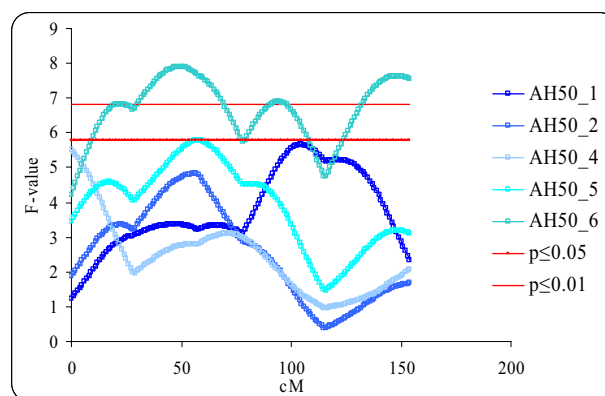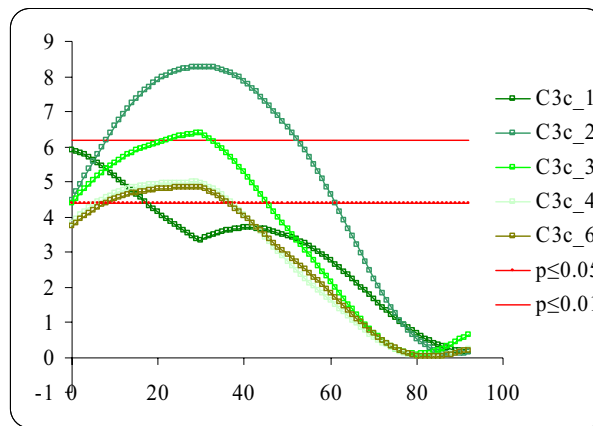

Figure 18:   Relative position of QTL for C3c at T1, T2, T3, T4, and T6 on SSC3

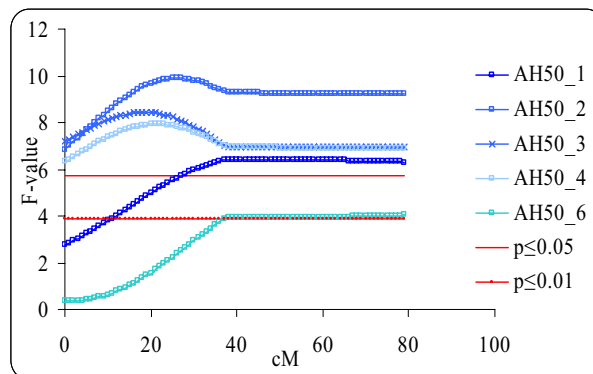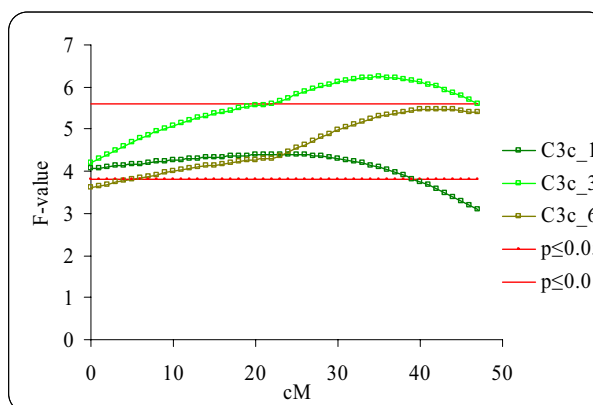Figure 19:   Relative position of QTL C3c at T1, T3, and T6 on SSC11
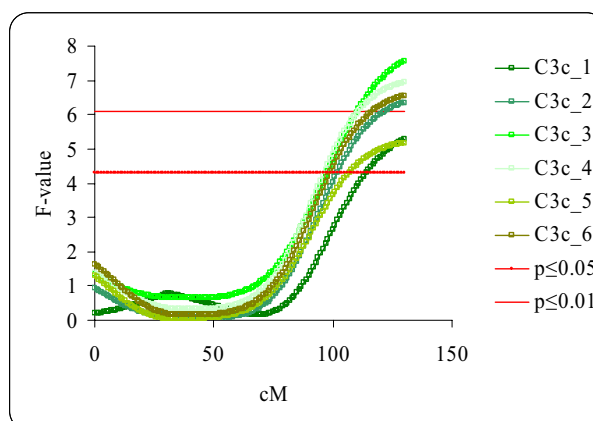
Figure 20:   Relative position of QTL for C3c at T1-T6 on SSC18

For Hp concentration, significant and highly significant F-values were detected for all measurements distributed over half of the autosomes. Only for SSC17, QTL could be detected for two different measurements (T4 and T5) indicating the region next to marker *SW335* at 0 cM as interesting region comprising genes with possible effects on the Hp concentration. Moreover, significant F-values for one measurement on SSC3, 5, 7, 8, 11, 14, 15 and 16 could be found as shown in table 23.

Table 23:   Location and estimated effects of putative QTLs affecting the concentration of Hp at different time points

Trait	SSC	Position (cM)	F-value	Additive effect	S.E.	Dominant effect	S.E.	Variance (%)[1]
Hp								
T1	3	92	6.25*	413.06	163.57	-187.47	187.33	25.41
T2	7	127	5.44*	616.3	187.1	-419	195.4	29.22
T2	8	27	7.4**	975.03	279.89	-1348.43	361.92	28.97
T3	11	12	5.63*	546.98	189.19	-758.12	226.21	24.67
T3	14	120	9.18**	1065.85	265.73	-1706.11	506.79	24.30
T4	15	0	4.23*	-643.68	225.67	563.18	267.23	24.08
T4	17	0	6.05**	-1132.21	375.94	843.77	411.04	24.21
T5	5	89	4.11*	-271.01	182.03	638.98	236.31	28.58
T5	16	79	14.88**	-175.92	290.07	1781.91	414.57	32.83
T5	17	0	3.67*	-872.06	444.67	1239.33	486.18	28.69
T6	18	114	6.46**	441.73	573.49	438.74	623.39	25.49

** p≤0.05 ** p≤0.01 at chromosome-wide level,

[1]the fraction of phenotypic variance in the backcross explained by each QTL

For the different Hp concentration in this study, only QTL affecting two different time points, T4 and T5 could be detected on SSC17. Figure 21 shows the effect on SSC17.
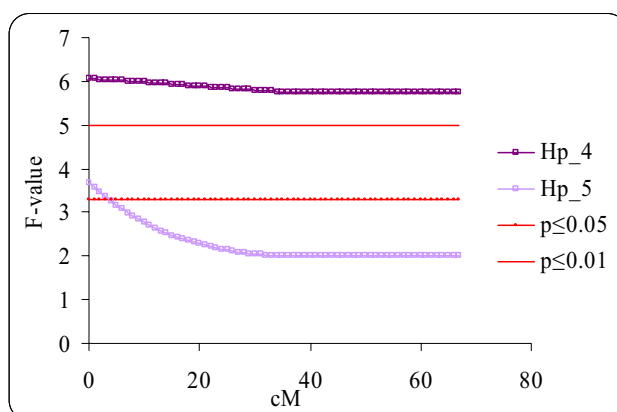
Figure 21:   Relative position of QTL for Hp levels at T4 and T5 on SSC17

4.2.4.3   QTL for antibodies responses to vaccinations

For the trait of antibody response of Mh vaccination, four QTL on two different chromosomes could be detected. There was a chromosome-wide significant QTL detected on SSC10 at 100 cM with a peak at the position of marker *S0070* for all three time points of measurement. The results are shown in table 24.

Table 24:   Location and estimated effects of putative QTLs affecting Mh-Ab

Trait	SSC	Position (cM)	F-value	Additive effect	S.E.	Dominant effect	S.E.	Variance (%)[1]
Mh								
T1	10	100	4.43*	513.31	194.41	-732.9	251.44	33.56
T2	10	100	4.76*	522.74	190.98	-746	247.01	32.97
T3	6	16	4.53*	551.67	195.17	-695.3	239.01	33.01
T3	10	100	4.18*	513.15	191.5	-684.2	247.67	33.06

** $p \leq 0.05$ ** $p \leq 0.01$ at chromosome-wide level,

[1] the fraction of phenotypic variance in the backcross explained by each QTL

Regarding the antibody response to Mh vaccination, the most interesting chromosome for the detected of QTL affecting this trait was SSC10, as seen in table above and in figure 22.
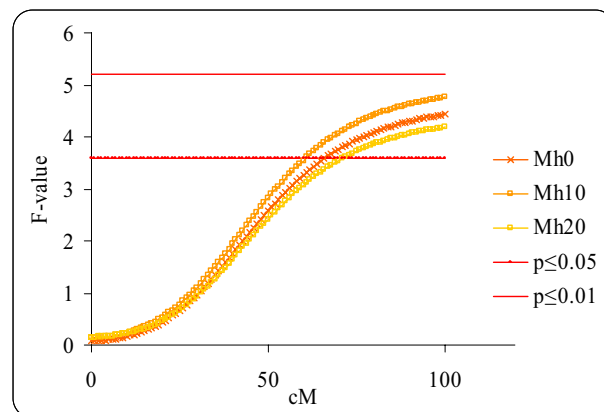
Figure 22:   Relative position of QTL for the antibody response to mycoplasma
                vaccination T1-T3 on SSC10

On SSC6, one significant QTL could be detected for the last measurement. The measurements of antibody response to Mh vaccination nearly reach the significant F-values of 4.4 and 4.3 respectively as demonstrated in figure 23.
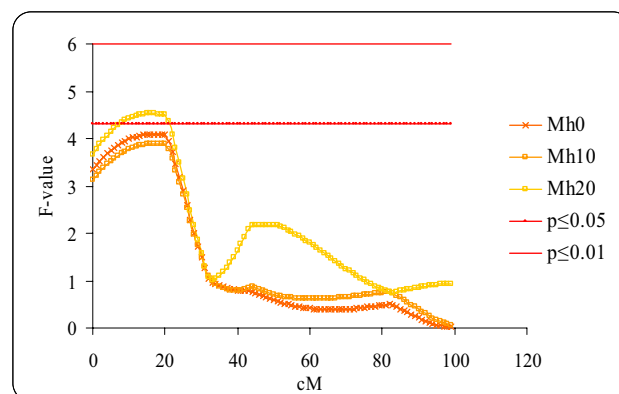
Figure 23:   Relative position of QTL for the antibody to mycoplasma vaccination at
                T1-T3 on SSC6

By using the 2-QTL model, no good evidence for two QTL on SSC6 for the measurement of the antibody response to Mh vaccination could be found.

For the measurement of the antibody response to TT vaccination, there was no QTL found for the first measurement (T3). For the second measurement (T4), there was a significant QTL on SSC17 at 0 cM. A highly significant QTL could be detected at 30 cM at the locus of the marker *S0026* for the last measurement (T5) during TT vaccination. Data are shown in table 25.

Table 25:   Location and estimated effects of putative QTLs on the antibodies to tetanus toxoid at different time points of measurement

Trait	SSC	Position (cM)	F-value	Additive effect	S.E.	Dominate effect	S.E.	Variance (%)[1]
TT								
T4	17	0	4.15*	-859.81	377.96	565.0	413.25	24.34
T5	16	30	9.98**	-16.11	167.78	676.19	223.48	28.45

** p≤0.05 ** p≤0.01 at chromosome-wide level,

[1]the fraction of phenotypic variance in the backcross explained by each QTL

The F-values of other time point measurements for antibody response to TT vaccination were too low and no nearly significant level could be reached. One QTL could be detected affecting the trait of measurement of antibody to PRRS vaccination. A highly significant linkage was detected on SSC3 at the position of 14 cM at T6. The detected peak was seen between the loci of the marker *SW72* and *S0164* as shown in table 26.

Table 26:   Location and estimated effects of putative QTLs on the antibodies to PRRS vaccination at different time points of measurement

Trait	SSC	Position (cM)	F-value	Additive effect	S.E.	Dominant effect	S.E.	Variance (%)[1]
PRRS								
T6	3	14	6.35**	73.69	212.78	450.98	258.97	30.36

** p≤0.05 ** p≤0.01 at chromosome-wide level,

[1]the fraction of phenotypic variance in the backcross explained by each QTL

The following figure 24 illustrates QTL affecting antibody response to PRRS vaccination that could be detected on SSC3.
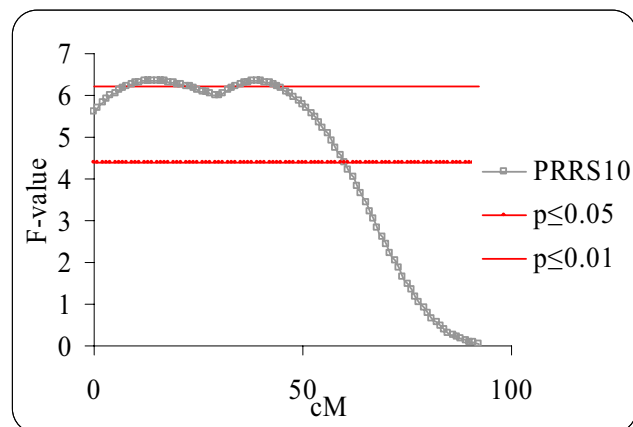
Figure 24:   Relative position of QTL for antibody to PRRS vaccination on SSC3

For the traits regarding antibody to different vaccinations, SSC3, SSC6, SSC10, SSC16 and SSC17 could be detected as interesting chromosomes. The most important locus seems to be located nearby the marker *S0070* on SSC10 where QTL affecting the antibody response to Mh vaccination at all three different measurements were detected.

4.3   Molecular genetic analyses of porcine MBL genes

4.3.1   Expression study of porcine MBL genes

The expression of *MBL1* and *MBL2* genes in different porcine tissues were investigated using mRNA from ten tissues of adult animals of the third generation of the backcross. The results showed differential expression of the porcine MBL genes as demonstrated in figure 25. Both MBL genes were highly expressed in liver. Low *MBL1* expression was also found in lung, testis and brain, while low expression of *MBL2* was detected in testis and kidney.
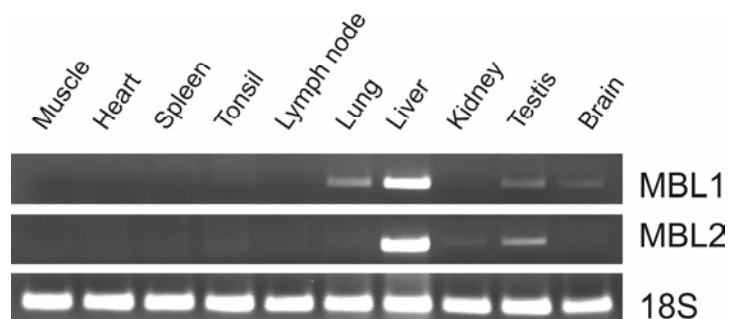
Figure 25:   Tissues specific expression patterns of *MBL1* and *MBL2* genes assayed
               by RT-PCR. The RT-PCR of ribosomal 18S was performed to provide an
               internal reference.

4.3.2   Single nucleotide polymorphism detection

Two SNPs were found at the position 579 (G to A) and 645 (G to A) of porcine *MBL2* mRNA (GenBank accession no. NM_214125) in F2 DUMI population, one of these was confirmed by enzyme digestion in PCR-RFLP analysis. This two SNPs were located at codon 193 (AAG) and 215 (GTG) of the predicted amino acid sequence (GenBank accession no. AAD45377), but did not effect amino acid composition in the translated protein (*Lys* and *Val* at codon 193 and 215, respectively). A SNP (at codon 215) affecting an *Ade*I restriction site was found to be segregating in the F2 DUMI resource population. The *Ade*I PCR-RFLP includes fragments of 319 bp (allele A), 286 bp, and 33 bp (allele G) as illustrated in figure 26.
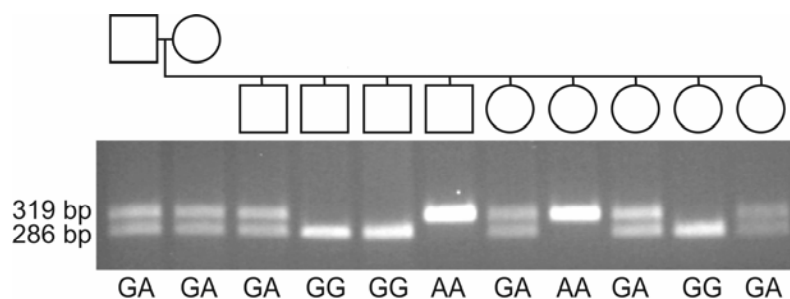
Figure 26:   Mendelian inheritance of the G>A SNP at position 645 of porcine *MBL2* in the F2 DUMI resource population. (The 33 bp fragment was not visible in this figure.)

4.3.3   Genetic and physical mapping of porcine MBL genes

The results of RH mapping as shown in table 27 assigned both porcine *MBL* genes to chromosome 14 (SSC14) with retention frequencies of 16% for both genes. The most significant linked markers (with two-point analysis) for porcine *MBL1* and *MBL2* were *SW210* (89 cR; LOD = 3.32) and *SW1552* (35 cR; LOD =10.66), respectively.

Genetic mapping by 'two point' linkage analysis using CRIMAP 2.4 (Green et al. 1990) was also performed. The linked marker was *S0007* with two-point recombination frequencies and LOD scores for porcine *MBL1* and *MBL2* being 0.32, 3.34 and 0.23, 8.26, respectively.

Table 27:   Relative distances, LOD scores, linked markers and chromosomes of the
                corresponding 'two point analysis' for MBL genes by radiation hybrid
                panel (118 hybrids)

| Gene | Map Order | Chromosome | Marker 1 | P(BR)[a] 1 | Distance 1 | LOD 1 |
			Marker 2	P(BR) 2	Distance 2	LOD 2
MBL1	1	14	*SW1536*	0.62	0.97	2.87
			SW1556	0.67	1.11	2.22
	2	15	*SW1263*	1.05		0
			SW120	0.7	1.2	1.8
	3	14	*SW210*	0.59	0.89	3.33
			SW1536	0.62	0.97	2.87
	4	15	*SW1339*	0.78	1.5	1.02
	5	8	*S0178*	1.01		0
			Sw2521	0.8	1.59	0.85
MBL2	1	14	*SW1552*	0.29	0.35	10.67
			SW2122	0.77	1.48	1.07
	2	14	*SW1082*	0.43	0.56	6.33
			SW1552	0.29	0.35	10.67
	3	14	*SW2001*	0.67	1.1	2.09
			SW1082	0.43	0.56	6.33
	4	4	*SW1364*	0.56	0.82	4.05
			SW589	0.76	1.43	1.2
	5	12	*SWR1021*	0.81	1.64	0.75

[a] P(BR) Estimate of breaking probability

4.3.4   Genotyping of the porcine MBL genes

The results from 347 F2 DUMI pigs using *Hinf*I enzyme digestion in PCR-RFLP
showed allele C and allele T frequencies of *MBL1* gene of 0.67 and 0.33, respectively.
The genotype frequencies were 0.48, 0.38 and 0.14 for C/C, C/T, and T/T, respectively.
For *MBL2*, the results from 284 F2 DUMI pigs using the *Ade*I enzyme digestion in
PCR-RFLP showed allele G and allele A frequencies of 0.41 and 0.59, respectively. The

genotype frequencies were 0.21, 0.40, and 0.39 for G/G, G/A, and A/A, respectively (Table 28).

Table 28:   Genotype and allele frequencies of porcine *MBL1* (C to T substitution at intron 1) and *MBL2* (G to A substation at codon 215) in the F2 DUMI population

	Animals	Allele frequencies		Genotype frequencies		
		C	T	C/C	C/T	T/T
MBL1	304	0.67	0.33	0.48	0.38	0.14
		G	A	G/G	G/A	A/A
MBL2	284	0.41	0.59	0.21	0.40	0.39

4.3.5   Phylogenetic analyses of MBL genes

The predicted amino acids sequences of MBL-A and MBL-C across species (porcine, human, rhesus monkey, mouse, rat and chicken) were aligned. The unrooted neighbour-joining tree showing phylogenetic relationships of MBL based on the neighbour-joining (NJ) method reconstructed by MEGA3 (figure 27) indicated the three distinct forms of MBL among all species investigated (MBL-A and MBL-C in mammals and MBL in chicken). In total 12 predicted amino acid sequences were used for constructing the tree. The MBL-A and MBL-C branches each comprise of three different sub-branches, i.e. rodent (mouse and rat), primate (rhesus and/or human) and non-primate (porcine and cattle). The non-primate sub-branch of both MBLs indicates highest identity (76% and 98% bootstrap values for MBL-A and MBL-C) of porcine to bovine. The result also indicates a closer relationship of the non-primate to primate than to the rodent group.
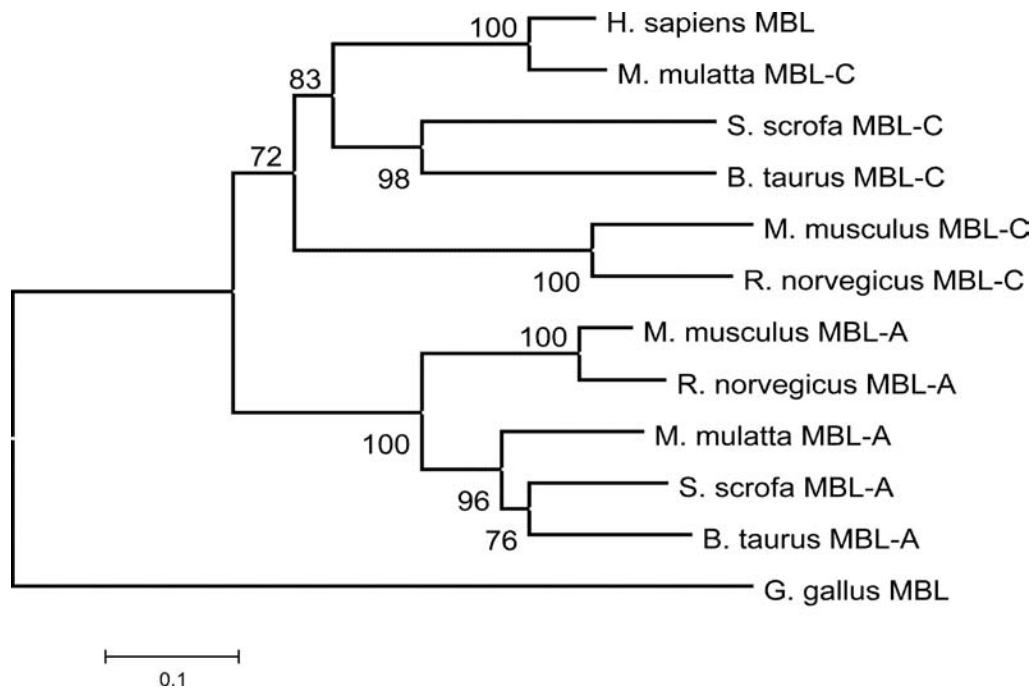
Figure 27:   Unrooted neighbour-joining tree showing phylogenetic relationships of
               MBL in mammals and chicken. The numbers at the nodes are the
               bootstrap scores (percentage of 1000 replicates).

4.3.6   Association analyses

The model application including the *MBL1* and *MBL2,* and their interactions with time
points revealed that the genotype of both genes showed no effect on haemolytic
complement activity in classical and alternative pathways as shown in table 29. The
haemolytic activities of both pathways depend on sequences of the complement cascade
that do not directly involve *MBL1* or *MBL2*. Thus, finding of no-association could have
been expected. However, the outcome emphasises and highlights the specificity of the
results obtained for C3c serum concentration that reflects *In vivo* complement activation
after the vaccination that may act on the lectin pathway controlled by MBL. In fact, the
C3c protein level, which reflects *In vitro* complement activity, tended to be higher in
MBL1 heterozygous genotypes (C/T) than in the homozygous genotypes (C/C and T/T)
(p=0.067). There was a highly significant effect of time points of measurement
(P<0.001).

Table 29:   Least square means of the haemolytic complement activities (AH_{50}, CH_{50}) and C3c concentration for the effect of *MBL1*and *MBL2* genotypes in the F2 DUMI population

Genotype		AH50	CH50	C3c
MBL1	C/C	56.29 ± 2.47	68.12 ± 3.31	0.190 ± 0.004
	C/T	58.13 ± 2.60	65.25 ± 3.55	0.198 ± 0.005
	T/T	54.27 ± 3.40	62.90 ± 4.31	0.192 ± 0.005
Effect (P):	*MBL1*	0.355	0.313	0.067
	Time	< 0.001	< 0.001	< 0.001
	MBL1*Time	0.690	0.479	0.056
MBL2	G/G	58.24 ± 3.14	67.59 ± 4.36	0.201 ± 0.006
	G/A	60.58 ± 2.60	68.89 ± 3.50	0.201 ± 0.005
	A/A	56.69 ± 2.63	65.54 ± 3.61	0.194 ± 0.005
Effect (P):	*MBL2*	0.141	0.499	0.136
	Time	< 0.001	< 0.001	< 0.001
	MBL2*Time	0.664	0.723	0.967

Interactions of time points and MBL genotypes in the repeated measures model reflect the dependency of the profile of complement activity along the experiment on the genotype. For *In vivo* complement activity, a slight *MBL1* genotype dependent deviation of the profiles of C3c concentration over time points was found (p=0.056). This deviation is most prominent late after Mh vaccination. The profiles of the haemolytic complement activities between different genotypes of porcine *MBL1* and *MBL2* were similar over time points as shown in figure 28. No significant effect of interaction between genotypes and time points on haemolytic complement activity was found in the alternative and classical pathway.
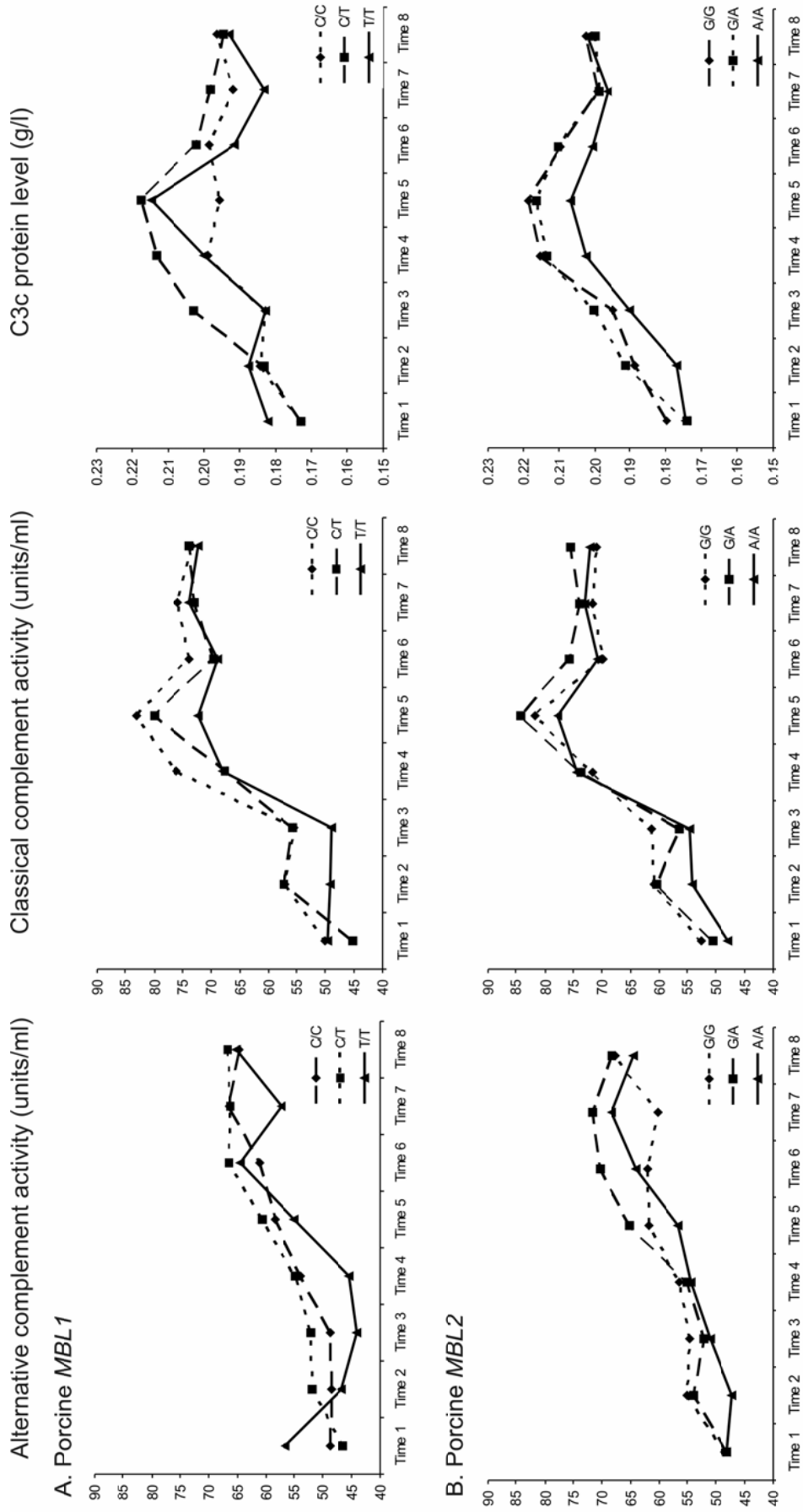
Figure 28: Plots of least square means of CH50, AH50 and C3c at different time points (A: *MBL1*, B: *MBL2*)

5   Discussion

5.1   Immunological analyses

5.1.1   Complement activity

The complement system plays an important role in host defence against infection and in most inflammatory processes. Assessing the functional integrity of the complement system (classical pathway) has been accomplished in the clinical laboratory by such assays for many years (Jaskowski et al. 1999). The assay is quantitative, with the result expressed as the reciprocal of the serum dilution required to produce lysis of 50% of defined numbers of red cells under standard conditions. The pathway is initiated by IgM on the surface of sheep RBCs (early antigen, EA, antibody sensitized sheep erythrocytes) and the assay is performed in the presence of calcium and magnesium ions (required for classical pathway activation). This assay needs to be controlled with positive control samples that are known to contain all the components and by a negative control, which controls spontaneous lysis of the erythrocytes. The amount of haemolysis (measured by determining the absorbance of the cell supernatant) can be compared with a known serum or using the von Krogh equation (North and Whaley 2005).

In this study, haemolytic activities of classical and alternative pathways of experimental pigs under different vaccinations were altered from 28.44 U/ml prior vaccinations to 35.27 U/ml after three vaccination treatments in the classical pathway and from 27.95 to 56.09 U/ml in the alternative pathway. Mekchay et al (2003) reported a range of the haemolytic complement activity between 39.17 to 59.63 U/ml for the classical pathway, and 49.50 to 63.06 U/ml for the alternative pathway in the F2 DUMI population. Moreover, it has been reported that the porcine haemolytic complement activity varied from 3.6 to 210 U/ml for the classical pathway (Ish et al. 1993, Renshaw and Gilmore 1980), and 8 to 33 U/ml for the alternative pathway (Mallard et al. 1989, Wimmers et al. 1999a). The variation of the haemolytic activity is associated with many factors, including age (Bernoco et al. 1994, Tanaka et al. 1986), vaccination (Mallard et al. 1989, Wimmers et al. 1999a), nutritional status (Sakamoto et al. 1998), genetics (Mallard et al. 1989, Vaiman et al. 1978, Wimmers et al. 1999a) and sample storage temperature (Demey et al. 1993, Pandey et al. 1993).

For the alternative pathway activity, sheep red cells are inefficient, so rabbit erythrocytes are used for the AH_{50} assay. Ethyleneglycol bis (b-aminoethyl ether)-N,N-tetraacetic acid (EGTA) chelates calcium, but not magnesium ions, will prevent any concomitant activation of the classical pathway while permitting activation of the alternative pathway (North and Whaley 2005).

5.1.2   Antibody response to vaccinations

The antibodies were measured with assays designed to detect naturally acquired antibodies. A rise in antibody response to Mh and TT vaccinations was clearly found over the time points of measurement (14.59 to 37.40 % for Mh antibodies, and 0.09 to 0.92 of ELISA S/P ratio result for TT antibody). It is difficult to estimate the trend of PRRS antibodies over vaccination time due to the single measurement in the study.

M. hyopneumoniae is the primary pathogen responsible for swine enzootic pneumonia, which is thought to be the most important contributor to disease-associated economic losses in swine production worldwide (Kristensen et al. 2004). The study from Feld et al. (1992) suggested that using the blocking ELISA appears to be a valuable and reproducible tool in the surveillance and serodiagnosis of *M. hyopneumoniae* infections in pigs. Comparing to the indirect hemagglutination assay (IHA), the ELISA give earlier detection and less cross reaction results than the IHA assay. This conclusion was confirmed by the previous study. Armstrong et al. (1983) indicated that ELISA is potentially very attractive as a practical serodiagnostic test for *M hyopneumoniae* in pigs, and their study indicated that it is extremely sensitive for detecting antibodies to *M. hyopneumoniae*. Furthermore, it lends itself to automation and thus would be economical for herd testing.

The measurement of antibodies response to TT in this study indicated the increase of antibodies over the time. In the measurement for this antibody, the tetanus toxoid was used as an antigen and the conjugated rabbit antibody against pig immunoglobulin was used as a second antibody. It can be concluded from the results, that the increased antibody level detected reflects the total immunoglobulin due to the detection antibody used. It has been, however, reported that TT antibody levels decreased significantly with time after the last TT vaccination but did not lead to nonprotective levels of TT antibodies (Aboud et al. 2001, Cook et al. 2001, Simonsen et al. 1987a).

PRRS has been recognized as an economically important disease of pigs in many parts of the world in the early 1990s. This disease causes reproductive failure in pregnant sows and respiratory problems in piglets (Bilodeau et al. 1991, Christianson et al. 1992, Collins et al. 1992, Wensvoort et al. 1991, Yoon et al. 1992). To determine the antibody response to PRRS, ELISA technique was found to contribute to a better performance in the combination of sensitivity (96.1%) and specificity (100%) relative to the reference classification of the serum samples and repeatability (kappa value 98%). It is technically superior to an indirect immunofluorescent assay (IFA) and an immunoperoxidase monolayer assay (IPMA), time efficient, cost effective and suitable for testing of a large number of samples over a short period of time. These would bring the ELISA to a preferred assay for routine detection of antibody response to PRRS in pig sera (Cho et al. 1996, 1997b).

5.1.3 Complement C3c concentration

C3c is the split product derived from proteolysis of inactive C3b (iC3b) in serum and cell surfaces (Davis et al. 1984, Lachmann et al. 1982). The measurement of C3c concentration in serum has been performed in many studies for determining the complement activity of the major component in the complement system, C3 (Asghar et al. 1984, Borque et al. 1983, Gonzalez-Ortiz et al. 2002, Kallman et al. 1998, Mekchay et al. 2003, Wimmers et al. 1999a, Wimmers et al. 2003). In this study, C3c was measured by indirect ELISA, and a polyclonal rabbit anti-human C3c was used for determining the C3 component in the sera. The results show an increase of the C3c concentration after each period of Mh, TT and PRRS vaccination.

A previous study, conducted by Mekchay (2003), showed a significant effect of the interaction between the C3 genotype and the time point of C3c measurement on the C3c concentration. The result also showed an increase of C3c after 10 days of Mh vaccination in heterozygous animals which was higher than in homozygous animals in this study. In contrast, the C3c concentration in homozygous animals was significantly higher than in heterozygous animals after 4 days of Aujeszky vaccination. However, the C3c concentration was not different between genotypes at 10 days after PRRS vaccination. Another study indicated that the variation of the C3c is associated with sire

and dam factors as well as the vaccination effects (Wimmers et al. 1999a, Wimmers et al. 1999b).

5.1.4   Haptoglobin concentration

Estimations of changes in acute phase protein (APP) levels are useful, since they generally reflect the presence and intensity of an inflammatory process (Gabay and Kushner 1999). The Serum concentration of the APP including haptoglobin changes dramatically during the acute phase response. They are present at substantially different concentrations in the blood and can be classified according to the magnitude of their increase. Hp is a positive APP in serum concentrations during an acute phase response (Hiss et al. 2003, Petersen et al. 2004). An acute phase response was developed in the different diseases studies, this response was higher in animals with clinical signs and concurrent bacterial processes (Parra et al. 2006).

The Hp concentration obtained from backcross DUMI population in this study (range between 1.23 ± 0.92 to 2.18 ± 1.07 mg/ml) were similar to experimental pigs (1.19 ± 0.24 to 1.42 ± 0.02 mg/ml) that were raised in two traditional farms in the study of Chen et al. (2003), but higher than the values obtained from conventional and specific pathogen free herds in their report (0.9 and 0.3 mg/ml, respectively).

5.2   QTL analysis

The objective of the QTL study was to identify QTL that affect the immune traits and to further identify candidate genes underlying the detected QTL which might play an important role on the immune traits. In this study, different measurements of the immune competence were used as phenotypes. As the regions of QTL are too large, genes were identified by searching functional or positional candidate genes in the region of the QTL detected in this study. For that reason, we first identified genes, already mapped in the QTL regions in the pig. Further, because of many genes are already mapped in human, we identified genes, involved in the immune system on the homologous chromosomes in human. Table 30 is showing the homologous human chromosomes, to the chromosomes where QTL for the traits, measured in this study, were detected. Only the most interesting QTL for each chromosome are mentioned.

For further discussion, the single measurements were defined as times, so they were named as trait_1, trait_2, trait_3, trait_4, trait_5 and trait_6 for the measurements of AH_{50}, CH_{50}, C3c and Hp following the treatments with vaccinations of Mh, TT and PRRS. Also the single measurement of antibody response to vaccination was defined as day of vaccination and they were named as Mh_0, Mh_{10}, Mh_{20}, TT_0, TT_{20}, TT_{40} and $PRRS_{10}$ for Mh, TT and PRRS vaccination at different time points, respectively.

The interesting results are the QTL for C3c_1 on SSC3 and SSC18, for antibody to PRRS vaccination on SSC3, the QTL for AH_{50}_2 on SSC7 and SSC16 and the QTL for antibody response to Mh vaccination on SSC10.

Table 30:   Homologous regions in the human genome for detected porcine QTL

SSC	Trait	Porcine QTL position (cM)	Homologous region on HSA
1	AH_{50}_(3, 4)	74	HSA9 or HSA15q
3	$PRRS_{10}$	14	HSA16p or HSA7 or HSA2p
	C3c_(1, 2), C3c_(3, 4, 6), AH_{50}_6	0 to 30	
	CH_{50}_1, AH_{50}_6	75 to 95	
4	AH_{50}_(1, 3, 5, 6)	50 to 62	HSA8q
6	Mh_{20}	16	HSA16q or HSA19q
7	AH_{50}_(1, 2, 4, 5, 6), CH_{50}_6, Hp_2	0 to 105	HSA6p or HSA14q or HSA15q or HSA19q
10	Mh $(_{0, 10, 20})$	100	HSA10p
11	C3c_(1, 3, 6)	23 to 42	HSA13p or HSA13q12-21
16	AH_{50}_(1, 2, 3, 4), CH_{50}_5, Hp_5, TT_{40}	18 to 79	HSA5p or HSA5q11-14
17	Hp_(4, 5), C3c_4, CH_{50}_(4, 6), TT_{20}	0	HSA20p [or HSA5]
18	C3c_(1, 2, 3, 4, 5)	130	HSA7q22-36

5.2.1   QTL for haemolytic complement activity traits

There is no clear evidence of QTL for the CH_{50} levels measurements on any chromosome. The only region harbouring a QTL affecting two different measurements (CH_{50}_4 and CH_{50}_6) was found on SSC17 at 0 cM. Most QTL were detected for the measurements of AH_{50} on SSC1 (AH_{50}_3 and AH_{50}_4), SSC4 (AH_{50}_2, AH_{50}_3, AH_{50}_4 and AH_{50}_6), SSC7 (AH_{50}_1, AH_{50}_2, AH_{50}_4, AH_{50}_5 and AH_{50}_6), and SSC16 (AH_{50}_1, AH_{50}_2, AH_{50}_3, AH_{50}_4 and AH_{50}_6). Two of the most interesting chromosomal regions contain QTL for AH_{50} on SSC7 and SSC16, where QTL for five measurements could be detected. According to the literatures, several genes that found lie on SSC7 are involved in the immune response, including complement factor B properdin (*BF*), genes belonging to the major histocompatibility (MHC) class (i.e. DR beta2 (*DRB*) or DQ alpha (*DQA*) or beta (*DQB*) (Hradecky et al. 1982, Hruban et al. 1976, Smith et al. 1995, Vaiman et al. 1979)), immunoglobulin alpha (*IGA*) (Fronicke et al. 1996, Thomsen et al. 1998), mannose phosphate isomerase (*MPI*) (Christensen et al. 1985, Gellin et al. 1981), T cell receptor alpha (*TCRA*) and transforming growth factor beta 3 (*TGFB3*) (Rettenberger et al. 1996). While on SSC16, the complement component 9 gene (*C9*) (Thomsen et al. 1998, Wintero et al. 1998) is also involved in the immune response. For further identification of genes located in the porcine QTL regions in this study, the comparative maps of human genome were applied. HSA6p, HSA14q, HSA15q, and HSA19q are homologous to SSC7, while HSA5 is homologous to SSC16 (Meyers et al. 2005). Examples of genes involved in the immune response found in the comparative maps at the homologous porcine regions as obtained by NCBI GenBank are shown in table 31.

Table 31:   Comparative human positional candidate genes and regions for SSC7 and SSC16

SSC	HSA	Gene
SSC7	HSA6p21.3	Immune response to synthetic polypeptide-irglphe 1 (*IGLP1*)(Chan et al. 1984, Hsu et al. 1981)
SSC7	HSA6p21.3	Major histocompatibility complex, class I, A (*HLA-A*) (Bakker et al. 1979)
SSC7	HSA6p21.3	Complement component 4A (*C4A*) (Teisberg et al. 1976)

Table 31 (continued)

SSC	HSA	Gene
SSC7	HSA6p21.3	Major histocompatibility complex, class II, DR alpha (*HLA-DRA*) (Levine et al. 1984)
SSC7	HSA6p21.3	major histocompatibility complex, class II, DQ alpha-1 (*HLA-DQA1*) (Levine et al. 1984)
SSC7	HSA14q32.1	Protease inhibitor (*PI*) (Schroeder et al. 1985)
SSC7	HSA14q32.33	IgG heavy chain locus (*IGHG1*) (Cox et al. 1982)
SSC7	HSA19p13.3	CD209 antigen (*CD209*) (Soilleux et al. 2000)
SSC7 SSC16	HSA6p21.2-p12 HSA5q33.2 HSA5q32	IgE responsiveness, Atopic (*IGER*)
SSC16	HSA5q31	Interleukin 13 (*IL13*) (Morgan et al. 1992)
SSC16	HSA5q31.1	Interleukin 4 (*IL4*) (Sutherland et al. 1988)
SSC16	HSA5q31.1	Interleukin 5 (*IL5*) (Sutherland et al. 1988)
SSC16	HSA5q31.1-q33.1	Interleukin 12B (*IL12B*) (Sieburth et al. 1992)

5.2.2   QTL for complement component C3c and Hp concentrations

The results indicated at least three interesting QTL affecting the measurements for C3c that could be detected on SSC3, SSC11, and the most interesting on SSC18. Genes already mapped on porcine chromosomes are involved in traits related to the immune system are T cell receptor beta (*TCRB*), and inhibin beta A (*INHBA*) (Lahbib-Mansais et al. 1996, Rettenberger et al. 1996).

A number of genes locating on the HSA7, a homologous chromosome region to SSC18, that involved in the immune response could be identified and proposed as a candidate gene. The following genes are interesting regarding the immune system: T-cell antigen receptor, gamma subunit (*TCRG*) at 7p15-p14 (Bensmana et al. 1991), mucopolysaccharidosis type VII (*GUSB*) at 7q21.11 (Ward et al. 1983), interleukin 6 (*IL6*) at 7p21, zinc finger protein, subfamily 1A, member 1 (*ZNFN1A1*) at 7p12, Shawachman-diamond syndrome (*SDS*) at 7q11, carboxypeptidase, vitellogenic-like (*CPVL*) at 7p15-p14, and receptor activity-modifying protein 3 (*RAMP3*) at 7p13-p12.

On the SSC3, different genes, including interleukin alpha and beta (*IL1A* and *IL1B*), immunoglobulin kappa light chain (*IGKC*) and the B-cell genes, B-cell CLL/lymphoma 7B (*BCL7B*) and B-cell maturation factor (*BCMA*) are already mapped. High conserved regions of SSC3 are mentioned to be HSA16p, HSA7 and HSA2p (http://www.toulouse .inra.fr/lgc/pig/compare/compare.htm). Many genes could be identified derived from NCBI database which are involved in the immune system e.g. immunoglobulin, variable region genes of kappa light chain (*IGKV*), immunoglobulin kappa constant region (*IGKC*) on HSA2p12 (Malcolm et al. 1982), and T-cell antigen receptor gamma subunit (*TCRG*) on HSA7p15-p14 (Bensmana et al. 1991).

5.2.3   QTL for antibodies responses to vaccinations

The most interesting chromosome for antibody-related traits in our study is SSC3 for antibody response to PRRS vaccination and SSC10 for antibody response to Mh vaccination. The detected QTL affecting the antibody response to Mh vaccination is interesting, as for all three measurements the same region could be identified. The human genes on the HSA10p region are homologous to those regions that were found by QTL analysis on porcine SSC10 at 100 cM. Genes in this region are gata-binding protein 3 (*GATA3*) at HSA10p15 (Joulin et al. 1991) and neuropilin1 (*NRP1*) at HSA10p12 (Rossignol et al. 1999). In pig, different genes were mapped on SSC10q which are involved in immune response, including EST Z81203 of macrophage inflammatory protein beta 1 (*MIP1B*) at 10q11-q12, porcine EST BF713707 of inter-alpha-inhibitor heavy chain H2 at 10p17, integrin beta 1 subunit (*ITGB1*) at 10p17, porcine EST Z84039 of iron responsive element binding protein (*IREB1*) at 10q11-q12, and porcine EST of cathepsin L (AJ301219; *CTSL*) at SSC10q11-q12.

5.2.4   Comparison of detected QTLs in DUMI F2 and backcross population

The following figure 29 shows the detected QTL of F2 DUMI population by Wimmers (2002). Based on this result, the QTL analysis in backcross was performed in order to confirm the previous detection. Similarities were found on: SSC3, SSC4, SSC5, SSC8 and SSC10. There was no QTL detected on SSC13 in both populations. In backcross and F2, QTL for traits related to the measurement of C3c level were also found.
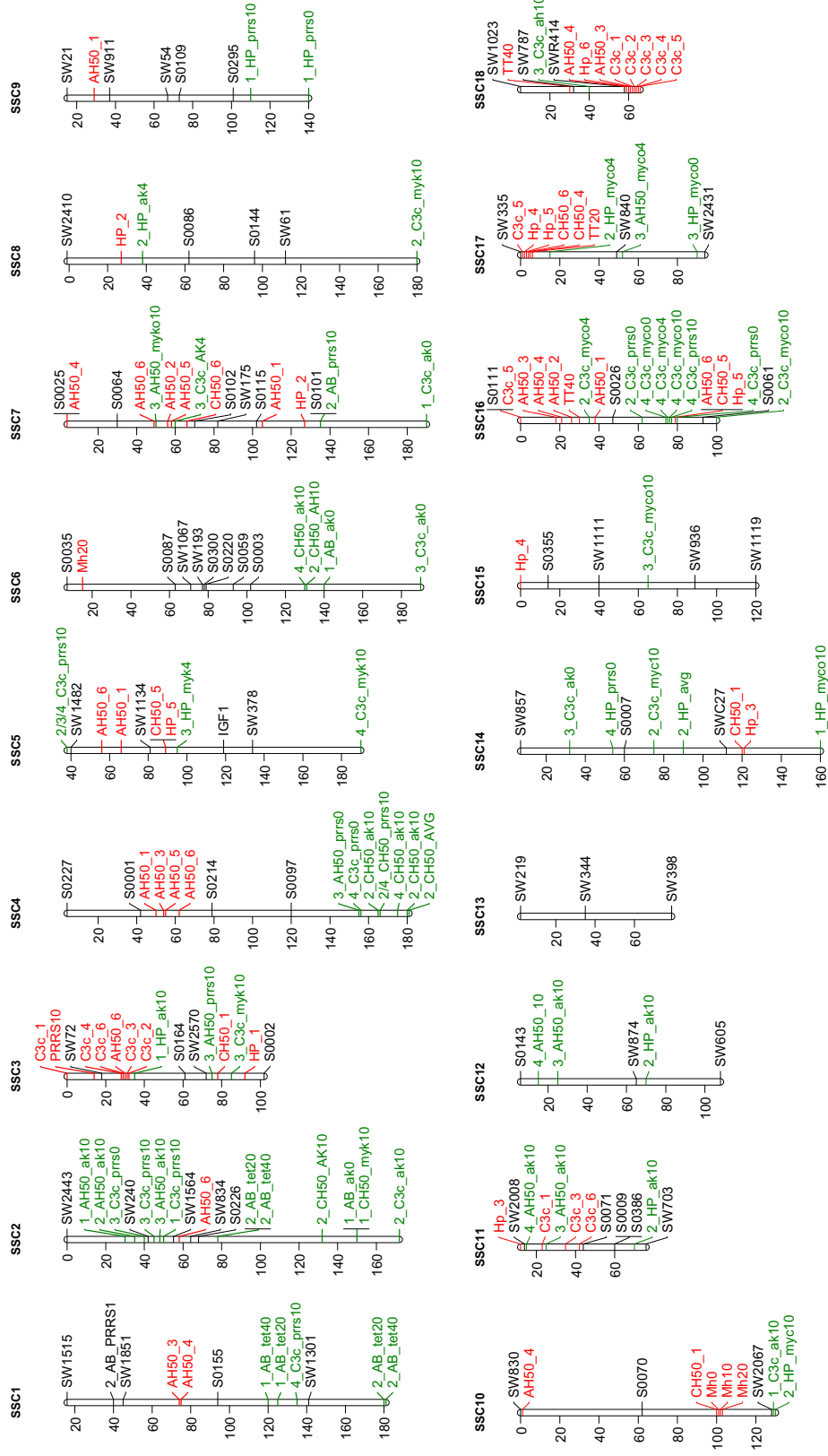
Figure 29:   Overview of QTL for haemolytic complement activities (AH_{50} and CH_{50}), antibodies to Mh, TT and PRRS vaccinations, Component complement C3c and haptoglobin traits in F2 and backcross DUMI populations (green: F2 DUMI (Wimmers 2002) red: backcross DUMI).

5.2.5   Conclusions from QTL identification

A number of QTL for the immune traits could be detected in the backcross population in this study. Many QTL regions affecting the immune traits were also found in different species such as mouse, human and chicken (de Buhr et al. 2006, Hall et al. 2002, Siwek et al. 2003a, Siwek et al. 2003b). In mouse, the number of candidate genes that play an important role for inflammatory processes and immune response could be increased by the combination of QTL and microarray approach (de Buhr et al. 2006). These results, found in other species, suggest further work including additional methods, which seem to be promising for the detection of candidate genes involved in the development of the immune system.

Only few reports exist on QTL affecting traits related to the immune capacity in pigs. Edfors-Lilja et al. (1998) identified four QTLs with significant effects on the immune functions in pigs. These four QTL consist of a QTL affecting total leukocyte counts on SSC1 at 78 cM ($p \leq 0.05$), mitogen-induced proliferation on SSC4 at 75 cM ($p \leq 0.05$), prevaccination levels of antibodies response to *E. coli* Ag K88 on SSC5 at 64 cM ($p \leq 0.01$) and antibody response to the O149 antigen on SSC6 at 69 cM ($p \leq 0.01$). Additionally, the other three putative QTL ($p \leq 0.10$), affecting mitogen-induced proliferation on SSC7 at 73 cM, spontaneous proliferation on SSC13 at 107 cM, antibody response to the O149 antigen on SSC6 at 67 cM could be identified in their study. The identification of QTL with effect on stress induced alterations of porcine immune function were also done by Edfors-Lilja et al.(2000). Their results indicate several significant and suggestive QTL for a number of immune capacities. These suggestive QTL consist of highly significant QTL ($p \leq 0.01$) affecting 'stress' induced alterations in mitogen induced interleukin 2 activity on SSC12 at 109 cM and number of neutrophils on SSC8 at 47 cM. Significant QTL ($p \leq 0.05$) affecting spontaneous proliferation after stress on SSC2 at 109 cM and mitogen induced interleukin 2 activity after stress on SSC6 at 127 cM were also detected. A suggestive QTL ($p \leq 0.10$) from this study was the QTL affecting mitogen induced proliferation after stress on SSC11 at 3 cM. Wattrang et al. (2005) verified and confirmed the involvement of the chromosomal regions on SSC1 and SSC8 reported by Edfors-Lilja et al (1998, 2000). The QTL affecting hematocrit and haemoglobin levels, leucocyte numbers and in vitro leukocyte functions were also detected on SSC1 and SSC8 at the similar regions.

In cattle, many QTL could be detected and published on the QTL database (http://www.animalgenome.org/cgi-bin/QTLdb/) (Hu et al. 2005). QTL for general disease resistance were found on BTA9, BTA11, BTA15, and BTA25 in animals from the 10 largest dairy cattle half-sib families in Sweden (Holmberg and Andersson-Eklund 2004). In chicken, many QTL could be identified for antibody response to different pathogens (Siwek et al. 2003a, Yonash et al. 2001).

Many interesting QTL regions as well as candidate genes are also detected in chicken for different immune traits and are mostly related to the immune response. The interleukin genes are found to be candidate genes for immune response (Zhou et al. 2001, Zhou and Lamont 2003b). Significant association was also found for the *TGFB2*, *TGFB3*, and *TGFB4* genes to multiple immune response parameters. The *TGFB* SNPs might be applied in MAS to improve antibody production. Different QTL were detected for genetic regulation of antibody response to two different T-cell dependent antigens on GGA2, GGA3, GGA14, GGA18, and GGA27 (Siwek et al. 2003a). The regions on GGA3, GGA5, GGA6, and GGAZ were found to contain QTL affecting antibody kinetics in hens (Zhou et al. 2003). QTL for different traits related to immune system parameters were detected on GGA3, GGA5, GGA6, GGA9, GGA10, GGA15, GGA16, GGA23 and GGA27. These QTL are involved in the regulation of the primary and secondary immune response to sheep red blood cells (Siwek et al. 2003b).

According to the comparative mapping of chicken and human chromosome regions by Groenen et al. (2000) and human to pig by Meyers et al. (2005), the homologous of detected QTL affecting immune response in the backcross in this study to chicken QTL affecting immune capacity traits could be identified as described in table 32.

Table 32: Homologous chromosomal segments of detected porcine QTL regions in human and chicken

GGA	HSA	SSC	QTL in pig
	HSA3p	SSC13	-
	HSA7q	SSC18	AH_{50}_3, AH_{50}_4, C3c_1-C3c_6, Hp_6
	HSA10p	SSC10	AH_{50}_4, CH_{50}_1, Mh_0, Mh_{10}, Mh_{20}
	HSA7p	SSC18	AH_{50}_3, AH_{50}_4, C3c_1-C3c_6, Hp_6
GGA2	HSA9q	SSC1	AH_{50}_3, AH_{50}_4
	HSA6p	SSC7, 1	AH_{50}_1-2, $AH_{50}4$-6, CH_{50}_6, Hp_2, AH_{50}_3, AH_{50}_4
	HSA18p	SSC1,6	AH_{50}_3, AH_{50}_4; Mh_{20}
	HSA18q	SSC1, 6	AH_{50}_3, AH_{50}_4; Mh_{20}
	HSA8p	SSC4, 14	AH_{50}_1, AH_{50}_3, AH_{50}_5, AH_{50}_6; CH_{50}_1, Hp_3
	HSA8q	SSC4, 14	AH_{50}_1, AH_{50}_3, AH_{50}_5, AH_{50}_6; CH_{50}_1, Hp_3
	HSA20p	SSC17	CH_{50}_4, CH_{50}_6, TT_{20}, C3c_4, Hp_4, Hp_5
	HSA1q	SSC14	CH_{50}_1, Hp_3
	HSA16p	SSC3	AH_{50}_6, CH_{50}_1. $PRRS_{10}$, C3c_1-C3c_4, C3c_6, Hp_1
GGA3	HSA4p	SSC8	Hp_2
	HSA6q	SSC1, 7	AH_{50}_3, AH_{50}_4; AH_{50}_1, AH_{50}_2, $AH_{50}4$-6, CH_{50}_6, Hp_2
	HSA19q	SSC6, 7	Mh_{20}, AH_{50}_1, AH_{50}_2, AH_{50}_4-6, CH_{50}_6, Hp_2
	HSA2p	SSC2	AH_{50}_6
	HSA11p	SSC2	AH_{50}_6
	HSA15q	SSC1	AH_{50}_3, AH_{50}_4
GGA5	HSA1p	SSC6	Mh_{20}
	HSA14q	SSC7	AH_{50}_1, AH_{50}_2, AH_{50}_4-6, CH_{50}_6, Hp_2
GGA6	HSA10q	SSC10	AH_{50}_4, CH_{50}_1, Mh_0, Mh_{10}, Mh_{20}
GGA9	HSA15q	SSC1	AH_{50}_3, AH_{50}_4
	HSA4q	SSC8	Hp_2
GGA10	HSA1	SSC3, 6	AH_{50}_6, CH_{50}_1, $PRRS_{10}$, C3c_1-C3c_4, C3c_6, Hp_1; Mh_3
	HSA6p	SSC7	AH_{50}_1, AH_{50}_2, AH_{50}_4-6, CH_{50}_6, Hp_2
	HSA19q	SSC6	Mh_{20}

Table 32 (continued)

GGA	HSA	SSC	QTL in pig
GGA14	HSA16p	SSC3	AH_{50}_6, CH_{50}_1. $PRRS_{10}$, C3c_1-C3c_4, C3c_6, Hp_1
GGA15	HSA22q	SSC14	CH_{50}_1, Hp_3
GGA16	HSA5q	SSC2	AH_{50}_6
	HSA19p	SSC2	
GGA23	HSA11q	SSC9	AH_{50}_1
	HSA1q	SSC9	
GGA27	HSA5q	SSC2	AH_{50}_6

The comparison of QTL regions found in chicken with the results of DUMI backcross shows mostly QTL detected for AH_{50} and CH_{50} which are mapped in similar regions to those found for different immune traits in chicken. The result from this study indicated that the QTL found in this study could be confirmed by several QTL and candidate gene studies in other species.

Different candidate genes, either mapped in the pig genome or in the homologous chromosome segments of human genome could be identified. The previous studies in human, pig, cattle, mouse and chicken may give a better evidence of the regions detected (in their own study). De Buhr et al. (2006) found the candidate genes monocyte antigen Cd14 on SSC2, guanylate binding protein 1 (*Gbp1*) on SSC14q26, and phospholipase A2, group IIA (*Pla2g2a*) on SSC6q28 as major candidate genes that play an important role in inflammatory processes and immune response.

Further, studies in other species suggest a combination of microarray and QTL analysis are useful tools for combining functional with positional data to refine candidate gene selection (de Buhr et al. 2006). Additionally, fine mapping or confirmation studies using other populations lead to narrowing the candidate gene (Siwek et al. 2003a, Siwek et al. 2003b, Wattrang et al. 2005).

5.3   Molecular genetics analyses of porcine MBL genes

5.3.1   Expression study of porcine MBL genes

The porcine *MBL1* expression in this study is similar to the results reported by Lillie et al. (2006) showing also high expression of *MBL1* in liver. Differential expressions of

MBL1 and *MBL2* in murine tissues were reported by Wagner et al. (2003). The semiquantitative expression study revealed that the liver is the major site of expression for both *MBL* genes. Low expression was also found in kidney, brain, spleen and muscle, but only murine *MBL1* is expressed in testis (Wagner et al. 2003).

5.3.2   Comparison of porcine MBL gene

The sequences of the two porcine *MBL*s are homologous to each other as shown in figure 30. The locations of putative structural domains are indicated based on porcine MBL-A (Lillie et al. 2006). The amino acid sequences of the two porcine MBL isoforms are homologous to each other. The identity of these two lectins is about 56.6%, allowing 9 gaps. Both porcine MBLs have three cysteine residues at the N-terminal domain which are involved in oligomerization. A collagen domain can be recognized from the amino acid sequence with its characteristic *Gly-X-Y* repetitive pattern, where *X* and *Y* can be any amino acid (Håkansson and Reid 2000). In animal MBL, the collagen regions comprise 19 *Gly-X-Y* triplets (Holmskov et al. 2003). Both porcine MBL-A and MBL-C have the same repetitive pattern as the MBL of other species, including a single interruption at the middle of the collagen domain as found in rat MBLs. The single interruption in the *Gly-X-Y* repeat pattern of this protein falls in the same part of the collagen domain suggesting a significance of interaction between this MBL with a common effector protein (Drickamer et al. 1986). The amino acid consensus sequence GEKGEP, which is involved in C1q receptor (C1qRp) interaction (Arora et al. 2001) is present in both porcine MBL. This suggests that porcine MBL has the same ability as C1q to stimulate phagocytosis in the complement system (Holmskov et al. 2003). The PGKXGP a sequence representing part of a putative MASP-binding motif is found in the porcine MBL-A, suggesting the potential to activate the lectin-complement pathway (Lillie et al. 2006). Although this motif is altered in MBL-C (PGMVGP) its function is retained, which is confirmed by the result of the porcine MBL-C to functionally activate the lectin pathway in MBL-deficient human sera (Agah et al. 2001). The most variable domain in MBL which contains hydrophobic amino acids necessary for forming the triple helical coil (Kawai et al. 1998) is also present in the porcine MBLs. It is interesting to note that porcine MBL-C misses 9 amino acids at this neck-region compared to MBL-A. This difference between porcine MBL-A and MBL-C may be

correlated with their α-helical-coiled coil formation ability. The carbohydrate recognition domain (CRD) of both porcine MBLs shows a high homology to each other. Furthermore, porcine MBL-A and MBL-C contain a mannose-binding EPN motif (Glu-Pro-Asn) in a CRD. This indicates the ability of porcine MBLs to recognize the mannose sugar (Stahl and Ezekowitz 1998).


```
                                                        Gly-X-Y repeat interuption
                                                        C1qRp interaction site
              Cys              Cys        Cys  Cys
pMBL-A  MLLFSSLPVLLLCVVTASYSEIKTCEDAQKTCSVITCGIPVTNGTPGRDGRDGPKGEKGEPGP  63
pMBL-C  MSLFPSLHLLLLIVMTASHTETENCEDIQNTCLVISCDSPGINGLPGKDGLDGAKGEKGEPGQ  63
        [--Signal peptide--][------N-terminal-----][----------------→

                  MASP-binding motif
pMBL-A  GFRGSQGPPGKMGPPGNIGETGPLGPKGQKGDPGDTSGVEAKLANLEGQIRILKSELDHVKKL  126
pMBL-C  GLIGLQGLPGMVGPQGSPGIPGLPGLKGQKGDSGIDPG--NSLANL-------RSELDNIKKW  117
        -Collagen domain------------------][------Neck-region-------]

                                        Cys
pMBL-A  QTFSLGKKSRKKLYVTNGEMMPFSKVKTLCAELQATVATPKNAEENKAIQDMAPDVAFLGITD  189
pMBL-C  LIFAQGKQVGKKLYLTNGKKMSFNGVKALCAQFQASVATPTNSRENQAIQELAGTEAFLGITD  180
        [--------------------------------------------------CRD----→

            Mannose sugar specificity
                                   Cys        Cys      Cys
pMBL-A  EVTEGQFMYVTGGRMTYSNWKSNEPNDHGSGEDCVILQRDGLWNDISCSSSFLAVCEFPA     249
pMBL-C  EYTEGQFVDLTGKRVRYQNWNDGEPNNADSAEHCVEILKDGKWNDIFCSSQLSAVCEFPA     240
        -----------------------------------------------------------]
```
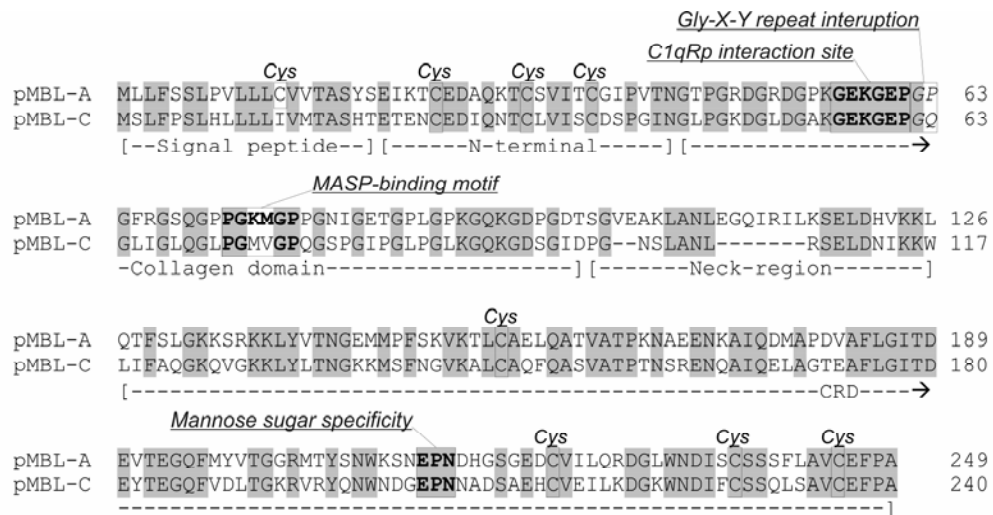
Figure 30:   Alignment of two full-length porcine MBL cDNA-deduced amino acid sequences, MBL-A and -C sequences. Shaded boxes represent identical amino acid residues. Dash (-) in a sequence indicates a gap.

5.3.3 Genetic and physical mapping of porcine MBL genes

The *MBL2* chromosomal location shown by RH mapping in this study is confirmed by gene assignments reported by Meyers et al. (2005) and Yasue et al. (2006), indicating that the porcine *MBL2* is located at the 3226.0 cR on SSC14; its nearest gene and marker is *DKK1* and *SW1552*, respectively. It has been known that a large portion of the porcine chromosome SSC14 is represented in human chromosome HSA10. Rearrangement of gene order on SSC14 contains three regions (46-51, 74-81, and 82-88 Mb-regions) of human chromosome HSA10 (Nonneman and Rohrer 2004). In addition, Yasue et al. (2006) also found the HSA10q arm corresponded to SSC14q24-qter and their study also demonstrated the occurrence of intra-chromosomal rearrangements. Collectin genes including *SFPTA, SFPTD* and *MBL1* were found located in the collectin locus or cluster in human (Guo et al. 1998), mouse (Akiyama et al. 1999) and cattle (Gjerstorff et al. 2004). The *MBL2* gene in human is located on the same chromosome

as the collectin cluster, whereas in mouse and bovine it is located on another chromosome (Gjerstorff et al. 2004). The results in this study reveal the porcine *MBL1* to be located between *SFPTA* and *SFPTD* genes on SSC14. Figure 31 demonstrates the map of SSC14 at the position 46.3-60 cM homologous to human and mouse. Gene positions on SSC14 were taken from Meyers et al. (2005), Yasue et al. (2006) and van Eijk et al.(2000), while the position used for human and mouse genes are from NCBI database, Build 36.2 and 36.1, respectively (http://www.ncbi.nlm.nih. gov/mapview/). Dash lines (----) indicate chromosomal rearrangement. The human *MBL1* in this comparison represents the MBL pseudogene (*MBL1P1)*.
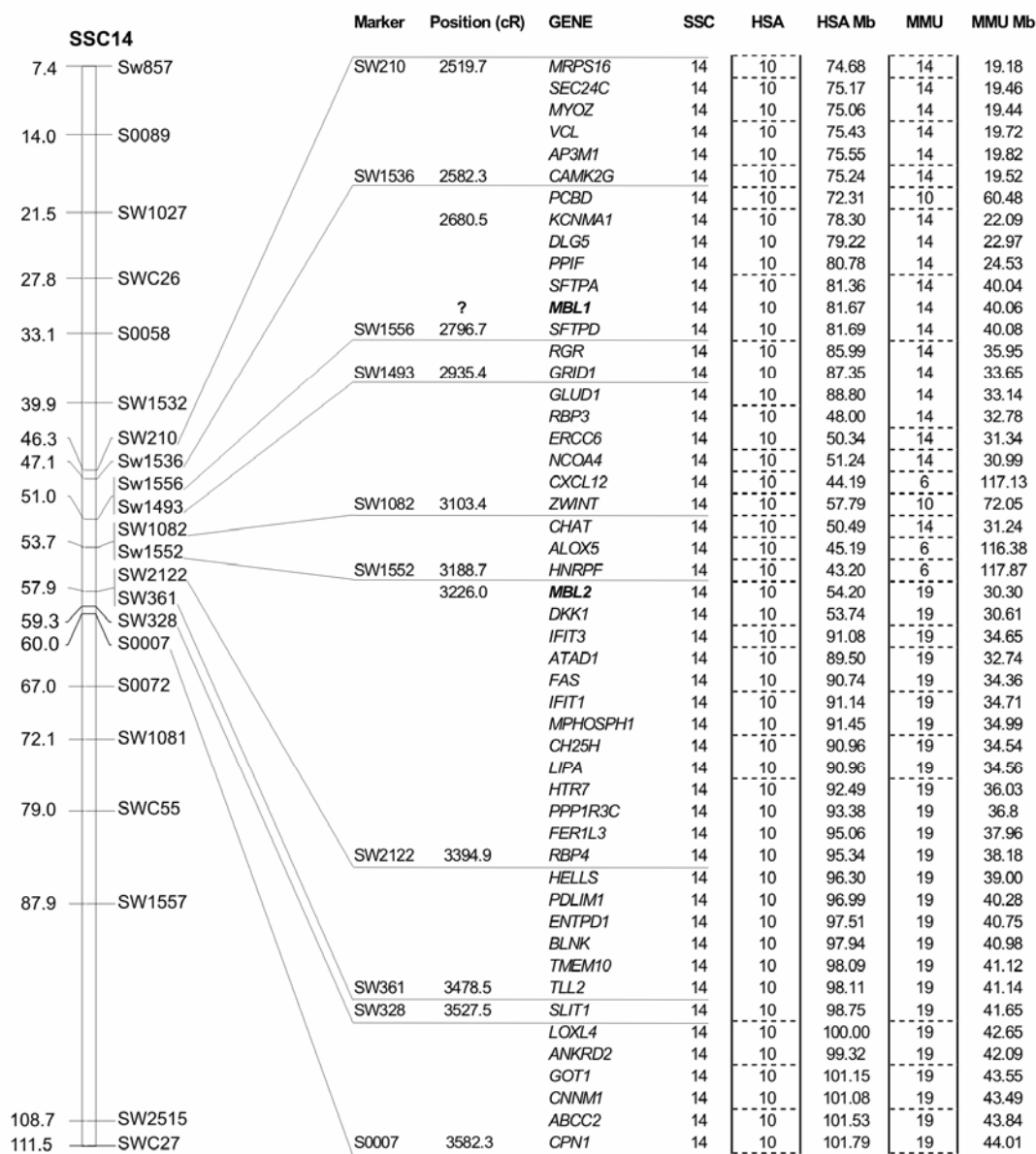

Figure 31: Comparative mapping of the 46.3-60 cM region (flanking markers *SW210* and *S0007*) of SSC14 with the human and mouse genome maps.

5.3.4   Association analyses

Through the activation of complement system, MBL has been found to trigger the opsonic activity of complement resulting in deposition of C3 on target pathogen and stimulation of phagocytic uptake via the C3 receptors (Presanis et al. 2003). Many studies revealed that mutations of MBL affect the complement activity in human. Three SNPs have been identified in exon 1, at codons 52 (Arg → Cys), 54 (Gly →Asp) and 57 (Gly→Glu) (Lipscombe et al. 1993, Madsen et al. 1994, Sumiya et al. 1991), showing association with many innate immunological factors in human (Holmskov et al. 2003). Furthermore, the point mutations in exon 1 of the human MBL gene are frequently described as being associated with MBL plasma concentration, reduced ligand binding capacity and failure to activate complement (Larsen et al. 2004). Comparative sequencing of the porcine *MBL1* and *MBL2* cDNA sequences revealed no non-synonymous nucleotide polymorphisms that may affect the protein function.

Moreover, linkage analysis revealed a significant QTL for C3c serum concentration late after Mh vaccination (T4) on SSC14 in the interval of *MBL1* and *MBL2* as shown in figure 32. In this figure, the x-axis indicates the relative position on the linkage map. Arrows on the x-axis indicate the position of markers. The y-axis represents the F-value. Lines indicate the 5% genome-wide and 5% chromosome-wide significance thresholds. Significant dominance effects were found at the QTL site, corresponding to the finding of differences between heterozygous and homozygous *MBL1* genotypes. The plot of the F-ratio from least square interval mapping for evidence of QTL for C3c serum concentration represents, at each position, a sum up of effects depending on the flanking markers. For instance, in mouse there are an increasing number of QTL studies where large QTL, when fine mapped, turned out to be due to multiple linked loci. Thus the position of the QTL might be a symptom of effects of the two MBL genes. Association analyses of the intronic and synonymous SNPs of *MBL1* and *MBL2* failed to consistently reveal significant effects, probably due to the fact, that they are not in linkage disequilibrium with a causative polymorphism.
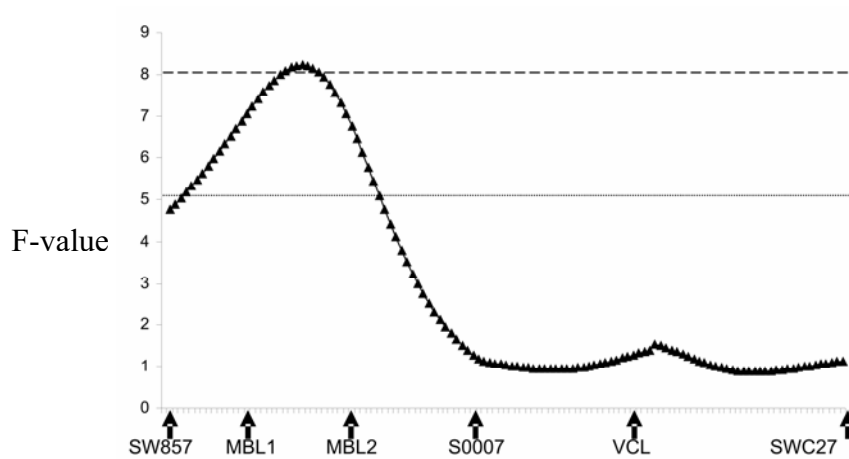
Figure 32:   Plot of the F-ratio from least square interval mapping for evidence of
               QTL for C3c serum concentration after Mh and prior to ADV
               vaccination (time 4) on SSC14.

QTL at 24 cM with: F-value=8.2; LOD=3.5; additive genetic effect=0.004±0.006;
dominance effect=0.051±0.012; fraction of phenotypic variance in the F2 explained by
the QTL=4%

6   Summary

This study was carried out in two Duroc × Berlin miniature pig (DUMI) populations, the F2 and backcross population. The piglets from F2 population were immunized with mycoplasma, Aujeszky's and with a PRRS vaccines at 6, 14 and 20 wk of age, while the backcross were immunized with mycoplasma, tetanus and PRRS vaccination at 6, 9 and 15 wk of age, respectively. Blood samples were collected for 8 and 6 different time points in F2 and backcross populations for the evaluation phenotypes. The immune competence traits measured in this study comprise of the haemolytic complement activity in the alternative and classical pathway, antibodies response to mycoplasma, tetanus toxoid and PRRS, complement component C3c and haptoglobin in the serum. Three different approaches including immunological studies, genetic linkage analysis and candidate gene study were employed. The evaluation of immune competence traits were performed using various assays, including haemolytic complement activity, enzyme-linked immunosorbent assays (ELISA) from both commercial and in housed developed assays. The information obtained from phenotypic evaluation in the first approach was further utilized in the quantitative trait loci (QTL) linkage mapping in the backcross population. In parallel, a candidate gene approach was employed to investigate the association between genes of interest and immune competence traits in the F2 population.

In the immune competence traits evaluation in the backcross, the haemolytic complement activity of classical and alternative pathways was elevated after each immunisation. The results indicated that the haemolytic complement activity of both pathways was increased over the time point of measurements (28.44 to 35.27 U/ml and 27.95 to 56.09 U/ml for classical and alternative pathways, respectively). There were substantial differences in the development and level of activity within the population. Similar to complement activity, the antibody response to different vaccinations including Mh, TT and PRRS vaccinations were measured and revealed an increase of antibodies in the experimental animals over the time points of measurements. Antibody responses to Mh vaccination were 37.40, 19.86 and 14.59% at 10 and 20 days after vaccination, respectively. The samples with a value less than 50% were considered positive, and therefore, an increase in the antibody level would be seen as a decrease in

the test results. The antibody response to TT was measured using an in house developed ELISA and tetanus toxoid was used as an antigen in the assay. The result showed an increase of antibodies over three time points of measurement, which expressed as S/P ratio, were 0.09, 0.65 and 0.92 at prior, after 10 and 20 days of vaccination, respectively. The antibody response to PRRS vaccination was measured at only time point at 10 days after vaccination, and the result revealed positive status of antibody which is expressed at 0.84 of S/P ratio according to the Herdchek® ELISA kit instruction. The complement component C3c was measured at all 6 time points and expressed as S/P ratio. The results indicated the complement C3c concentration was increased after each period of vaccination (0.84 to 0.87, 0.78 to 0.82 and 0.79 to 0.82 during mycoplasma, TT and PRRS vaccination, respectively). Hp concentrations were measured according to the method described by Hiss et al. (2003), by using a competitive ELISA and expressed as mg/ml of Hp in blood serum. Hp concentration in this study was found to be decreased over the first three time points of measurement (1.76 to 1.23 mg/ml, at T1 to T3, respectively). An increase of Hp was observed over the last three time points of measurement and reached the highest value at T6 or 10 days after PRRS vaccination (1.68 to 2.18, at T4 to T6, respectively).

For QTL mapping in the backcross population, the immune competence traits including complement activity, antibody response to vaccination, complement component C3c and haptoglobin were used as phenotypic values in this study. Microsatellite marker genotyping were employed for QTL detection. By genotyping, the seventy-four markers (an average of 4.11 markers per chromosome) were investigated. The microsatellite markers had between 2 to 7 alleles with an average of 4.19 alleles per marker. A total of 220 backcross animals were genotyped. The average heterozygosity and information content of the markers were 0.57 and 0.52. The lowest information content was 0.16 at the locus of the marker *S0155* on SSC1. In total, twenty (27% of all used) markers showed information content less than 0.5. Marker positions were calculated, using CRIMAP 2.4 software by using two possible options *twopoint* and *multipoint* to assign the relative position of the markers on each chromosome. It was concluded that the map used in this study was in good agreement with the map of the F2 population in a previous study in the F2 DUMI population (Wimmers 2002). Moreover, accordance of QTL regions in the studies of the F2 population and the present study could be found. Similarities were found on: SSC3, SSC4, SSC5, SSC8 and SSC10 in both, F2 and

backcross DUMI populations. There was no QTL detected on SSC13 in both populations. Forty-two significant and twenty-four highly significant QTL could be detected for all immune traits in the backcross. Most QTL were detected on SSC3, SSC16, and SSC18 (nine significant F-values on each chromosome). No significant F-value was detected on SSC12 and SSC13. Most highly significant QTL could be detected for antibody response to mycoplasma, TT and PRRS vaccination, C3c and Hp concentration. For AH_{50} and CH_{50}, twenty-two significant and nine highly significant QTL could be detected.

As the third approach, a candidate gene study was employed using the F2 population, the two porcine mannose-binding lectin genes, *MBL1* and *MBL2*, were selected and investigated as functional genes. Regarding the phylogenetic analysis, the porcine *MBL* genes were found to be highly identical with bovine rather than primate and rodent sequences. Radiation hybrid panel mapping positioned both porcine *MBL* genes to chromosome 14 (SSC14), with retention frequencies of 16% for both genes. The most significantly linked markers (with *twopoint* analysis) for porcine *MBL1* and *MBL2* were *SW210* (89 cR; LOD = 3.32) and *SW1552* (35 cR; LOD = 10.66), respectively. Genetic mapping by two-point linkage analysis using CRIMAP 2.4 was also performed and indicated as linked marker *S0007* with two-point recombination frequencies and LOD scores for porcine *MBL1* and *MBL2* of 0.32, 3.34 and 0.23, 8.26, respectively. The expression studies revealed that both *MBL* genes were found to be highly expressed in liver. Low *MBL1* expression was also found in lung, testis and brain, while low expression of *MBL2* was detected in testis and kidney. New single nucleotide polymorphisms in the porcine *MBL2* gene were found and genotyped in an experimental F2 pig population together with a previously reported SNP of *MBL1*. A SNP found in *MBL2* affecting an *AdeI* enzyme restriction site and a SNP that was published in *MBL1* affecting a *HinfI* enzyme restriction site were found to be segregating in the F2 DUMI resource population. The results from 347 F2 DUMI pigs using *HinfI* enzyme digestion in PCR-RFLP showed allele C and allele T frequencies in the *MBL1* gene of 0.67 and 0.33, respectively. The genotype frequencies were 0.48, 0.38 and 0.14 for C/C, C/T, and T/T genotypes, respectively. For *MBL2*, the results from 284 F2 DUMI pigs using an *AdeI* enzyme digestion in PCR-RFLP showed allele G and allele A frequencies of 0.41 and 0.59, respectively. The genotype frequencies were 0.21, 0.40, and 0.39 for G/G, G/A, and A/A genotypes, respectively. The result from association study showed that

only *MBL1* genotypes affected the C3c serum concentration, i.e. the *In vivo* complement activity, at p<0.1. Correspondingly, linkage analysis revealed a QTL for C3c serum level close to the position of the *MBL* genes. The study thus promotes the porcine *MBL* genes as functional and positional candidate gene for *In vivo* complement activity mediated via the lectin pathway.

7   Zusammenfassung

Diese Untersuchung wurde in zwei Duroc × Berliner Miniatur Schwein (DUMI) Populationen, der F2 und der Rückkreuzungspopulation durchgeführt. Die Schweine der F2 Population wurden im Alter von 6, 14 und 20 Wochen mit Impfstoffen gegen Mycoplasma, Aujeszky's und PRRS geimpft. Die Tiere der Rückkreuzung wurden im Alter von jeweils 6, 9 und 15 Wochen gegen Mycoplasma, Tetanus und PRRS geimpft. Den Tieren der F2 Population wurden Blutproben zu 8 Zeitpunkten, den Tieren der Rückkreuzungspopulation zu 6 Zeitpunkten für die Feststellung der Phänotypen entnommen. Die in dieser Arbeit untersuchten Merkmale der Immunkompetenz umfassten die hämolytische Komplementaktivität des klassischen und alternativen Signalweges, die Antikörperreaktion auf Mycoplasma, Tetanus und PRRS, die Komplement Komponente C3c und den Haploglobingehalt im Serum. Drei verschiedene Ansätze, eine immunologische Untersuchung, eine genetische Kopplungsanalyse und ein Kandidatengenansatz wurden angewandt. Die Auswertung der Merkmale der Immunkompetenz wurden mit verschiedenen Methoden, der hämolytischen Komplementaktivität, Enzyme-Linked Immunosorbent Assays (ELISA) mit kommerziellen und intern entwickelten Ansätzen durchgeführt. Die Informationen aus den phänotypischen Erhebungen des ersten Ansatzes wurden darüber hinaus für die Quantitative trait loci (QTL) Kopplungskartierung verwendet. Parallel wurde ein Kandidatengenansatz zur Untersuchung der Assoziation zwischen interessanten funktionellen Genen und den Merkmalen der Immunkompetenz in der Versuchspopulation durchgeführt.

Die hämolytische Komplementaktivität des klassischen und des alternativen Signalwegs wurde durch jede Immunisierung erhöht. Diese Ergebnisse zeigen, dass die hämolytische Komplementaktivität beider Signalwege über die Zeitpunkte der Messungen erhöht wurde (28,44 bis 35,27 U/ml und 27,95 bis 56,09 U/ml für jeweils den klassischen und alternativen Signalweg). Es gab erhebliche Unterschiede in der Entwicklung und im Level der Komplementaktivität innerhalb der Population. Ähnlich der Komplementaktivität wurde auch die Antikörperreaktion auf verschiedene Impfstoffe einschließlich Mycoplasma, Tetanus (TT) und PRRS Impfung beobachtet, welche eine Erhöhung der Antikörper in den Versuchstieren über die Zeitpunkte der

Messungen erkennen ließ. Die Antikörperantwort auf die Mykoplasmen Impfung betrug jeweils vor, 10 und 20 Tage nach der Impfung 37,40, 19,86 und 14,59%. Dabei wurden Proben mit einem Wert von weniger als 50% als positiv angesehen, daher ist ein Anstieg der Antikörperlevel als ein Sinken der Testergebnisse zu interpretieren. Die Antikörperantwort auf TT wurde mit einem selbst entwickelten ELISA untersucht und der Tetanusimpfstoff in diesem Ansatz als Antigen verwendet. Die Ergebnisse zeigten eine Erhöhung der Antikörper über drei Zeitpunkte der Messung, welche als S/P Verhältnis angegeben wurde. Diese waren zu den Messungen bei der Impfung, 10 und 20 Tage nach der Impfung jeweils 0,09, 0,65 und 0,92. Die Antikörperantwort auf die PRRS Impfung wurde nur zu einem Zeitpunkt 10 Tage nach der Impfung erfasst. Das Ergebnis ergab einen positiven Status der Antikörper, welches als 0,84 des S/P Verhältnisses entsprechend der Anweisung des Herdchek$^®$ ELISA Kit, angegeben wurde. Die Komplementkomponente C3c wurde zu allen 6 Zeitpunkten der Messungen erfasst und als S/P Verhältnis ausgedrückt. Die Ergebnisse zeigten, dass die Komplement C3c Konzentration nach jeder Periode der Impfung erhöht war (0,84 bis 0,87, 0,78 bis 0,82 und 0,79 bis 0,82 jeweils nach Mycoplasma, TT und PRRS Impfung). Die Hp Konzentrationen wurden anhand der von Hiss et al. (2003) beschriebenen Methode, unter Verwendung eines vergleichenden ELISA gemessen und in mg/ml Hp im Blutserum ausgedrückt. Die in dieser Untersuchung gefundenen Hp Konzentrationen waren während den ersten drei Zeitpunkten der Messung verringert (1,76 bis 1,23 mg/ml, jeweils bei T1 bis T3). Die Erhöhung der Hp Konzentration wurde über den letzten drei Zeitpunkten der Messung beobachtet, sie erreichte der höchsten Punkt bei den Messungen zu den Zeitpunkten T6 oder 10 Tage nach der PRRS Impfung (1,68 bis 2, 18, jeweils bei T4 bis T6).

Die Merkmale der Immunkompetenz einschließlich Komplementaktivität, Antikörperantwort auf Impfungen, Komplementkomponente C3c und Haptoglobin wurden als phänotypische Merkmale für eine QTL-Kartierung verwendet. Die Genotypisierung wurde mit Mikrosatellitenmarkern durchgeführt. Es wurden 74 Marker (im Durchschnitt 4,11 Marker pro Chromosom) typisiert. Die Loci hatten zwischen 2 und 7 Allele mit einem Durchschnitt von 4,19 Allelen je Marker. Insgesamt wurden 220 Rückkreuzungstiere für die QTL Analyse genotypisiert. Die durchschnittliche Heterozygotie und der durchschnittliche Informationsgehalt der Marker betrugen 0,57 und 0,52. Der niedrigste Informationsgehalt war 0,16 am Genort des Markers *S0155* auf

SSC1. Die Positionen der Marker wurden mit der CRIMAP 2.4 Software mit Hilfe der beiden Optionen *twopoint* und *multipoint* ihren relativen Positionen auf den Chromsomen zugeordnet. Die in dieser Untersuchung verwendete Genkarte hatte eine guter Übereinstimmung zu der Genkarte der F2 Population aus einer früheren Untersuchung in der F2 DUMI Population (Wimmers 2002). Es wurden darüber hinaus auch Übereinstimmungen der QTL Regionen in den Arbeiten zu der F2 Population und der vorliegenden Arbeit gefunden. Ähnliche Regionen wurden auf SSC3, SSC4, SSC5, SSC8 und SSC10 in beiden, der F2 und der Rückkreuzung, Populationen gefunden. Es wurde in beiden Populationen kein QTL auf SSC13 gefunden. In der Rückkreuzungs- und der F2 Population wurden QTL für Merkmale, welche im Verhältnis zu den Messungen der C3c Level stehen, gefunden. In der Rückkreuzungspopulation konnten 42 signifikante und 24 hoch signifikante QTL für alle Immunmerkmale gefunden werden. Die meisten QTL wurden auf SSC3, SSC16 und SSC18 (neun signifikante F-Werte auf jedem Chromosom) detektiert. Es wurden keine signifikanten F-Werte auf SSC12 und SSC13 gefunden. Die am höchsten signifikanten QTL wurden für die Antikörperantwort auf Mykoplasmen, TT und PRRS Impfung, C3c und Hp Konzentration gefunden. Für die Merkmale AH_{50} und CH_{50} wurden 22 signifikante und 9 hoch signifikante QTL detektiert.

In dieser Untersuchung wurden die beiden porcinen mannose-binding protein (MBL) Gene *MBL1* und *MBL2* ausgewählt und mit dem Kandidatengenansatz untersucht. Im Hinblick auf die phylogenetische Analyse konnte festgestellt werden, dass die porcinen *MBL* Gene eine höhere Identität mit den Sequenzen vom Rind im Vergleich zu denen von Primaten und Nagern haben. Die Kartierung mit einem Radiation Hybrid Panel zeigte, dass beide porcinen *MBL* Gene mit Retentionsfrequenzen von jeweils 16% dem Chromosom 14 (SSC14) zugeordnet werden können. Die am signifikantesten gekoppelten Marker (mit einer Zweipunkt- Analyse) für die porcinen *MBL1* und *MBL2* waren SW210 (89 cR; LOD = 3.32) und SW1552 (35 cR; LOD = 10.66). Eine genetische Kartierung mit einer Zweipunkt Kopplungsanalyse mit CRIMAP 2.4 wurde ebenfalls durchgeführt und ergab, dass S0007 der am höchsten gekoppelte Marker war, die Rekombinationsfrequenzen und LOD Scores für die porcinen *MBL1* und *MBL2* Gene betrugen jeweils 0,32 und 3,34 sowie 0,23 und 8,26.

Eine Expressionsstudie ergab, dass beide *MBL* Gene in der Leber hoch exprimiert waren. Eine niedrige Expression von *MBL1* wurde in Lunge, Hoden und Gehirn

gefunden, während sich *MBL2* in Hoden und Niere als niedriger exprimiert zeigte. Single nucleotide polymorphisms (SNP) des porcinen *MBL2* Gens wurden gefunden und in einer porcinen F2 Versuchspopulation parallel mit einem vorher beschriebenen SNP in *MBL1* genotypisiert. Ein im *MBL2* Gen gefundener SNP veränderte die Restriktionsstelle des *AdeI* Enzyms, ein im *MBL1* Gen gefundener SNP die des Enzyms *HinfI*. Diese SNP segregierten in der F2 DUMI Versuchspopulation. Die Ergebnisse von 347 untersuchten F2 DUMI Schweinen in einer PCR-RFLP mit einem *HinfI* Enzym Verdau ergab Frequenzen der Allele C und T von 0,67 und 0,33. Die Genotypenfrequenzen für C/C, C/T und T/T waren jeweils 0,48, 0,38 und 0,14. Für die Untersuchung des porcinen *MBL2* in 284 F2 DUMI Schweinen in einer PCR-RFLP mit dem *AdeI* Enzym Verdau ergaben sich Frequenzen für die Allele G und A von jeweils 0,41 und 0,59. Die Genotypenfrequenzen waren für G/G, G/A und A/A jeweils 0,21, 0,40 und 0,39.

Die Ergebnisse der Assoziationsstudien zeigten, dass sich nur die *MBL1* Genotypen in der C3c Serumkonzentration unterschieden, d.h. die *In vivo* Komplementaktivität (p<0,1). Entsprechend ergab die Kopplungsanalyse einen QTL für das C3c Serumlevel nahe der Position der *MBL* Gene. Diese Untersuchung bestätigt somit, dass die porcinen *MBL* Gene funktionelle und positionelle Kandidatengene für die *In vivo* durch den Lectin Signalweg vermittelte Komplementaktivität sind.

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Publications

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