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Mapping of quantitative trait loci for immune response traits and expression patterns of Toll-like receptors in lymphoid tissues in pigs

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Dedicated to my parents and my family

Mapping of quantitative trait loci for immune response traits and expression patterns of Toll-like receptors in lymphoid tissues in pigs

The aim of this research was to identify the quantitative trait loci (QTL) affecting antibody and innate immune response traits. For this purpose, Duroc-Pietrain (DUPI) pigs (n = 319) were genotyped with 122 genetic markers and phenotypes of serum antibodies for *Mycoplasma hyopneumoniae* (Mh) and tetanus toxoid (TT), and interferon-gamma (IFNg) levels were measured following vaccinations (Mh, TT, Porcine Reproductive and Respiratory Syndrome Virus [PRRSV]). Line-cross and imprinting QTL analysis were performed using QTL Express. A total of 30 QTL (12, 6, and 12 QTL for Mh, TT antibody, and IFNg, respectively) were identified, of which 28 QTL were detected by line-cross and 2 QTL by imprinting model. The serum concentration of interleukin 2 (IL2), IL10, IFNg, Toll-like receptor (TLR2) and TLR9 were measured in another group of DUPI population (n = 334) following vaccinations that were genotyped with 82 genetic markers. A total of 33 single QTL were detected, of which eight, twelve and thirteen QTL were identified for immune traits in response to Mh, TT and PRRSV vaccine, respectively. All immune traits are influenced by multiple chromosomal regions implying multiple gene action. Furthermore, expression stability of nine commonly used housekeeping genes (HKG) was studied using qRT-PCR in most lymphoid tissues at different ages (newborn, young and adult) of pigs. This study found that HKG becomes heterogeneous with age and the geometric mean of the *RPL4*, *PPIA* and *YWHAZ* seem to be the most appropriate combination of HKG for accurate normalization of gene expression data in pigs. Moreover, the expression patterns of ten TLRs (1-10) were studied in the same tissues used for HKG study. This study revealed that TLRs mRNA expressions were affected by age and organs. Most of the TLRs expression was higher at young pigs compared to adult and newborn pigs. *TLR3* gene was the highest abundant among all TLRs in most tissues. The western blot results of TLR2, 3 and 9 in selected tissues appeared to be consistent with the mRNA expression. The protein localization showed that TLRs expressing cells were abundant in lamina propria, Peyer's patches in intestine, around and within the lymphoid follicles in the mesenteric and cervical lymph node, within the white pulp in spleen and on the lining cells in bronchioles in lungs. This expressions study first shed light on the expression patterns of all TLR genes in important lymphoid tissues including gut-associated lymphoid tissues in different ages of pigs.

QTL-Kartierung von Immunreaktionsmerkmalen und Expressionsmuster des Toll-like Rezeptors in lymphatischen Gewebe beim Schwein

Das Ziel dieser Studie war Quantitative Trait Loci (QTL), die Einfluss auf Antikörper und Merkmale der angeborenen Immunreaktion haben, zu identifizieren. Zu diesem Zweck wurden Duroc×Pietrain Schweine (DUPI) (n = 319) mittels 122 genetischen Markern genotypisiert. Die Phänotypen der Antikörperspiegel von *Mycoplasma hyopneumoniae* (Mh), Tetanus Toxoid (TT) sowie von Interferon-gamma (IFNg) wurden nach der Impfung (Mh, TT, Porcine Reproductive and Respiratory Syndrome Virus [PRRSV]) im Serum gemessen. Die QTL-Analysen wurden mit Hilfe der Software QTL-Express ausgeführt. Insgesamt wurden 30 QTL (jeweils 12, 6 und 12 QTL für Mh, TT-Antikörper und IFNg) identifiziert, wobei bei 2 QTL der Einfluss von Imprintingeffekten nachgewiesen wurde. In einer weiteren Gruppe der DUPI-Population (n = 334), welche mittels 82 Markern genetisch erfasst wurden, wurden die Serumkonzentrationen des Interleukins 2 (IL2), IL10, IFNg, Toll-like Rezeptor 2 (TLR2) und TLR9 nach der Impfung gemessen. Dabei wurden insgesamt 33 QTL detektiert, von denen jeweils 8, 12 und 13 QTL mit der Immunreaktion auf Mh, TT und PRRSV Impfungen assoziierten. Alle Immunmerkmale wurden durch mehrere chromosomale Regionen beeinflusst, was multiple Genaktionen impliziert. Darüber hinaus wurde die Expressionsstabilität von 9 häufig verwendeten ‚house keeping' Genes (HKG) mit Hilfe der qRT-PCR inngerhalb von lymphatischen Geweben von Tieren unterschiedlichen Alters (Neugeborene, Jungtiere, Adulte) untersucht. Die Ergebnisse dieser Studie zeigten, dass die Expression der HKG mit dem Alter heterogen werden. Somit scheint das geometrische Mittel von *RPL4*, *PPIA* und *YWHAZ* am geeignetsten für die Normalisierung von Genexpressionsdaten beim Schwein zu sein. Dasselbe Gewebe der Referenzgenanalyse wurde für die Expressionsanalyse von zehn TLRs (1-10) verwendet. Diese Analyse zeigte, dass die TLRs mRNA-Expression vom Alter und Organen abhängig war. Dabei konnte eine höhere TLRs Expression bei Jungtieren und eine geringere bei Adulten und Neugeborenen detektiert werden. Das Gen TLR3 hatte das höchste Expressionsniveau in der Mehrzahl an Geweben von allen TRL Genen. Die Ergebnisse des Western Blot von TLR2, 3 und 9 in ausgewählten Geweben stimmten mit den Genexpressions-Analysen überein. Die Protein-Lokalisierung zeigte, dass TLRs in Zellen von *lamina propria*, Peyer's Drüsen im Darm, in und um die lymphoiden Folikel des mesenterial und zervikalen Lymphknotens, im weißen Zellengwebe der Milz und in den Bronchialepithelien der Lunge exprimiert werden. Diese Expressionsstudie lieferte die ersten Erkenntnisse über die Expressionsmuster aller TLR in Lymphgeweben einschließlich der Lymphgewebe des Darms bei verschiedenen Alterstufen beim Schwein.

Contents

List of abbreviations

$A_{260/280}$	:	Absorbance at 260/280 mm wavelength ratios
ACTB	:	Actin, beta
APC	:	Antigen presentic cells
AUC	:	Area-Under-Curve
B2M	:	Beta-2-microglobulin
BI	:	BestKeeper Index
BKS	:	Bovine calf serum
BLM	:	Bloom syndrome RecQ helicase-like
Bp	:	Base pairs
BSA	:	Bovine serum albumin
cDNA	:	Complementary DNA
CFA	:	Complete Freund's adjuvant
CLN	:	Cervical lymph node
cM	:	Centimorgan
CpG	:	Cytidine-phosphate guanosine
Ct	:	Cycle threshold
CW	:	Chromosome-wide
DAPI	:	4',6-diamidino-2-phenylindole
DNA	:	Deoxynucleic acid
dNTP	:	Deoxyribonucleoside triphosphate (usually one of dATP, dTTP, dCTP and dGTP)
DUPI	:	Duroc × Pietrain cross
E. coli	:	*Escherichia coli*
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-linked immunosorbent assay
EP	:	Enzootic pneumonia
Fig	:	Figure
FITC	:	Fluorescein isothiocyanate
GALT	:	Gut-associate lymphoid tissues
GAPDH	:	Glyceraldehyde 3- phosphate dehydrogenase
GeXP	:	GenomeLab expression analysis

GIT	:	Gastrointestinal tract
GW	:	Genome-wide
H_2O	:	Water
HKGs	:	Housekeeping genes
HPRT1	:	Hypoxanthine phosphoribosyltransferase 1
Hsp	:	Heat shock protein
IEC	:	Intestinal epithelial cells
IFNg	:	Interferon gamma
LOD	:	Logarithm of odds
MALP-2	:	Macrophage-activating lipopeptide-2
MBL	:	Mannan binding lectin
MDF2β	:	Murine β-defensin 2
Mg	:	Milligrams
MgCl2	:	Magnesium chloride
Mh	:	*Mycoplasma hypopneumoniae*
min	:	Minute
ml	:	Milliliters
MLN	:	Mesenteric lymph node
mM	:	Mili mole
mRNA	:	Messenger RNA
NaCl	:	Sodium chloride
ng	:	Nanograms
NTC	:	No template control
^oC	:	Degree celsius
Pam2Cys	:	S-[2,3-bis(palmitoyloxy)propyl]-cysteine
Pam3Cys	:	N-palmitoyl-(s)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-cysteine
PAMP	:	Pathogen associated molecular patterns
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffer saline
PBST	:	Permiabilisation solution-Tritonx-100
PGN	:	Peptidoglycan
pH	:	pH value
poly I:C	:	Polyriboinosinic-polyribocytidilic acid

PPIA	:	Peptidylprolyl isomerase A (cyclophilin A)
Pps	:	Peyer's patches
PRR	:	Pathogen recognition receptors
PRRS	:	Porcine resproductive and respiratory syndrome
PRRSV	:	Porcine resproductive and respiratory syndrome virus
qRT-PCR	:	Quantitative real-time reverse transcriptase polymerase chain reaction
QTL	:	Quantitative trait loci
RPL4	:	Ribosomal protein L4
S.D.	:	Standard deviation
SDHA	:	Succinate dehydrogenase complex subunit A flavoprotein
TBP	:	TATA-box binding protein
TLR	:	Toll-like receptors
TNFa	:	Tumor necrosis factor
TRITC	:	Tetramethyl rhodamine isothiocyanate
TT	:	Teteanus toxoid
U	:	Units
UV	:	Ultra-violet light
v/v	:	Volume per volume
vs.	:	Versus
YWHAZ	:	Tyrosine 3/tryptophan 5-monooxygenase activation protein zeta polypeptide

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1.1 Introduction

Quantitative trait loci mapping method is a statistical method for identifying loci associated with a quantitative phenotype. The current release of the Pig Quantitative Trait Locus (QTL) database (Pig QTLdb) (July 8, 2011) contains 6,344 QTLs representing 593 different traits from 281 publications (http://www.animalgenome.org/cgi-bin/QTLdb/SS/index). These QTL are mostly for economically important traits like growth, carcass and meat quality, and reproduction (Hu et al. 2010). 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 and the antibody response was one of the first immune competence traits to be examined by QTL analysis (Cho et al. 2011, Edfors-Lilja et al. 2000, Edfors-Lilja et al. 1998, Lu et al. 2010b, Reiner et al. 2002, Wattrang et al. 2005, Wimmers et al. 2008). Immune competence comprises the ability of an individual to protect itself against any pathogen by using innate and humoral immunity. It also means the immune responsiveness of an animal in response to vaccines or antigens or pathogens. QTL underlying immune response variations have been detected in mouse, chicken, and humans (Almasy and Blangero 2009, Biscarini et al. 2010, Hall et al. 2002, Siwek et al. 2003). Only few studies were devoted to detect the QTL regions for immune response traits in pigs. The QTL detection is performed in underlying experimental crosses between lines that differ in their innate and specific immune responses. Duroc and Pietrain are reported to be divergent regarding bacterial disease resistance trait such as postweaning diarrhea due to *E. coli* F18 infections (FUT1 gene responsible for the resistance to postweaning diarrhea is differentially expressed in Duroc and Pietrain intestine) (Vrtková et al. 2007). With regards to general immune responses, Duroc and Pietrain are differentially responding to sheep erythrocytes (Buschmann et al. 1974). Differences between Duroc and Pietrain in response to stress are also reported previously by Rosochacki et al. (2000) and stress is an important predisposing factor for animal to increase susceptibility to different infectious diseases (Hicks et al. 1998, Morrow-Tesch et al. 1994, Rosochacki et al. 2000). Moreover, immunological traits are reported to have the potential to improve selection of pigs for resistance to clinical and subclinical disease (Henryon et al. 2006). Therefore, QTL for immune response traits were detected in two different group of Duroc-Pietrain (DUPI) population. A DUPI

population was genotyped with 122 genetic markers and the serum titer of antibody response to *Mycoplasma hyopneumoniae* (Mh) and tetanus toxoid (TT), and interferon gamma (IFNg) in response to Mh, TT and porcine reproductive and respiratory syndrome virus (PRRSV) vaccine were measured as phenotypes (Chapter 1). The other DUPI population was genotyped with 82 genetic markers and the innate immune traits i.e. the serum titer of interleukin 2 (IL2), IL4, IL10, IFNg, toll-like receptor 2 (TLR2) and TLR9 in response to Mh, TT and PRRSV vaccines were measured as phenotypes (Chapter 2).

The immune system is highly organized and is a very complex system working synergistically to protect the host from any infections or insults and to maintain homeostasis (Cooper and Herrin, 2010). It comprises two functional types of responses, innate or cellular or non-specific and adaptive or humoral or acquired responses. The innate immune system comprises the cells and mechanisms that protect the host from any pathogenic infectious agents in a non-specific manner. Its does not confirm long-lasting or life-long protection to the host. This non-specific immune system provides immediate defense against infection, and can be found in all classes of plant, living organisms, animals and humans (Beutler 2004). The cells of the immune system utilize germ-line encoded receptors termed as pattern recognition receptors (PRRs) to recognize pathogen specific patterns termed as pathogen associated molecular patterns (PAMPs) (Medzhitov and Janeway 1997). After recognizing the PAMPs by PRRs, there is induction of opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways and cytokines production and induction of apoptosis (Akira and Takeda 2004, Medzhitov and Janeway 1997). The innate immune system is highly developed in its ability to discriminate between self and foreign pathogens. This discrimination relies, to a great extent, on a family of evolutionarily conserved receptors, known as the Toll-like receptors (TLRs), which have crucial roles in early host defense against invading pathogens (Akira and Takeda, 2004). Toll-like receptors recognize conserved molecular patterns (PAMPs), which are shared by large groups of microorganisms (Akira and Takeda 2004). 11 TLRs have been identified in humans and 13 in mice, whereas in other mammals including pigs, there are 10 members of TLRs are recognized. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface, while TLR3, 7-9 are located in the endosomes/lysosome (Akira and Takeda, 2004). Notably, soluble forms of TLRs are reported to present in

human plasma, breast milk and saliva (Buduneli et al. 2011, LeBouder et al. 2003). Activation of the TLR leads not only to the induction of inflammatory responses but also to the development of antigen-specific adaptive immunity (Akira and Takeda 2004). So TLRs are considered as critical proteins linking innate and adaptive immunity. It is important to note that each TLR has its specific ligands. TLR2 is essential for the recognition of microbial lipopeptides; TLR4 recognizes lipopolysaccharide (LPS) of gram negative bacteria. TLR9 is the CpG DNA receptor, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 is a receptor for flagellin, the main protein of flagellar bacteria. Thus, the TLR family discriminates between specific patterns of microbial components (Takeda and Akira 2004). The TLRs play important roles in B-cell activation and antibody production *in vivo* and generation of T-dependent antigen-specific antibody responses requires activation of TLRs in B cells (Pasare and Medzhitov 2005). Expression of TLRs in response to *Mycoplasma hyopneumoniae* vaccine (Muneta et al. 2003, Regia-Silva et al. 2011) and porcine reproductive and respiratory syndrome virus (PRRSV) (Liu et al. 2009, Miller et al. 2009) are reported in pigs. TLR ligands depended adjuvants in vaccines against some infectious diseases are proven (such as in BCG [Bacillus Calmette-Guérin]) and are reported to be possible in case of several diseases (such as in DTP [diphtheria toxoid, tetanus toxoid, pertussis], rabies etc) (van Duin et al. 2006). Therefore, this study was devoted to identify the chromosomal regions influencing the TLRs in response to Mh, TT and PRRSV vaccine (Chapter 2).

Cytokines are the cell-signaling protein molecules secreted by the cells of the immune system for intercellular communications in response to any pathogens or inflammations. Cytokines as intercellular signaling molecules form complex networks to orchestrate and coordinate immune responses. Cytokines include interleukins (IL), chemokines, interferons (IFN), colony stimulating factors, tumor necrosis factors (TNF), and other proteins (Scheerlinck and Yen 2005). Now over 100 cytokines have been identified in mammals, including 49 interleukins and 50 cytokines are reviewed by Scheerlinck and Yen (2005). The Th1 cell secretes mainly IL2 and interferon gamma (INFg) and the Th2 cell secretes mainly IL4, IL10, and IL13. It is important to keep balance between Th1 (pro-inflammatory) cytokines (such as IFNg) and Th2 (anti-inflammatory) cytokines (such as IL4 and IL10). Each cytokine has its specific function as well as they work synergistically to perform a particular task. Cytokines work through complex

networks because all cytokines, to a greater or lesser extent, exhibit pleiotropy (multiple biological actions) and redundancy (shared biological actions) (Nicola 1994). As a part of the innate immune system, cytokines are produced by lymphocytes in response to antigens including vaccine antigens immediately after recognizing by the TLRs. The cytokines then lead to the inflammatory response and subsequently B cell activation and antibody production. Finally, B cells are divided and form memory cells that can recognize the antigen or vaccine antigen in later invasion and produce antibodies. Interferon gamma (IFNg) produced from Th1 cells and NK cells, is responsible for the activation of macrophages and NK cells, induction of MHC-I & -II expression, and inhibits Th2 activity. IFNg has immunomodulatory functions, possesses antiviral activity and protects swine from diseases (Danilowicz et al. 2008, Scheerlinck and Yen 2005, Yao et al. 2008). Interleukins are mainly produced by leukocytes and are involved in mounting the immune response. IL1 and IL2 are responsible for the activation of T and B lymphocytes by stimulating their growth and maturation. IL4 plays a role in increasing antibody secretion by B lymphocytes. IL12 is responsible to increase the number of cytotoxic T cells and natural killer cells to kill the invading pathogens. Importantly, IL10 is an important anti-inflammatory cytokine that not only suppresses inflammation but also modulates the survival time of infected animals (Scheerlinck and Yen 2005). The use of recombinant cytokines as adjuvants in vaccines is attracting considerable attention (Asif et al. 2004) and pig IL2 is reported to enhance immunity when used as vaccine adjuvant in mice (Xie et al. 2007). IL2, IL12, IL4, IL6, IL8 and IL10 as inflammatory cytokines play important roles in porcine enzootic pneumonia caused by *M. hyopneumoniae* in pigs (Lorenzo et al. 2006, Rodriguez et al. 2007, Rodriguez et al. 2004). Tetanus toxin (TT) selectively inhibited IFNg production (Blasi et al. 1990) and the IFNg level is considered as the indicator for immune responsiveness of cells in response to TT (Tassignon et al. 2005). It has been reported that recombinant IL2 treatment is able to potentiate the antibody response to tetanus toxoid in humans (Fagiolo et al. 1997). Recently, it has been documented that the protection by PRRS vaccines depends on the ability of the vaccine to induce an IFNg response (LeRoith et al. 2011). Interleukins including IFNg are reported to be linked to PRRS virus clearance in pigs (Lunney et al. 2010). Due to the important roles of cytokines, one of the aim of this study was to indentify the chromosomal regions affecting cytpokines (IL2, IL10 and IFNg) in pigs.

The acquired immune response is a specific immune 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 to the agent and for the recall memory (Bishop et al. 2010). 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 or killed whole organisms (Bahr 2001). Additionally, the modern vaccine technology is very close to develop vaccines using TLR ligands as vaccine adjuvant which will lead to produce more safe and effective vaccine (Duthie et al. 2011, van Duin et al. 2006). 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 2007). *Mycoplasma hyopneumoniae* (Mh) is the principal aetiological agent of enzootic pneumonia (EP), a chronic respiratory disease that affects pigs (Sibila et al. 2009). It is an important bacterial disease in pig industry because of its high prevalence of up to 80 % in pigs worldwide. It is characterized as a chronic disease with high morbidity and low mortality rates (Fano et al. 2005). This disease causes high economic loss due the retardation of growth and production loss (DeBey et al. 1992, Thacker et al. 1999). Active immunization using inactivated *M. hyopneumoniae* bacteria, has been recommended as vaccine to protect animals from mycoplasmal pneumonia (Okada et al. 1999). The presence of infection as well as the antibody titer of *M. hyopneumoniae* could be monitored using specific ELISA (Sibila et al. 2009). The ELISA tests have a higher sensitivity at the individual level compared to other methods (e.g. indirect hemagglutination) (Armstrong et al. 1983, Sheldrake et al. 1990, Sorensen et al. 1992). Tetanus is caused by the bacterium *Clostridium tetani*. It produces toxins (tetanospasmin, a neurotoxin) that affect the central nervous system. In the suckling pig, the most common route of infection is the wound in case of unhygienic castration. In the favourable condition, these bacteria appear in vegetative form and produce tetanospasmin. The common clinical symptoms are hypersensitivity, pig shows stiffness of legs and muscles, an erected tail, muscular spasms of the ears and face and coincide with high mortality. The ELISA test is commonly used to determine TT antibody titers (Aybay et al. 2003, Gupta

and Siber 1994). One of the aims of this research was to detect the chromosomal regions influencing the antibody production for Mh and TT in response to their vaccine. Although the PRRSV specific antibody induced by PRRSV vaccine was not measured in this study, the PRRSV vaccine induced innate immune responses traits (production of IFNg, IL2, TLR2 and TLR9) were considered for quantitative trait loci (QTL) analyses. PRRS is one of the most important and prevalent viral diseases in pig industry (Dee et al. 1997). It is distributed world wide and has a high economic impact. Importantly, PRRSV can persist in the host's body for long time and continues to shed virus (Wills et al. 1997). It has been reported that PRRSV persist in the host by suppressing TLR3 (Sang et al. 2008).

Beside the QTL study for immune response traits, the organs related to immune functions were considered for gene expression study. The expression of commonly used housekeeping genes (HKGs) (Chapter 3) and TLR family genes (TLR1-10) (Chapter 4 and 5) were investigated in different immune organs of pigs. The mammals' body possesses highly developed and sophisticated immune organs that protect the organism from any infectious pathogens and that maintain homeostasis. Gut-associated lymphoid tissues (GALT) are highly organized immune compartments, are intimately associated with the gut epithelium, lymphoid cells in lamina propria, Peyer's patches and mesenteric lymph node (MLN). GALT constitute the largest mass of immune cells in the body. The gut immune system protects swine against infectious and non-infectious environmental insults and discriminates ingested nutrients, food, and commensal microflora from pathogenic agents (reviewed by Artis 2008, Burkey et al. 2009, Dvorak et al. 2006, Neutra et al. 2001). The gut epithelium provides the physical barrier as well as the mucosal immune system protects the organism and mediates subsequent innate and adaptive immune responses. Mesenteric lymph nodes (MLN) synergistically with GALT fight against the pathogens entering through the oral route (Burkey et al. 2009). The GALT are armed with TLRs and are reported to recognize the pathogens as well as to discriminate between pathogens and probiotics or beneficial microflora via a cross talk through TLRs (Kitazawa et al. 2006, Tohno et al. 2006, Uenishi and Shinkai 2009). Cervical lymph nodes (CLN), thymus, liver, spleen, lung, heart, skin and peripheral blood mononuclear cells (PBMC) are vital lymphoid organs in animals and humans that protect the host from pathogens. CLN play vital roles in defence against respiratory virus in pigs (Bailey et al. 2000). Thymus is an important immune organ where T-

lymphocytes development occurs and thymic B cells produce immunoglobulin (Cukrowska et al. 1996). Spleen is the largest secondary immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens. The unique function of spleen is filtering the blood for foreign material and removing old or damaged red blood cells. It aids in the development of white blood cells are reviewed by Cesta (2006). Although the liver is mainly focused on detoxification, it has also important immune functions. Liver is the resident for macrophages (Kupffer cells), dendritic cells, liver natural killer (NK) cells and responses to different pathogens in pigs (Skovgaard et al. 2009). Liver produces acute phage proteins (haptoglobin, serum amyloids) as part of innate immune response. Alveolar epithelial cells in lung provide a barrier between circulation and external air. Lung is an important immune organ harbouring huge numbers of lymphocytes, macrophages (alveolar macrophages) fighting against most respiratory pathogens in pigs (reviewed by Pabst and Binns 1994). The surfactant protein also functions in pulmonary host defense (Crouch et al. 2000, Wright et al. 2001). Skin is the largest organ of the body and is exposed to the highest number of pathogens, allergens, mechanical and physical insults and it is involved in the regulation of body temperature. Skin is the interface between the internal milieu and the external environment and acts as a mechanical, physical and biological protective organ are reviewed by Schmitt (1995). Pig's skin is exposed to numerous bacteria, virus and fungus. Peripheral blood mononuclear cells (PBMC) include different cells (such as lymphocytes, monocytes and macrophages) playing important immune functions in mammals. PBMC are essential for subsequent analyses in immune monitoring and are used as a cell line to study the effect of different antigens, mutagens or vaccines (Hornung et al. 2002, Siednienko and Miggin 2009, Yancy et al. 2001). Therefore, all these organs were considered for the gene expression studies (Chapter 4 and 5).

Today's, quantitative real-time PCR (qRT-PCR) is the most frequently used method for gene quantification. qRT-PCR is an efficient method for quantification of mRNA transcript expressions due to its high sensitivity, reproducibility and large dynamic range. It is fast, easy to use and provides simultaneous measurement of gene expression in many different samples for a limited number of genes (Arya et al. 2005, Nolan et al. 2006, Nygard et al. 2007). When analyzing data for relative quantification in qRT-PCR, results are normalized to a reference. The most accepted approach to quantification is normalisation of the expression level of a gene of interest (target gene) to the expression

level of an internal stably expressed gene (control or reference gene) (Huggett et al. 2005, Radonic et al. 2004, Vandesompele et al. 2002). The reference gene is a stably expressed gene that is experimentally verified in given species and tissues under given experimental conditions (Erkens et al. 2006, Lovdal and Lillo 2009, Maroufi et al. 2010, Nygard et al. 2007). Since the reference gene is exposed to the same preparation steps as the gene of interest, the normalisation adjusts for differences in the quality or quantity of template RNA or starting material and differences in RNA preparation and cDNA synthesis. A variability or alteration in the chosen reference gene by the experiment, however, may change the obtained results entirely and could be incorrect. It is therefore necessary to validate the expression stability of reference genes prior to their use in an experimental protocol. An ideal reference gene should be stably expressed and unaffected by experimental protocol or status (Schmittgen and Zakrajsek 2000). However, recent studies showed that the expression of housekeeping genes (HKGs) differs between tissues (Maroufi et al. 2010, Nygard et al. 2007, Pierzchala et al. 2011), breeds (Pierzchala et al. 2011), experimental condition (such as treatment or disease) (Beekman et al. 2011, De Boever et al. 2008, Maccoux et al. 2007, Penning et al. 2007) and age (Al-Bader and Al-Sarraf 2005, Pierzchala Mariusz et al. 2011, Touchberry et al. 2006). Set of reference genes are suggested on the basis of their stability over tissues in pigs (Erkens et al. 2006, Nygard et al. 2007, Pierzchala et al. 2011, Piorkowska et al. 2010), but studies with regards to the expression stability of commonly used house keeping in different porcine tissues collected from different ages of pigs are scare. Therefore, the expression stability of the HKGs was investigated in different organs of pigs with different ages (Chapter 3).

It is necessary to know which TLRs are expressed in tissues and by specific cell types in order to understand the TLRs functions. The immune responsiveness of individuals is reported to depend on the variation of TLR expression (Jaekal et al. 2007). The tissue, cellular, and sub cellular localization and distribution of TLRs influence the type of immune response elicited. Thus, the first step in understanding the role of TLRs is to determine which TLRs are expressed by specific tissues, organs and cells of interest. The immune responsiveness of different lymphoid organs is not the same. An organ could be more responsive to a pathogen while an other organ could be immunologically more reactive to another pathogen. In order to gain an understanding of how responsive tissues and cells are likely to be involved at detecting pathogens, TLR mRNA

expression patterns have been determined in different species. Expression studies of the complete TLR family (1-10) have been reported in humans (Garrafa et al. 2010, Hornung et al. 2002, Siednienko and Miggin 2009), cattle (Menzies and Ingham 2006), sheep (Chang et al. 2009, Nalubamba et al. 2007, Taylor et al. 2008) and chicken (Iqbal et al. 2005). For pigs, there is no such complete study of TLRs1-10 expression reported. Notably, the immune responsiveness to antigens or vaccine varies according to the age of the individuals (Panda et al. 2010, van Duin and Shaw 2007) and is assumingly associated with TLRs expression (Dunston and Griffiths 2010, Renshaw et al. 2002, van Duin and Shaw 2007). Furthermore, the expression and function of TLRs are reported to vary with age (Renshaw et al. 2002, Tohno et al. 2006, van Duin and Shaw 2007). Age-associated changes of the adaptive immune system are documented in pigs (Dickie et al. 2009, Hoskinson et al. 1990); however, data on the impact of aging on the innate immune system especially on the TLR expression pattern is rare in pigs. Since TLRs are vital immune components, it is important to study their expression pattern in tissues or organs related to immune functions. Therefore, the aim of this research was to study the expression patterns of all porcine TLR (1-10) genes in selected immunologically important lymphoid organs or tissues collected from pigs of three different ages (Chapter 4 and 5).

With this background, several experiments were conducted in this thesis to achieve the following aims:

1. Evaluation of the porcine immune competence on the basis of antibody production, cytokines and Toll-like receptors expressions in response to vaccine antigens and identification of the quantitative trait loci affecting these immune response traits.
2. Identification of the expression stability of nine commonly used housekeeping genes in porcine organs to assemble an appropriate set of housekeeping genes for the normalization of gene expression in pigs.
3. Investigation of the expression patterns of all Toll-like receptors in lymphoid organs of pigs at different ages.

1.2 Materials and methods

To achieve the objectives of this research several materials and methods were used. The details materials and methods are described in details in the different chapters in this thesis. The importance of some methods and their descriptions are briefly summarized here.

1.2.1 Enzyme-linked immunosorbent assay (ELISA)

Immune competence of pigs can be monitored by measuring the immune response induced by infection or vaccine antigens. The most common diagnostic assays are based on detecting antibodies specific to the pathogen. Blood serum was used as a sample for ELISA. ELISA was used to quantify the antibody concentration of Mh and TT (Chapter 1) as well as to measure the cytokines and TLR proteins (Chapter 2) in response to vaccine antigens. ELISA is found to be potentially a very attractive and practical serodiagnostic test for mycoplasmal pneumonia in pigs. A study conducted by Armstrong et al. (1983) indicated an extremely high sensitivity of ELISA for detecting porcine antibodies to *M. hyopneumoniae*. In addition, the ELISA can be performed automatically and would thus be economical for testing when compared to indirect hemagglutination (IHA) and complement fixation (CF) methods. ELISA is reported to be an effective and sensitive method to detect the TT antibody when compared to ELISPOT, flow cytometry and real-time PCR (Tassignon et al. 2005). Besides measuring the antigen specific antibody, the immune responsiveness of animals can also be detected by measuring the serum concentration of immune response components like cytokines and TLRs (Edfors-Lilja et al. 1998, Lu et al. 2010b, Buduneli et al. 2011). For measuring the serum cytokines ELISA is the mostly practiced, easy and cost effective immunological assay for a large number of animals (Andreotti et al. 2003). The concentration of TLRs in body fluids is also possible to measure by ELISA (Buduneli et al. 2011). The uses of ELISA has increased dramatically in the immunological as well as in diagnostic research (reviewed by Lequin 2005).

1.2.2 QTL analysis

Quantitative trait locus (QTL) analysis is a statistical method that links two types of information-phenotypic data (trait measurements) and genotypic data (usually molecular markers)-in an attempt to explain the genetic basis of variation in complex traits (Kearsey, 1998). QTL analysis allows researchers in fields as diverse as agriculture, evolution, and medicine to link certain complex phenotypes to specific regions of chromosomes (reviewed by Miles and Wayne, 2008). QTL analysis is an important method applied to the genetic dissection of immune responses of population (de Koning et al. 2005). For this method, 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 studies (de Koning et al. 2005). For QTL analysis, the animals were genotyped with genetic markers as described earlier (Grosse-Brinkhaus et al. 2009, Liu 2005, Liu et al. 2007, Phatsara 2007, Wimmers et al. 2008). Allele and inheritance genotyping errors were checked using Pedcheck software (version 1.1) (O'Connell and Weeks 1998). The relative positions of the markers were assigned using the build, twopoint and fixed options of CRIMAP software (version 2.4) (Green et al., 1990). Recombination units were converted to map distances using the Kosambi mapping function (Kosambi 1944). Marker information content and segregation distortion were tested by following Knott et al. (1998). Using a regression approach, the QTL were calculated for immune traits. 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 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 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).

1.2.3 Quantitative real-time polymerase chain reaction

In recent years, real-time polymerase chain reaction (PCR) has emerged as a robust and widely used methodology for biological investigation because it can detect and quantify very small amounts of specific nucleic acid sequences. As a research tool, a major application of this technology is the rapid and accurate assessment of gene expression as a result of physiology, pathophysiology, or development (Valasek and Repa 2005). In this research the commonly used housekeeping genes were quantified in different porcine tissues using quantitative real-time polymerase chain reaction (qRT-PCR) (Chapter 3) following standard procedures. For this purpose, RNA was isolated from tissues using the phenol-chloroform method and was purified (Chapter 3). The RNA quality and quantity was measured using agarose electrophoresis and Nanodrop, respectively. cDNA was synthesized and purified (Chapter 3) for quantification in qRT-PCR. Nine-fold serial dilutions of plasmid DNA were prepared and used as template for the generation of the standard curve. In each run, beside each cDNA sample, plasmid standards for the standard curves and no-template control were used. A no-template control (NTC) was included in each run for each gene to check for contamination. For qRT-PCR, 1× Power SYBR Green I master mix with ROX as reference dye (Bio-Rad) was used in the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Melting curve analysis was constructed to verify the presence of gene-specific peak and the absence of primer dimer. Agarose gel electrophoresis was performed to test for the specificity of the amplicons. To ensure repeatability of the experiments, all reactions were executed in triplicate and the mean was used for further analysis (Chapter 3).

1.2.4 GenomeLab expression analysis

Although qRT-PCR is the most commonly practiced methods for gene quantification, it is not cost-effective for the quantification of a set of genes in a large numbers of tissues when compared to the GenomeLab Genetic Analysis System (GeXP). The qRT-PCR results are reported to vary according to the system (Lu et al. 2010a). The Beckman Coulter GeXP genetic analysis system allows for multiplexed detection and quantitation of up to 35 genes in 192 samples in a single analysis (Rai et al. 2009). The results of GeXP is comparable to quantitative real-time PCR (Raghunathan et al. 2009). Therefore, GeXP is a faster and cheaper method in this regard (Rai et al. 2009). The analytical procedure includes modified reverse transcription and PCR amplification,

followed by capillary electrophoretic separation (Rai et al. 2009). All the forward primers are mixed together (forward-plex) while 'reverse-plex' is prepared by mixing all the reverse primers (Chapter 4 and 5). Each of these primers is chimeric, having a 3′ gene-specific end and a 5′ end containing a quasi-T7 universal sequence, which serves as a template in subsequent amplification steps (Rai et al. 2009). The GeXP software matches each fragment peak with the appropriate gene, and reports peak height and area-under-the-curve (AUC) for all peaks in the electropherogram. Electrophoretic separation is needed to be done by GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Kanamycin RNA internal positive control is to be included and produces a peak at 326 bp when samples are separated via electrophoresis. All experiments usually included "no template" (i.e. without RNA) and "no enzyme" (i.e. no reverse transcriptase) as negative controls to confirm the absence of peaks at the expected target sizes. The "no template" sample produces a single peak at 326 bp, corresponding to the externally spiked-in kanamycin RNA. The data set is exported from the GeXP software after normalization to kanamycin, with area-under-the-curve (AUC) set to 1 which minimizes inter-capillary variation (Rai et al., 2009). This data were used for subsequent analyses after normalization against reference genes (Chapter 4 and 5).

1.2.5 Western blot

Western blotting (WB) is a powerful and important procedure for the immunodetection of proteins post-electrophoresis, particularly proteins that are of low abundance (reviewed by Kurien and Scofield 2006). WB allows the transfer of proteins from a sodium dodecyl sulfate (SDS) polyacrylamide gel to an adsorbent membrane. The blotted proteins form an exact replica of the gel and have proved to be the starting step for a variety of experiments. The subsequent employment of antibody probes directed against the nitrocellulose bound proteins has revolutionized the field of immunology. Transfer of proteins separated by SDS–polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) to an adsorbent membrane, is a powerful tool to detect and characterize a multitude of proteins, especially those proteins that are of low abundance. WB offers the following specific advantages: wet membranes are pliable and easy to handle, the proteins immobilized on the membrane are readily and equally accessible to different ligands, only a small amount of reagents is required for transfer analysis and multiple

replicas of a gel are possible. Prolonged storage of transferred patterns, prior to use, becomes possible and the same protein transfer can be used for multiple successive analyses (Kost et al. 1994, Kurien and Scofield 2006). In this thesis, the TLR2, TLR3 and TLR9 proteins were detected from several tissues (Chapter 4 and 5). The detailed procedure and specific chemicals and antibodies can be found in the respective chapters (Chapter 4 and 5).

1.2.6 Immunohistochemistry

Immunohistochemistry (IHC) technique is the visualization of a tissue or cellular component *in situ* by detecting specific antigens using antibody-antigen interactions where the antibody is tagged with a visible marker. The marker may be a fluorescent dye, colloidal metal, hapten, radioactive marker or an enzyme that digest a substrate to reveal the substrate color. Cells normally express specific proteins that can be detected by IHC. Immunohistochemistry involves a series of uniform steps, typically beginning with antigen retrieval. The art of IHC requires specialized procedures for the detection of protein. Fixation, tissue processing, immunoreactions and antigen retrieval methods are important elements of IHC. Methods of antigen retrieval vary in terms of reagents and methods (reviewed by Cregger et al. 2006). The first definitive step of IHC following antigen retrieval is the application of a specific primary antibody (typically produced by immunizing mice or rabbits with a peptide/antigen of interest), followed by extensive washing to remove excess amounts of the primary antibody. A species-specific secondary antibody is then applied, which binds to the primary antibody. The secondary antibody is typically conjugated to biotin, horseradish peroxidase, or some other tag. Finally, a detection reagent is applied that includes a chromagen substrate or a fluorescently tagged molecule to visualize the localization of the primary antibody (reviewed by Cregger et al. 2006). Several conditions and technical aspects are involved in the successful IHC (reviewed by Ramos-Vara 2005). The distribution of immunoreactive TLR2, TLR3 and TLR9 proteins in different tissues was characterized in this study (Chapter 4 and 5).

1.2.7 Data analysis

In this thesis different statistical methods are involved that are described in more detail in the respective chapters. The data were analyzed using the software package SAS (version 9.2). Generalized linear models (PROC GLM) were used to identify any possible effect of sire, dam, sex, birth weight, average daily weight gain, litter size, parity and month of birth on the blood concentration of immune components (Chapter 1 and 2) or on the gene expressions (Chapter 3, 4 and 5). For the expression stability analysis of HKGs, different publicly available web-based software package (such as geNorm, NormFInder and BestKeeper) were used (Chapter 3). Details of the logarithm used by geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004) are described in Chapter 3.

1.3 Results

To fulfill the objectives of this study, different work packages were carried out. The detailed results can be found in the respective chapters in this thesis. Some of the important results are very briefly described here. For better description with regards to the aims of this research and the chapters, the results are divided into several parts. First, to detect the QTL for mycoplasma and tetanus (TT) antibody, and for interferon gamma (IFNg), a Duroc × Pietrain F2 resource population (n = 319) was vaccinated with *Mycoplasma hyopneumoniae* (Mh), Tetanus toxoid (TT) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) at 6, 9 and 15 weeks of age, respectively (Chapter 1). Blood samples were collected at 6 different time points for the evaluation of phenotypes. The immune competence traits measured in this study comprise of the serum titer of Mh and TT antibodies and the concentration of IFNg. Antibody titers of Mh and TT were measured in blood samples collected just before vaccinations and two times after the vaccinations (10 and 20 days after Mh vaccination, and 20 and 40 days after TT vaccination). The IFNg blood concentration was measured from samples collected after the Mh, TT and PRRSV vaccinations (10 days after Mh and PRRSV, and 20 days after TT vaccination). The phenotypes of immune response traits were characterized using enzyme-linked immunosorbent assays (ELISA) from both commercial and in house developed assays. The information obtained from phenotypic evaluation was further utilized in the quantitative trait loci (QTL) linkage mapping using QTL Express. This population was genotyped with 122 genetic markers. The titers of Mh and TT antibodies were increased with age. The IFNg production was highest after Mh vaccination, was lowest after TT vaccination and was moderate after PRRSV vaccination. However this difference of IFNg was not significant. Antibodies and IFNg were found to be significantly affected by sex, litter size, parity, and month of birth in this study. A total of 18 QTL were identified for antibodies on nine chromosomes, of which 12 were for Mh antibody and six were for TT antibody (Chapter 1). Among these QTL, 12 were suggestive ($P < 0.05$, at chromosome-wide level), five were significant ($P < 0.01$, at chromosome-wide level) and a QTL for TT was highly significant ($P < 0.05$, at experiment-wide level). This highly significant QTL for TT antibody is very close to the location of *TLR6* on SSC8. Two QTL for Mh antibody were detected within a close region on SSC15 and on SSC18 which might be the QTL with pleotropic effect. 12 chromosomal regions were found to affect IFNg production. Two QTL were identified within close regions on SSC5 and SSC11, which

might be pleotropic QTL. Importantly, the confidence interval of the two QTL detected on SSC5 did incorporate the location of the IFNg gene. Additionally, this study identified two QTL on SSC5 using the two-QTL for IFNg approach, and a QTL for TT and IFNg with imprinting effect on SSC2 (Chapter 1).

Second, with the aim to detect the QTL affecting innate immune response traits, another Duroc × Pietrain F2 resource population (n = 332) was vaccinated with the same vaccines (Mh, TT and PRRSV) at the same ages (6, 9 and 15 week of age, respectively). Serum concentrations of IFNg, IL2, IL10, TLR2 and TLR9 were measured in blood samples collected after each vaccination (Chapter 2). For the measurement of these innate immune response components, commercial ELISA kits were used. This population was genotyped with 82 genetic markers. The serum concentrations of IFNg and IL10 were highest in response to PRRSV and were lowest after Mh vaccination. Serum concentrations of IL2, TLR2 and TLR9 were found to be lower in response to TT vaccination. Moreover, age, gender, litter size and parity were found to have an effect on these innate immune components. A total of 33 QTL were detected on almost all autosomes, of which four QTL were for each of IL2, IFNg and TLR2; eight and 13 for IL10 and TLR9, respectively. Additionally, six QTL were identified by the two-QTL approach (Chapter 2). The flanking region of some QTL did incorporate the chromosomal location of some genes with important immune function which are postulated to be important candidates for porcine immune responses.

Third, it is important to know the expression stability of commonly used housekeeping genes across tissues of pigs at different ages. Due to the normalization, in case of the gene expression study, the expression of target gene is greatly affected by the expression stability of the housekeeping genes. For this purpose, nine commonly used housekeeping genes were selected, cloned and expression study was performed using qRT-PCR. The expression of these nine housekeeping genes (B2M, BLM, GAPDH, HPRT1, PPIA, RPL4, SDHA, TBP and YWHAZ) were performed in 13 different lymphoid tissues (cervical lymph node, duodenum, heart, ileum, jejunum, kidney, liver, lung, mesenteric lymph node, skin, spleen, stomach and thymus) including peripheral blood mononuclear cells (PBMC) collected from newborn (one day old), young (2 months old) and adult (5 months old) pigs (Chapter 3). In order to determine the expression stability and to select the most stable housekeeping gene, these expression data were analysed using geNorm, NormFinder and BestKeeper analysis programs. geNorm found that *RPL4*, *PPIA* and *YWHAZ* were the most stable housekeeping genes in newborn and adult, whereas *B2M*, *YWHAZ* and *SDHA* were the most stable in young

pigs. According to the NormFinder, *TBP* is the most stably expressed housekeeping gene in newborn and young pigs, whereas *PPIA* is the most stable in adult pigs. Moreover, geNorm suggested that the geometric mean of the three most stable genes should be used for the appropriate normalization. In all cases, *GAPDH* was detected as the least stable housekeeping gene by geNorm (Chapter 3). The housekeeping genes were affected by age and organs. However, the first three most stable reference genes in most cases were consistently the same when using geNorm and NormFinder, even if they were not in the exact same ranking order.

Fourth, the study aimed to reveal the expression patterns of all TLRs (TLR1-10) in gut-associated lymphoid tissues (GALT) (stomach, duodenum, jejunum and ileum) including mesenteric lymph node (MLN) (Chapter 4). TLRs mRNA analysis was preformed in these tissues collected form newborn (one day old), young (2 months old) and adult (5 months old) pigs. For this purpose, GenomeLab Expression Analysis (GeXP) was used. Moreover, the expression patterns of TLR2, TLR3 and TLR9 proteins were detected using Western blot and the immunoactive distribution of these three TLRs was characterized using immunohistochemistry. In most tissues, TLRs mRNA abundances were higher in young (2 months old) and adult (5 months old) pigs than in newborn (one day old) piglets. Among all the TLRs, TLR3 mRNA was found to be higher expressed across tissues. However, all the TLRs did not exhibit the same patterns of expression: in most of the cases TLRs increased with age. mRNA abundance of all TLRs was affected by age and organs. The protein expression patterns of TLR2, TLR3 and TLR9 seemed to be consistent with the mRNA expressions. Immunoreactive proteins of TLR2, TLR3 and TLR9 were detected in intestinal epithelial cells, in the lymphoid cells in lamina propria and in the lining cells of villi. Higher signals were detected in the Peyer's patches in intestine (Chapter 4). These proteins were also remarkably higher expressed in the lymphoid follicles, sinus and trabeculae compared to the red pulp in the MLN.

Lastly, the expression patterns of TLRs (TLR1-10) were characterized in lymphoid tissues other than GALT (cervical lymph node, heart, kidney, liver, lung, skin, spleen, thymus and PBMC) collected form newborn (one day old), young (2 months old) and adult (5 months old) pigs (Chapter 5). For the mRNA expression study, the GeXP was used. Additionally, TLR2, TLR3 and TLR9 protein expression and distribution were characterized in selected tissues (lung, spleen and cervical lymph node) using Western blot and immunohistochemistry, respectively. In most tissues, TLRs mRNA abundance was higher in young animals compared to adult and newborn animals. In all tissues,

TLR3 mRNA expression was higher than other TLRs. These expression differences may indicate the immune responsiveness of these organs with regards to the age of the animals. The mRNA abundance of all TLRs was affected by age and organs. The protein expression of TLR2, TLR3 and TLR9 was detectable in all tissues. TLR2, TLR3 and TLR9 immunoreactive proteins were stained in the alveolus and lining cells of bronchioles in lungs, in the lymphoid cells in white pulp in spleen, and in the lymphoid cells in the cervical lymph node especially in the lymphoid follicle in the cortex (Chapter 5).

1.4 Conclusions

Herein, we identified the chromosomal region associated with immune response traits in pigs in response to vaccine antigens. To obtain the results, a Duroc x Pietrain resource population was genotyped using genetic markers and as phenotypes vaccine antigen induced immune response components were measured for quantitative trait loci analysis. Several QTL were recorded on all autosomes that were affecting both adaptive and innate immune responses traits. With regards to number and magnitude of their impact, QTL for immune response traits behave like those for other quantitative traits such as for meat quality and carcass traits, production and reproduction traits. Some of the identified QTL coincided with previously reported QTL for immune response and disease resistance traits (Edfors-Lilja et al. 1998, Reiner et al. 2008, Reiner et al. 2007, Wimmers et al. 2008), and the newly identified QTL are potentially involved in immune functions. This study focused on some putative candidate genes (such as *TNFa* [tumor necrosis factor alpha], *TLR6*, *MPO* [myeloperoxidase], MBL2 [mannose-binding lectin 2], NRAMP1 [natural-resistance-associated macrophage protein 1], LBP [lipopolysaccharide binding protein], BPI [bactericidal/permeability-increasing protein]) for immune traits located on the peak of the QTL regions which could be interesting candidates for further study through association analysis (Zhou et al. 2001, Zhou and Lamont 2003). Polymorphisms in TNFa (Mellick 2007), TLRs (Uenishi et al. 2011a, 2011b), MPO (He et al. 2009), NRAMP1 (Wu et al. 2008), LBP (Liu et al. 2008) and BPI (Shi et al. 2003) are reported to be associated with diseases. Since the confidence interval was higher due to the limited number of markers, fine mapping using SNP chips could be beneficial to detect particular candidate genes (de Koning et al. 2005). Importantly, cytokines and TLRs orchestrated through a very complex network and production of antibody are mediated by a complex and sophisticated process which may explain a couple of pleotropic QTL identified in this study. By QTL detection, the linkage between loci is calculated to localize the chromosomal region including the candidate genes. However, it is necessary to analyze the association of these candidate genes with the traits. Polymorphisms of IFNg located on the QTL region affecting immune response are reported to be associated with primary and secondary antibody response to different antigens in chicken (Zhou et al. 2001). A similar finding was reported for transforming growth factor beta 2 (*TGFB2*) gene in

chicken and *TGFB2* is suggested as a candidate gene to be applied in marker-assisted selection to improve antibody production (Zhou and Lamont 2003). Similarly in pigs, several QTL for immune traits were identified close to the mast/stem cell growth factor receptor (*KIT*) gene and candidate gene analysis showed significant effects of this gene on the immune response traits (Wattrang et al. 2005). In this study, the QTL analysis was used to detect the chromosomal regions influencing immune traits and scanning of putative candidate genes regarding the innate and adaptive immune response traits in pig. Furthermore, follow-up research is needed to further characterize these QTL in animal populations challenged with infection and in other crosses. However, this discovery of the QTL regions will facilitate to understand the genetic basis underlying immune traits and to identify the candidate genes for immune competence.

The expression stability of nine commonly used HKGs was analysed using different normalization programs in order to detect a stable set of HKGs across lymphoid organs at different ages of pigs. It could be seen that the HKGs are affected by both the organs and age of individuals suggesting that source of samples and age of population should be taken into account for selecting appropriate HKGs. The mostly used popular HKG *GAPDH* is found to be the least stable which is reported previously by several researchers in pigs (Barber et al. 2005, Jung et al. 2007, Oczkowicz et al. 2010, Piorkowska et al. 2010, Svobodova et al. 2008). This study suggested a set of HKGs which could be beneficial for the research community to select appropriate HKGs for expression studies in pigs. Although tissues were not the same, our result is in agreement (Oczkowicz et al. 2010, Piorkowska et al. 2010, Svobodova et al. 2008) while is in contradictory (Erkens et al. 2006, Kuijk et al. 2007) with some previous reports in pigs. This study suggested that the combination of the three most stable HKGs should be used for gene normalization (Chapter 3). Instead of discrepancies in the ranking order of reference genes obtained by different analysing software methods, the geometric mean of the *RPL4*, *PPIA* and *YWHAZ* was identified to be the most appropriate combination of HKGs for accurate normalization of gene expression data in different porcine tissues at different ages (Chapter 3).

The expression patterns of TLRs were determined in different lymphoid organs collected from newborn, young and adult pigs. The lymphoid organs including gut-associated lymphoid tissues (GALT) expressed all TLRs mRNA indicating that these organs are armed with all TLRs in order to fight against varieties of pathogens. All ten

porcine TLRs were influenced by age and organs which as reported previously in humans and mice (Renshaw et al. 2002, Tohno et al. 2006, van Duin and Shaw 2007). Among the TLRs (TLR1-10), TLR3 was found to be the most abundant in lymphoid tissues indicating that the pigs used in this study might have received antibodies passively through colostrum or might have antibodies against PRRSV due to previous low grade exposure to PRRSV (Sang et al. 2008). Selected TLRs proteins (TLR2, TLR3 and TLR9) were detected using Western blot and their distribution was investigated in selected tissues (stomach, duodenum, jejunum, ileum, mesenteric lymph node, lung, spleen and cervical lymph node) using immunohistochemistry (Chapter 4 and 5). This study is the first detecting all TLRs mRNA in porcine lymphoid tissues and revealed the expression patterns of TLRs in pigs with different ages thus helping to understand the immune responsiveness of these organs.

1.5 References

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2. Chapter 1 (Published in: *Mammalian Genome*, 2010. 21(7-8):409-418)

Mapping of quantitative trait loci for mycoplasma and tetanus antibodies and interferon-gamma in a porcine F_2 Duroc x Pietrain resource population

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Summary

The aim of the present study was to detect quantitative trait loci (QTL) for innate and adaptive immunity in pigs. For this purpose, a Duroc x Pietrain F_2 resource population (DUPI) with 319 offspring was used to map QTL for immune traits blood antibodies and interferon-gamma using 122 microsatellites covering all autosomes. Antibodies response to *Mycoplasma hyopneumoniae* and tetanus toxoid vaccine and the interferon-gamma (IFNg) serum concentration were measured at three different time points and were used as phenotypes. The differences of antibodies and interferon concentration between different time points were also used for the linkage mapping. Line-cross and imprinting QTL analysis including two-QTL were performed using QTL Express. A total of 30 QTL (12, 6 and 12 for mycoplasma, tetanus antibody and IFNg, respectively) were identified at the 5% chromosome wide level significant of which 28 were detected by line-cross and 2 by imprinting model. Additionally, two QTL were identified on chromosome 5 using the two-QTL approach where both loci were in repulsion phase. Most QTL were detected on pig chromosomes 2, 5, 11 and 18. Antibodies were increased over time and immune traits were found to be affected by sex, litter size, parity and month of birth. The results demonstrated that antibody and IFNg concentration are influenced by multiple chromosomal areas. The flanking markers of the QTL identified for IFNg on SSC5 did incorporate the position of the porcine *IFNg* gene. The detected QTL will allow further research in these QTL regions for candidate genes and their utilization in selection to improve the immune response and disease resistance in pig.

Introduction

The current release of the Pig QTLdb (May 05, 2010) contains 5732 QTL representing 558 different traits (http://www.animalgenome.org/QTLdb/pig.html) mostly for economically important traits like growth, carcass and meat quality, and reproduction. Differences in immune status and variation in immune response depending on the genetic background have been reported (Edfors-Lilja et al. 1994, 1998) and medium high to high heritabilities (h^2 = 0.3 - 0.8) have been estimated for several of the immune traits in pigs (Edfors-Lilja et al. 1998). However, little is known about the genetics underlying these traits especially in swine. Antibody response is one of the first immune competence traits to be examined by QTL analysis (Edfors-Lilja et al. 1998) but a very

limited number of QTL analyses have been devoted to health, disease resistance, immune capacity and immune response traits (Edfors-Lilja et al. 1998, Reiner et al. 2007, Wimmers et al. 2008, Wimmers et al. 2009). QTL underlying the immune response variations have been detected in mouse, chicken and human (Almasy and Blangero 2009, Biscarini et al. 2010, Hall et al. 2002). Therefore the aim of the present study was to detect immune specific QTL for innate (interferon-gamma) and adaptive (tetanus and mycoplasma antibodies) immunity in pig.

Measurements of antibodies are of immense importance for the evaluation of health status of animals and herds, especially for the evaluation of vaccination efficiency and herd health programs (Regula et al. 2003). Interferon-gamma (*IFNg*) is one of the key molecules in the immune system and provides the first line defence against pathogens. It has immunomodulatory function, possesses antiviral activity and protects swine from diseases (Scheerlinck and Yen 2005, Yao et al. 2008). While *IFNg* is a component of the innate immune system, the antibodies belong to the adaptive / humoral immunity. Values of these immune parameters vary according to the individual's immune status which can be triggered by vaccine antigens. Therefore the antibody levels were measured before and after immunological stimulation by vaccines. IFNg was measured at three time points after each vaccination as an innate immune trait, which might not reflect vaccine effect but it is an important immune parameter and can be considered as an indicator of disease resistance (Scheerlinck and Yen 2005, Yao et al. 2008). With regard to number and magnitude of their impact, QTL for immune traits behave like those for other quantitative traits. Discovery of the chromosomal regions influencing these important immune traits including their production variations will facilitate the identification of candidate genes for antibodies and interferon production, disease resistance and immune competence in pigs.

Materials and methods

Experimental population and blood sampling

The animal population used for the evaluation of immune traits and the genome scan was based on a Duroc x Pietrain cross. A detailed description of the population structure has been reported earlier (Liu et al. 2007, 2008). In our study, genetic information of three generations P, F_1 and F_2 and phenotypes from 319 F_2 pigs were used. All pigs

were kept at the Frankenforst experimental research farm at the University of Bonn (Germany). The animals were fed an *ad libitum* diet during the whole test period and were slaughtered at approximately 105 kg live weight. Pigs were vaccinated with *Mycoplasma hyopneumoniae* (Mh), tetanus toxoid (Tet) and porcine reproductive and respiratory syndrome virus (PRRSV) vaccines at 6, 9 and 15 weeks of age, respectively. Blood samples were taken at six different time points (supplementary file 1). Antibody titres of Mh were measured in blood samples collected just before vaccination (6 weeks) and 10 and 20 days afterwards. The sample for tetanus antibody measurement was collected just before vaccination (9 weeks) and 20 and 40 days after vaccination. The IFNg blood levels were measured from samples collected at 10 days after Mh and PRRSV, and 20 days after tetanus vaccination.

Measurement of antibodies and interferon-gamma

Antibody response to Mh vaccination was determined by monoclonal blocking ELISA using the HerdChek M. hyo. antibody ELISA kit (IDEXX GmBH, Germany) following the manufacture's protocol. Tet antibody was determined by in-house developed indirect ELISA (Wimmers et al. 2008). The optical density (OD) was read at 650 nm and 490 nm for Mh and Tet, respectively by using a microplate reader (ThermoMax, Molecular Devices) and the result of antibodies were determined as S/P ratio. Serum IFNg was measured by sandwich ELISA using Swine INFγ CytoSet and CytoSet Buffer Set (Invitrogen). Absorbance was measured at 450 nm within 30 minutes after adding stop solution and results were calculated as pg/ml using a 4-parameter curve fitted in SoftMaxPro software (Molecular Devices). In all cases two replications of each sample were used for ELISA and the mean value was considered as the serum concentration of respective traits.

Statistical analysis

Single measurement of the antibodies and interferon at different time points as well as changes in titre between time points were considered as single trait and analysed in this study. The differences of titre between two time points describe the kinetics of these immune traits in response to vaccine antigen (Edfors-Lilja et al. 1998). The data were analysed using the SAS software package (version 9.2) for a detailed description of the data structure. Generalized linear models (PROC GLM) were used to identify any

possible obvious effect of sire, dam, sex, birth weight, average daily weight gain, litter size, parity and month of birth on the blood level of antibodies and interferon. The phenotypic data followed approximately a normal distribution and were used for linkage analysis.

Marker analysis

A linkage map with the total length of 2159.3 cM and an average marker interval of 17.7 cM was constructed. P, F_1 and F_2 animals of the DUPI population were genotyped at 122 markers loci covering all porcine autosomes. Marker positions and details of genotyping procedures were given in Liu et al. (2007) and for SSC1 in Grosse-Brinkhaus et al. (2009). Most of the markers were selected from the USDA/MARC map (http://www.marc.usda.gov). They are also available in Sscrofa5 (NCBI) and Sscrofa9 (Ensembl). Genotyping, electrophoresis, and allele determination were done using a LI-COR 4200 Automated Sequencer including the software OneDScan (Scanalytics). The CE8000 sequencer (BeckmanCoulter) was used for genotyping of SSC1 and SSC18. Allele and inheritance genotyping errors were checked using Pedcheck software (version 1.1) (O'Connell and Weeks 1998). The relative positions of the markers were assigned using the build, twopoint and fixed options of CRIMAP software (version 2.4) (Green et al. 1990). Recombination units were converted to map distances using the Kosambi mapping function. Marker information content and segregation distortion were tested by following Knott et al. (1998).

QTL analysis

QTL interval mapping was performed using the web-based program QTL Express (Seaton et al. 2002) based on a least square method. Single and two-QTL analyses were carried out and imprinting (ADI) models were applied. The basic QTL regression model used in the present study was:

$y_i = \mu + F_i + \beta\, cov_i + c_{ai}a + c_{di}d + \varepsilon_i$

where:

y_i = phenotype of the i^{th} offspring;

μ = overall mean;

F_i = Fixed effect ($parity_{2.....10}$, month of $birth_{1.....12}$);

β = regression coefficient on the covariate;

cov_i = covariate (litter size, age at blood sampling in days);

c_{ai} = additive coefficient of the i^{th} individual at a putative QTL in the genome;

c_{di} = dominant coefficient of the i^{th} individual at a putative QTL in the genome;

a = additive effects of a putative QTL;

d = dominant effects of a putative QTL; and

ε_i = residual error

The presence of imprinting effects was tested by adding a third effect (i) into the model (Knott et al. 1998) using QTL Express (Seaton et al. 2002). Chromosome- (CW) and experiment-wide (GW) significance thresholds were determined using 1000 permutations (Churchill and Doerge 1994). Chromosome-wide 1% and 5% significance thresholds became genome-wide significance thresholds after Bonferroni correction for 18 autosomes of the haploid porcine genome (de Koning et al. 2001). Methods for mapping a single QTL can be biased by the presence of other QTL (Meuwissen and Goddard 2004, Raadsma et al. 2009). To address this situation, two-QTL models were also fitted for all traits using QTL Express (Seaton et al. 2002). To control for false-positive QTL due to multiple testing, the permutation thresholds obtained in the single-QTL analyses were used to test for the significance of the two-versus one-QTL and two-versus no-QTL. Multiple QTL were declared on a chromosome if they were separated by at least 30 cM and exceeded 5% CW/GW level significance (Kim et al. 2005, Liu et al. 2008). The phenotype variation that was explained by a QTL was calculated by the following equation.

$$Var\% = \frac{MS_R - MS_F}{MS_R} \text{ x } 100$$

Where, MS_R was the mean of square of the reduced model; MS_F was the mean of square of the full model.

Results

Phenotypes distribution

It was found that antibodies for tetanus and mycoplasma were increased over time after vaccinations for most animals, but IFNg levels did behave differently (Fig. 1). Overall Mh antibody concentrations were increased significantly at 10 and 20 days after

vaccination in comparison with the concentration prior vaccination (Fig. 1.A). Tetanus antibody was significantly higher at 15 weeks of age in comparison to 9 weeks of age (Fig. 1.B). When different IFNg concentrations were compared, the concentration was higher at 7 weeks and lower at 12 weeks of age but no significant difference could be verified (Fig. 1.C). Antibodies and interferon were found to be significantly affected by sex, litter size, parity and month of birth (Supplementary file 2).

QTL for mycoplasma and tetanus antibodies

A total of 18 QTL were identified for antibodies, of which one was highly significant (experiment-wide, $P < 0.05$), five were significant (chromosome-wide, $P < 0.01$) and 12 were suggestive (chromosome-wide, $P < 0.05$) (Table 1). Two QTL for Mh3 and Mh2-1 respectively were identified on SSC2. A QTL for Tet3 was detected at 115 cM on SSC4 (CW, $P < 0.01$) on the marker *S0097*. Chromosomal regions on SSC7 influencing Mh1 and Mh2 were mapped at 19 cM and 33 cM, respectively. The QTL for Mh2 (CW, $P < 0.01$) at 33 cM was located on the marker *S0064* (Fig. 2.A). QTL for Tet3 (GW, $P < 0.05$) was identified at 0 cM on SSC8 very close to the marker *SW241* which explained 37.50% of the phenotypic variation. QTL (CW, $P < 0.05$) for Mh1 at 85 cM and for Mh2 at 101 cM were identified in this study close to the marker *SW398* on SSC13. Additionally, two chromosomal regions (CW, $P < 0.05$) at 59 cM and 53 cM on SSC15 were associated with Mh3 and Mh2-1. The QTL for Mh3 (CW, $P < 0.05$) was very close to the marker *SW936*. On the SSC16 a QTL (CW, $P < 0.01$) was found at 6 cM influencing Mh3-2, which explained 27 % of the phenotypic variation (Fig. 2.C). Five QTL regions were identified on SSC18 (Fig. 2.D). Among them, QTL (CW, $P < 0.05$) for Mh3 and Mh3-1 were located at the same position very close to the marker *SY4* and QTL (CW, $P < 0.05$) for Tet4 and Tet5-4 were located within 8 cM region around the marker *S0062*. Moreover, a paternally imprinted QTL was identified for Tet3 (CW, $P < 0.05$) on SSC2.

QTL for interferon-gamma

Interferon-gamma was found to be related to 12 chromosomal regions on 8 different porcine autosomes in this study (Table 2). A chromosomal region was identified for IFN4-2 at 68 cM on SSC4. Three QTL regions were detected on SSC5 influencing IFNg. Among them, chromosomal regions at 51 cM (CW, $P < 0.05$) and 54 cM (CW, P

< 0.01), very close to the marker *SW2425* were found to influence IFN4 and IFN4-2, respectively. Three suggestive QTL (CW, $P < 0.05$) were detected for interferon-gamma on SSC11. Among them, QTL for IFN2 and IFN4-2 was mapped at 29 cM and 23 cM, respectively close to the marker *S0071*. The remaining QTL (CW, $P > 0.05$) affecting IFN6-4 was identified at 7 cM on SSC11 (Fig. 2.B). Additionally, a QTL was identified on SSC16 exceeding the 1% CW significance threshold which explained 27.80% of the phenotypic variation (Fig. 2.C). Moreover, a paternally imprinted QTL (CW, $P < 0.05$) affecting IFN2 was identified at 16 cM on SSC2.

Two-QTL analyses for different traits

The two-QTL model was used to identify the presence of possible two QTL regions on the same chromosome. Results for the two-QTL model conducted with QTL Express are presented in Table 3. Significant evidence for an additional QTL under a two-QTL model was found in a case on SSC5 for IFN4-2, with a difference of 66 cM between two the loci. SSC5 was genotyped with 14 microsattelite markers and the average marker distance was 10.78 cM. In this case, several markers (such as *S0092*, *SW0005* and *SW1987*) were located in between the two QTL regions and one of the two chromosomal regions was identified in the single QTL approach (QTL A). The two loci on SSC5 for IFN4-2 in this study were in repulsion phase. The QTL affecting IFN4-2 at 51 cM and 117 cM jointly explained 39.63% of the phenotypic variation.

Discussion

Phenotype distribution

A rise in antibodies concentration in response to Mh and Tet vaccine antigen is found over the time points but it did not increase in all animals, which might be due to individual variation. Animals, specially having higher Mh antibody at 6 weeks of age (T1; before vaccination) are reduced values at T2 (10 days after vaccination) and again increased at T3 (20 days after vaccination). Hodgins et al. (2004) reported that maternally derived antibodies play a major negative role in response to Mh vaccines, by neutralizing vaccine antigen. Mh antibody concentration at T1 is found to be affected by sex but Moreau et al. (2004) did not find interaction between the effect of vaccine and sex in pigs. In this study, Mh antibody is significantly influenced by the effects of sire

and dam. Differences in patterns of colonization of *M. hyopneumoniae* between pigs sired by different boars was reported by Ruiz et al. (2002). Passive transmission of Mh antibody from dam to piglets through colostrum might be the evidence for dam influence. The Mh antibody concentration was significantly influenced by parity in this study, which is supported by the study of Calsamiglia and Pijoan (2000).

Age is found to have effect on Tet antibody in this study and Cook et al. (2001) reported that the tetanus antibody concentration decreased significantly with age. Antibody reached to the higher concentration after 6 week of vaccination (T5) in this study, but no such report is found in pigs. However, human peripheral blood mononuclear cells (PBMC) are reported to produce highest concentration of anti-tetanus antibody at 3 weeks after exposure to tetanus toxoid (Virella and Hyman 1991). IFNg concentration showed a trend to be reduced with age in this study, and the IFNg concentration was higher in younger (7 week of age) compared to older animals (12 and 16 weeks). Davis et al. (2006) reported that PBMCs collected from young pigs produced higher IFNg than the PBMCs collected from older pigs. Sire, litter and sex have effects on IFNg production which is supported by a previous report in pigs (Mallard et al. 1989). Outteridge (1993) stated that in addition to genetic causes, there are many causes for individual variation and immune responsiveness such as nutritional status, immunological maturation, antigenic competition and immunological priming.

QTL for mycoplasma and tetanus antibody traits

In the pig, genome-wide significant QTL for cellular and humoral immune traits are shown to segregate on chromosomes 1, 4, 5 and 6 in an experimental cross of wild boar and Yorkshire (Edfors-Lilja et al. 1998, 2000). QTL for the pseudorabies virus resistance/susceptibility are mapped to chromosomes 9, 5 and 6 (Reiner et al. 2002) and for the *Sarcocystis miescheriana* are detected on SSC7, 16, and 2 (Reiner et al. 2007) in pigs. QTL on SSC1, 2 and 6 were mapped for antibodies of PRRS and Aujeszky's disease virus (Wimmers et al. 2009); and on SSC3, 6, 16 and 17 for mycoplasma, tetanus, and PRRS antibodies (Wimmers et al. 2008) in pigs. At the recent past the NCBI released the draft assembly of the porcine genome Sscrofa5 (NCBI) include assemblies for chromosomes 1, 4, 5, 7, 11, 13, 14, 15, 17 and X, and the ENSEMBL released Sscrofa9 (Ensembl). These databases help to search for the immunologically important genes located on the identified QTL regions.

Two QTL with additive and dominant effects were detected on SSC2 to influence Mh antibody production. Very close to this regions, a QTL for leukocyte number is reported previously (Edfors-Lilja et al. 2000). In response to *M. hyopneumoniae* macrophage (leukocyte) activation and proliferation is reported in pig (Rodriguez et al. 2007) which is an evidence for possible QTL affecting Mh antibody. Moreover, leukocytes and monocytes are reported to phagocyte the mycoplasma pathogen (Marshall et al. 1995). A QTL for Tet antibody was mapped on SSC4 close to the marker *S0097* where Edfors-Lilja et al. (2000) reported a QTL affecting eosinophil numbers. The QTL on SSC7 for Mh antibody were in a similar region where a QTL for platelets number is detected earlier (Reiner et al. 2007). Choi et al. (2006) reported that *M. hyopneumoniae* causes thrombocytopenia by destroying platelets in pig. Moreover, the immunological important tumor necrosis factor (*TNF-α* and *β*), *MHC* (*I* and *II*), *C2* and *C4* genes are mapped on the same region (Ensembl; NCBI) on SSC7. *TNF-α* is reported to be highly expressed and responsible for cachexia in pigs experimentally infected with *M. hyopneumoniae* (Choi et al. 2006). The very important innate immune gene *TLR6* (Toll-like receptor 6) is located at the region on SSC8 affecting tetanus antibody production. Toll-like receptors (TLRs) play an essential role in the recognition of microbial components and are reported as critical proteins linking innate and humoral immunity (Takeda and Akira 2004). TLRs are speculated to be used in vaccines design including tetanus toxoid (van Duin et al. 2006). The natural resistance-associated macrophage protein 1 (*NRAMP1*) is mapped on SSC15q23-26 where two QTL regions (close to *SW936* marker) are detected for Mh antibody in this study. *NRAMP1* is a potential candidate gene in controlling pigs resistance to salmonella infection (Sun et al. 1998). Recent studies using knockout mice indicate that the *NRAMP1* gene, expressed in macrophages is capable to control resistance and susceptibility to *Mycobacterium bovis* (BCG), *Leishmania donovani* and *Salmonella typhimurium* (Stecher et al. 2006). *NRAMP1* might be suggested as a good candidate gene for Mh antibody production.

A QTL affecting Mh antibody was found on SSC16 close to the marker *S0111* where a QTL for *C3* was reported earlier (Wimmers et al. 2008). Wimmers et al. (2003) stated that *C3* is associated with Mh antibody concentration in pigs. Furthermore, two linkage regions were identified related to Mh antibody production close to the marker *SY4* on SSC18. Very close to this region T cell receptor beta variable 19 (*TRBV19*) is located (Ensembl). The T-cell receptors (TCRs) are a complex of integral membrane proteins

that participates in the activation of T-cells in response to the presentation of antigen and *TRB* is reported to be expressed by T-cell in response to the *Mycoplasma sp*. stimulation (Friedman et al. 1991). Additionally, two QTL were found to affect Tet antibody close the marker *S0062* where growth hormone-releasing hormone receptor (*GHRHR*) and acyloxyacyl hydroxylase (*AOAH*) are located (Ensembl). Growth hormone can acts as a cytokine which can influence lymphocyte proliferation and its receptors are located on lymphocytes and macrophages (Postel-Vinay et al. 1997). LeRoith et al. (1996) reported that GH administration elicited a marked activation of the immune system in response to tetanus toxoid. *AOAH* is reported to modulate host inflammatory responses in Gram-negative bacterial invasion (Feulner et al. 2004).

QTL for interferon-gamma trait

The cytokine network is complex and demonstrates redundancy and pleiotropism. *IFNg* is an important cytokine for inducing the macrophage killing activation and has been evaluated as marker for acute bacterial infection in swine (Yao et al. 2008). The significant QTL for IFNg on SSC4 was assigned close to the position of *CD1* and *CRP* (C-reactive protein). *CRP* plays an important role via monocytes to upregulate proinflammatory cytokines. One of the most interesting finding for the interferon QTL was the identification of two linkage regions influencing IFNg on SSC5 close to SSC5p11-12 where the *IFNg* gene is located. It implies that *IFNg* is influenced by region of its own location. However, more regions of other chromosomes are also affecting IFNg production, evidence of multiple gene effect. Previously reported QTL affecting neutrophil proliferation (Reiner et al. 2002) and IgG production (Edfors-Lilja et al. 1998) are also close to our identified QTL for IFNg on SSC5. Unique receptors to *IFNg* are located on the surface of the T- and B-lymphocytes, NK-cells, and neutrophils. Transforming growth factor β2 (*TGFB2*) is a potent anti-inflammatory cytokine located on SSC10 close to the marker *SW830* (Ensembl) has an antagonistic effect on IFNg (Ulloa et al. 1999). Kruppel-like factor 5 (*KLF5*) is mapped close to the marker *S0071* (Ensembl; NCBI) on SSC11. Chen et al. (2000) reported that *KLF5* is an immediate-early *IFNg* responsive gene and *IFNg* induces *KLF5* expression. *C9* gene (complement component 9) is an important component of the complement system and plays an important role in innate immune response. This gene is located within the flanking markers of the QTL identified for IFNg (CW, $P < 0.01$) on SSC16 (Ensembl).

Imprinted QTL

Most imprinted genes are identified in humans and mice (http://igc.otago.ac.nz/). Imprinted genes are considered as one culprit of phenotypic variation in pig (Bischoff et al. 2009) but only a small number of imprinted genes are identified in pigs (Zhang et al. 2007). A number of imprinting QTL are reported in pigs (de Koning et al. 2000, Holl et al. 2004, Nezer et al. 1999, Thomsen et al. 2004) but no imprinting QTL for immune traits is reported yet. Recently, imprinting QTL for immune response is reported in chicken (Pinard-van der Laan et al. 2009). The paternally imprinted QTL found in our study have its best position at 16 cM for IFNg and 0 cM for Tet antibody on SSC2. Imprinting QTL on SSC2 are reported to influence lean growth (Nezer et al. 1999), skeletal and cardiac muscle mass (Jeon et al. 1999), backfat (de Koning et al. 2000), teat number and coat colour (Hirooka et al. 2002), and reproduction (Holl et al. 2004). Notable imprinted genes in this region include *IGF2* (insulin-like growth factor 2), *H19* and *Wilms tumor*. QTL with imprinting effects are reported to be more appropriate for analyzing F_2 data than only single line-cross model (Holl et al. 2004). However, for most of the QTL showing imprinting effects, biological reasons for the inherited mode are difficult to derive. Evolutionary reasons behind the presence of parent-of-origin effects are also unclear, although several theories exist (Thomsen et al. 2004, Tycko and Morison 2002).

Conclusions

The results of this work shed new light on the genetic background of both innate and adaptive immune response in pigs. Mycoplasma and tetanus antibodies and interferon-gamma production are influenced by both environmental and genetic factors. This study has identified several new quantitative trait loci for immune traits on most autosomes. *TNFα*, *NRAMP1* and *TCRs* might be of good candidate genes for mycoplasma and *TLR6* for tetanus antibody production. Our results showed that *IFNg* is influenced by the chromosomal region to which it is mapped, and there might be more regulative genes along with multiple chromosomal regions. This study enforces that genomic imprinting might be important in livestock species. Despite the fact that candidate genes were identified, it must be considered before an interpretation of QTL results, that confidence regions of the QTL are large and can contain many of potential candidate genes for the QTL (de Koning et al. 2005). However, this discovery of the QTL regions

will facilitate identifying candidate genes for immune competence and disease resistance, which is the first step for marker assisted breeding efforts. Further, follow-up research is needed to further characterize these quantitative trait loci in other crosses and identify candidate genes by fine mapping using denser marker sets like large scale SNP assays.

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Table 1: Evidence of QTL for mycoplasma and tetanus antibody levels.

SSC[a]	Trait[b]	Pos[c]	F-value[d]	V(%)[e]	a ± SE[f]	d ± SE[g]	Closest Markers[h]
2	Mh2-1	168	6.05*	20.14	-0.26 ±0.08	-0.17 ±0.14	SWR2157 (168.7)
2	Mh3	129	6.31*	20.99	-2.99 ±0.93	3.08 ±1.06	SW1564(127.1)
2	Tet3[#]	0	16.38**	50.00	-0.02 ±0.01	0.07 ±0.02	SW2443(0.0)
4	Tet3	115	9.59**	25.00	-0.01 ±0.01	-0.07 ±0.02	S0097(115.6)
7	Mh1	19	5.65*	18.18	0.07 ±0.02	-0.02 ±0.04	S0025(0.0)-S0064(33.0)
7	Mh2	33	8.39**	26.97	0.05 ±0.09	-0.56 ±0.14	S0064(33.0)
8	Tet3	0	11.98***	37.50	0.02 ±0.01	-0.08 ±0.02	SW2410(0.0)-SW2611(0.1)
11	Tet4	6	7.73**	28.57	-0.05 ±0.01	-0.06 ±0.02	SW2008(0.0)
13	Mh1	85	7.15*	23.38	0.09 ±0.03	-0.1 ±0.05	TNNC(69.6)-SW398(100.9)
13	Mh2	101	6.81*	22.51	0.27 ±0.11	-0.4 ±0.15	SW398(100.9)
15	Mh2-1	53	5.63*	16.75	0.34 ±0.11	0.01 ±0.19	SW936(60.6)
15	Mh3	59	5.67*	17.26	0.35 ±0.11	0.11 ±0.18	SW936(60.6)
16	Mh3-2	6	8.46**	27.17	-0.35 ±0.09	-0.34 ±0.14	S0111(0.0)
18	Mh1	74	6.88*	22.08	0.03 ±0.02	0.1 ±0.03	SJ061(64.1)-SWR414(81.2)
18	Mh3	1	6.83*	22.60	0.22 ±0.09	-0.4 ±0.15	SY4(0.0)-SW1808(8.5)
18	Mh3-1	1	7.77*	25.29	0.2 ±0.09	-0.44 ±0.15	SY4(0.0)-SW1808(8.5)
18	Tet4	57	5.98*	21.43	0.01 ±0.01	-0.04 ±0.01	S0062(56.9)-SW1682(58.4)
18	Tet5-4	49	5.69*	19.35	-0.02 ±0.01	0.06 ±0.02	S787(43.2)-S0062(56.9)

[a] Sus scrofa chromosome.

[b] Trait abbreviations: Mh1: Mh antibody level at time point 1; Mh2: Mh antibody level at time point 2; Mh3: Mh antibody level at time point 3; Mh3-1: antibody difference between time point 3 and 1; Mh3-2: Mh antibody difference between time point 3 and 2; Mh2-1: Mh antibody difference between time point 2 and 1; Tet3: Tetanus antibody level at time point 3; Tet4: Tetanus antibody level at time point 4; Tet5-4: Tetanus antibody difference between time point 5 and 4.

[c] Chromosomal position in Kosambi cM.

[d] Significance of the QTL: *, significant on a chromosome-wide level with $P \leq 0.05$; **, significant on a chromosome-wide level with $P \leq 0.01$; ***, significant on a genome-wide level with $P \leq 0.05$.

[e] The percentage of phenotypic variance explained by the QTL

[f] Additive effect and standard error. Positive values indicate the Duroc alleles result in

higher values than Pietrain alleles; negative values indicate that Duroc alleles result in lower values than Pietrain alleles.

[g] Dominance effect and standard error.

[h] The closest markers were those markers around the peak, as near as possible (position of markers in cM)

[#] The imprinting effect and standard error was detected for T3 (-0.07±0.01) on SSC2. When both the additive and the imprinting effects are positive or negative, the paternal allele expresses (maternal imprinting); otherwise the maternal allele expresses (paternal imprinting).

Table 2: Evidence of QTL for interferon-gamma levels.

SSC[a]	Trait[b]	Pos[c]	F-value[d]	V(%)[e]	a ± SE[f]	d ± SE[g]	Closest Markers[h]
2	IFN2[#]	16	5.93*	26.99	429.06±176.32	-4314.5±1332.7	SW2623(12.9)-S0141(32.6)
4	IFN4-2	68	6.77*	22.39	166.17±67.1	300.08±112.21	S0214(66.3)
5	IFN2	2	5.87*	19.58	95.09±31.3	68.11±49.62	ACR(0.0)-SW413(2.6)
5	IFN4	51	6.59*	21.86	-126.57±45.01	139.17±65.96	SWR453(46.7)-SW2425(58.2)
5	IFN4-2	54	8.29**	26.71	-124.98±52.43	229.99±76.8	SWR453(46.7)-SW2425(58.2)
10	IFN6-4	21	6.25*	20.79	317.12±101.56	-375.88±330.1	SW830(0.0)-S0070(83.7)
11	IFN2	29	6.13*	20.41	-115.43±34.32	-94.55±44.44	S0071(28.8)
11	IFN4-2	23	6.7*	22.18	255.9±69.93	164.05±99.64	S0071(28.8)
11	IFN6-4	7	5.71*	19.08	-330.61±98.74	-240.03±137.5	SW2008(0.0)
16	IFN6	91	8.7**	27.80	43.63±39.17	335.75±80.6	S0026(70.7)-S0061(108.0)
17	IFN2	0	6.12*	20.37	24.98±49.00	348.14±114.5	SW335(0.0)
18	IFN6	48	5.6*	18.63	17.26±27.26	-137.01±41.3	SW787(43.2)-S0062(56.9)

[a, c, d, e f, g, h,] See footnotes for Table 1

[b] Trait abbreviations: IFN2: IFNg level at time point 2; IFN4: IFNg level at time point 4; IFN6: IFNg level at time point 6; IFN6-2: IFNg difference between time point 6 and 2; IFN6-4: IFNg difference between time point 6 and 4; IFN4-2: IFNg difference between time point 4 and 2.

[#] The imprinting effect and standard error was detected for IFN2 (3311.43±1081.97) on SSC2. When both the additive and the imprinting effects are positive or negative, the paternal allele expresses (maternal imprinting); otherwise the maternal allele expresses (paternal imprinting).

Table 3: Summary of significant QTL under a two-QTL model on SSC5 using QTL Express.

SSC[a]	Trait[b]	Position cM[c]		*F* –value[d]		V[e](%)	Effect A[g]		Effect B[g]		Sig[h]	
		QTL A	QTL B	2vs0	2vs1		A±SE	D±SE	A±SE	D±SE	2vs0	2vs1
5	IFN4-2	51	117	7.56	5.66	39.63	-129.11 ±50.60	206.00 ±70.40	113.55 ±63.02	-207.7 ±100.89	**	*

[a, c, d, e,] See footnotes for Table 1; [b] See footnotes for Table 2; [g]the QTL effect and the standard error (SE) of both QTL positions QTL A and QTL B; [h]significant threshold of the *F*-value (sign threshold) determines if the QTL reached the significance level under 2 vs 0 QTL (2 degrees of freedom), or 2 vs 1 QTL (1 degree of freedom); with *chromosome-wide $P < 0.05$; **chromosome-wide $P < 0.01$

Table 4: (Supplementary file 2) Analysis of variance of antibodies and IFNg response to vaccination at different time points (Proc GLM).

Traits	Time point	Mean±SE	Number	Mini-mum	Maxi-mum	R^2	Dam	Sire	Dam* Sire	Gender	Litter size	Parity	Birth month
Mh antibody	T1	0.2±0.02	142	0.01	1.93	0.19	*	***	ns	*	***	***	***
	T2	1.08±0.05	185	0.01	3.04	0.40	***	***	***	ns	***	*	**
	T3	1.5±0.07	113	0.01	3.25	0.41	***	***	**	ns	***	ns	ns
Tet antibody	T3	0.09±0.003	226	0.01	0.24	0.32	***	***	***	ns	***	***	***
	T4	0.10±0.002	249	0.01	0.22	0.19	***	***	ns	**	***	*	ns
	T5	0.11± 0.002	267	0.01	0.29	0.62	***	***	***	***	***	***	***
IFNg	T2	162.9±10.27	271	1.34	491.1	0.20	***	*	ns	ns	**	*	***
	T4	115.5±8.4	266	1.5	452	0.24	***	ns	**	ns	**	*	ns
	T6	148.7±7.6	243	1.7	467.3	0.32	***	***	***	*	**	*	ns

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

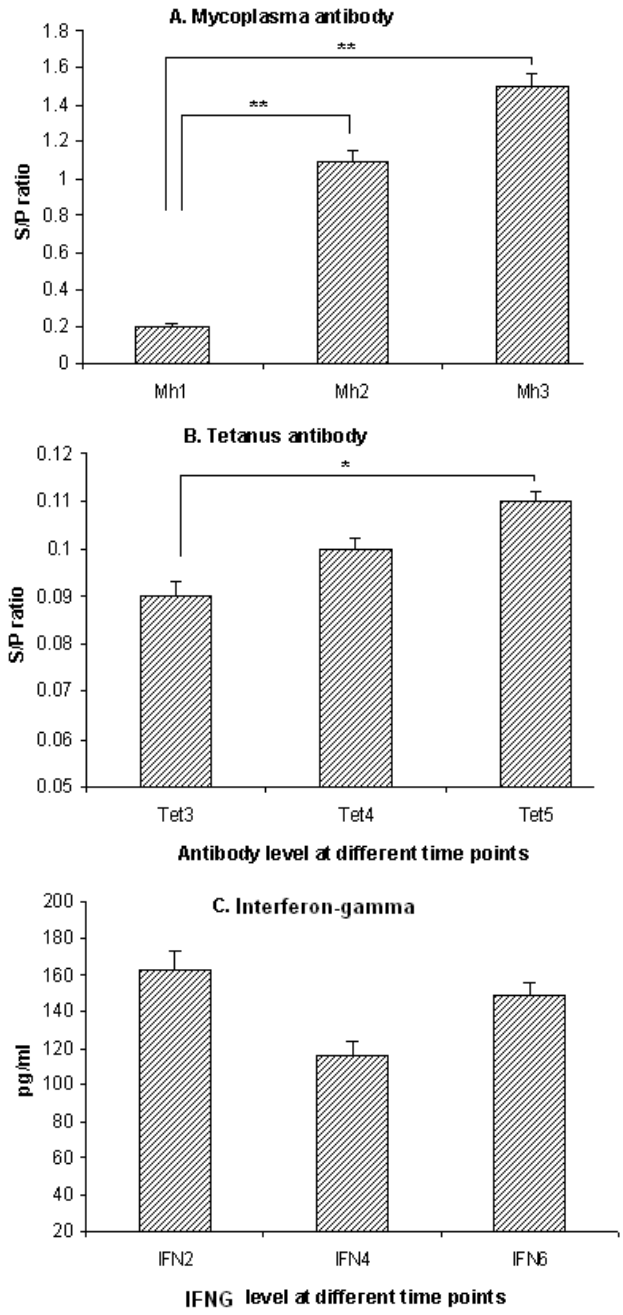

Figure 1: The concentration of antibodies and interferon-gamma at different ages. Mh1, Mh2 and Mh3 indicate mycoplasma antibody level at prior vaccination (at 6 weeks of age), 10 and 20 days afterwards, respectively; Tet3, Tet4 and Tet5 indicate tetanus antibody level at prior vaccination (at 9 weeks of age), 20 and 40 days afterwards, respectively; IFN2, IFN4 and IFN6 indicate interferon-gamma level at 52, 83 and 115 days of age, respectively. * $P < 0.05$; ** $P < 0.01$.

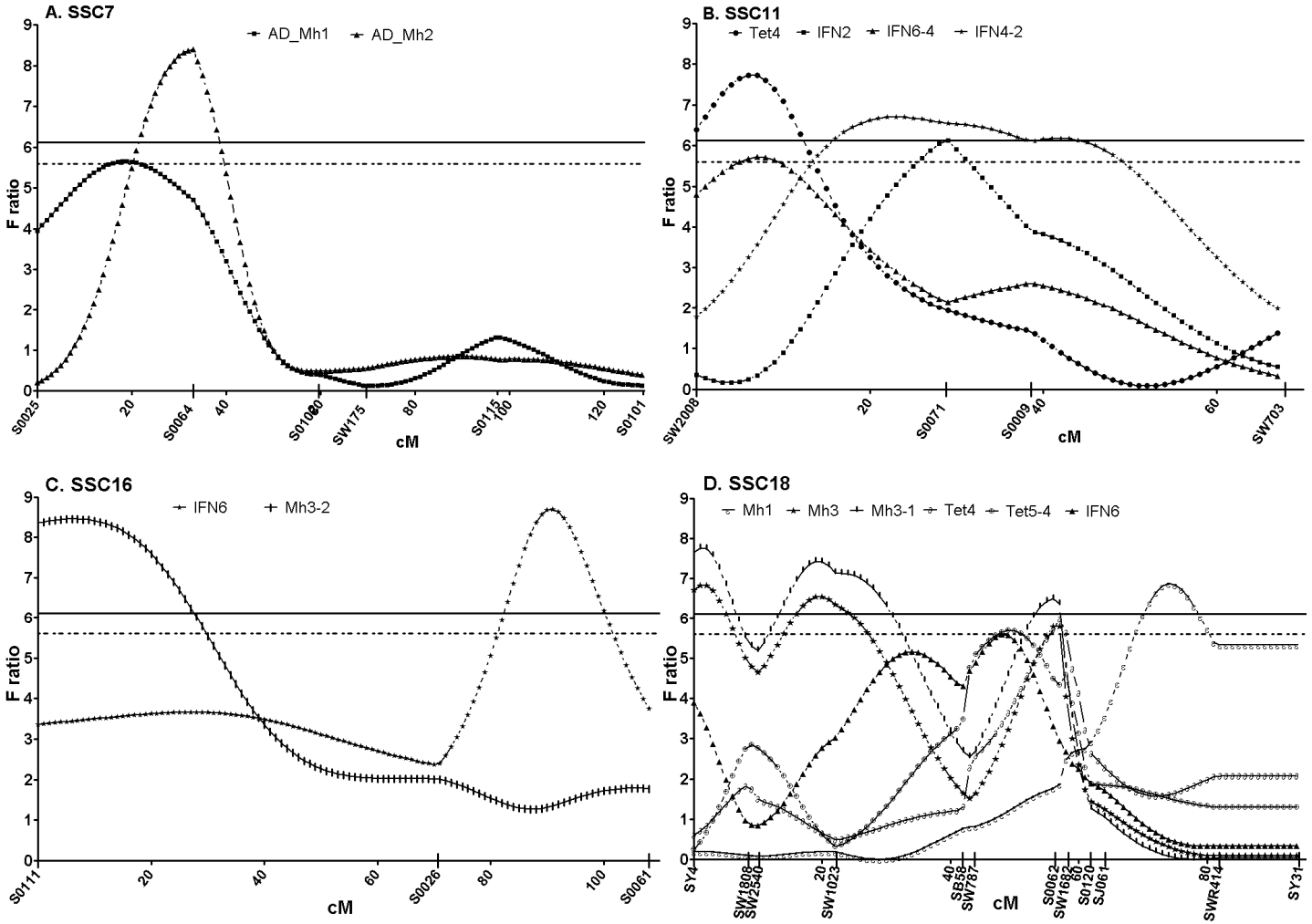

Figure 2: QTL results for immune traits on SSC7 (A), SSC11 (B), SSC16 (C) and SSC18 (D). Two threshold levels are shown: the dashed line is the suggestive (CW, 5%) and thick solid line is the chromosome-wide significance (CW, 1%). Genetic distances in Kosambi cM are given on the X-axis along with markers and their positions respectively, and *F*-values are at the Y-axis.

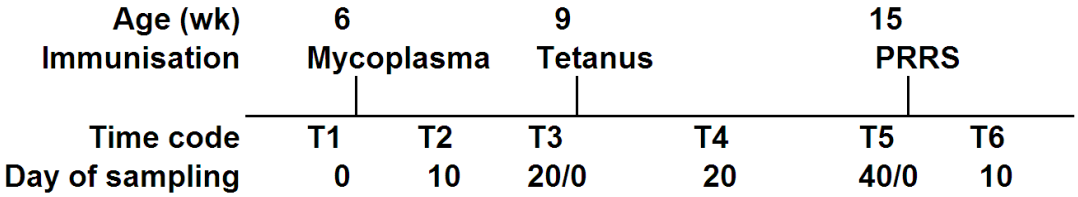

Figure 3: (Supplementary file 1) Schematic display of vaccination program and time point of blood sampling from F_2 DUPI population.

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Mapping quantitative trait loci for innate immune response in the pig

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Summary

The aim of the present study was to detect quantitative trait loci (QTL) for the serum levels of cytokines and Toll-like receptors as traits related to innate immunity in pig. For this purpose, serum concentration of interleukin 2 (IL2), interleukin 10 (IL10), interferon-gamma (IFNg), Toll-like receptor 2 (TLR2) and Toll-like receptor 9 (TLR9) were measured in blood samples obtained from F_2 piglets (n = 334) of a Duroc x Piétrain resource population (DUPI) after *Mycoplasma hyopneumoniae* (Mh), tetanus toxoid (TT) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccination at 6, 9 and 15 weeks of age. Animals were genotyped at 82 genetic markers covering all autosomes. QTL analysis was performed under the line cross F_2 model using QTL Express and 33 single QTL were detected on almost all porcine autosomes. Among the single QTL, eight, twelve and thirteen QTL were identified for innate immune traits in response to Mh, TT and PRRSV vaccine, respectively. Besides single QTL, six QTL were identified by a two-QTL model, of which two for TLR9_TT were in coupling phase and one for IL10_PRRSV was in repulsion phase. All QTL were significant at 5% chromosome-wide level including one and seven at 5% genome- and 1% chromosome-wide level significance. All innate immune traits are influenced by multiple chromosomal regions implying multiple gene action. Some of the identified QTL coincided with previously reported QTL for immune response and disease resistance, and the newly identified QTL are potentially involved in the immune function. The immune traits were also influenced by environmental factors like year of birth, age, parity and litter size. The results of this work shed new light on the genetic background of innate immune response and these findings will be helpful to identify candidate genes in these QTL regions related to immune competence and disease resistance in pigs.

Introduction

The current release of the Pig QTLdb (May 12, 2010) contains 5732 QTL representing 558 different traits (www.animalgenome.org/QTLdb/pig.html) mostly for economically important traits like growth, carcass and meat quality, and reproduction. Evidence of genetic variation in immune response has been found in livestock (Edfors-Lilja et al. 1994). Medium high (h^2 = 0.3 - 0.8) (Edfors-Lilja et al. 1998) to low (h^2 = 0.14 – 0.16) (Henryon et al. 2006) heritabilities have been estimated for several of the immune traits

in pig. A few reports are found regarding variation of humoral immune response (Edfors-Lilja et al. 1998, Wimmers et al. 2008, 2009, Uddin et al. 2010) but little is known about the genetics underlying innate immunity in pig. Innate immunity has considerable specificity and is capable of discriminating between pathogens and self (Takeda and Akira 2004). Activation of the innate immune response is the prerequisite for the triggering of humoral immunity. Pattern recognition receptors (PRRs) and cytokines are important components of the immune system; however, more than 26 serum proteins are described in pig (Miller et al. 2009) playing important roles in immune system. Cytokines as intercellular signalling molecules form complex networks to orchestrate and coordinate immune responses. *IFNg* and *IL10* have immunomodulatory functions, possess antiviral activity, protect swine from diseases and modulate the survival time of infected animals (Danilowicz et al. 2008, Scheerlinck and Yen 2005, Yao et al. 2008). Pig IL2 is reported to enhance immunity when used as vaccine adjuvant in mice (Xie et al. 2007). PRRs 'sense' conserved molecular patterns PAMPs (pathogen-associated molecular patterns), which are shared by large groups of microorganisms and lead to the immune response. Toll-like receptors (TLRs) function as PRRs for the recognition of microbial components and are considered as critical proteins linking innate and adaptive immunity (Takeda and Akira 2004). TLRs polymorphisms are reported to be associated with susceptibility to infectious diseases in man and pig (Shinkai et al. 2006). Both *TLR2* and *TLR9* are reported to contribute in porcine gut immunity (Uenishi and Shinkai 2009), to be involved in bacterial infections (Muneta et al. 2003) and are suggested to be used in vaccine design and disease-resistance breeding (Uenishi and Shinkai 2009). Moreover, levels of these immune parameters vary according to the individual's immune status which can be triggered by vaccine antigens. Therefore, determining the genetic basis of these immune parameters is of considerable interest, as this information could be used to select for animals with superior immune response.

A good understanding of the immune response is required to improve the health of pigs, which is an important issue in pig breeding. The identification of QTL for disease resistance in livestock is reported to be the next big frontier for the contribution of domestic animal genomics to the understanding of host-pathogen interaction and the subsequent improvement of both animal and human health (Womack 2005). Moreover, immune traits are suggested to be potentially useful as criteria to improve selection of

pigs for resistance to clinical and subclinical disease (Henryon et al. 2006). Therefore the aim of this study was to identify chromosomal regions associated with the variability of innate immune responses in response to vaccine antigens by QTL analyses as well as to detect the environmental and genetic factors affecting these immune traits. Three important vaccines (Mh, TT and PRRSV) were used to verify the innate immune responses. The immune traits were the serum levels of cytokines (*IFNg*, *IL2* and *IL10*) and TLRs (*TLR2* and *TLR9*) which are important innate immune proteins considered as the indicators of disease resistance (Danilowicz et al. 2008, Muneta et al. 2003, Scheerlinck and Yen 2005, Shinkai et al. 2006, Uenishi and Shinkai 2009, Xie et al. 2007, Yao et al. 2008). Cytokines and TLRs are found to influence each other and there are some QTL with pleiotropic effects detected since cytokines and TLRs interact in complex networks. Discovery of such QTL will contribute to the understanding of mechanisms influencing immune response, disease resistance and immune competence, which is the first step for marker assisted breeding efforts.

Methods

Animals

QTL analysis was performed using a resource population of Duroc / Piétrain cross described earlier (Liu et al., 2007, 2008) comprising three generations P, F_1 and F_2. A total of 334 F_2 pigs were used for phenotyping of immune traits. All pigs were kept at the Frankenforst experimental research farm at the University of Bonn (Germany). The F_2 pigs were given an *ad libitum* diet during the whole test period and were slaughtered at approximately 105 kg. The vaccination and sampling procedures were explained in details by Uddin et al. (2010). In brief, pigs were vaccinated with *Mycoplasma hyopneumoniae* (Mh), tetanus toxoid (TT) and porcine reproductive and respiratory syndrome virus (PRRSV) vaccines at 6, 9 and 15 weeks of age, respectively. Blood samples were collected at three different time points after each vaccination at 52 days, 83 days and 115 days of age with a period of 30 days interval. Serum was separated and kept in -80 ^{o}C until used.

Phenotypes

Serum IL2, IL10 and IFNg were measured by sandwich ELISA using swine IL2 CytoSet, IL10 CytoSet and INFγ CytoSet, (Invitrogen Corporation, CA, USA),

respectively, following manufacturer's protocol. In all cases, CytoSet Buffer Set was used as recommended. Absorbance was measured at 450 nm using a microplate reader (ThermoMax, Molecular Devices) within 30 minutes after adding stop solution and results were calculated as pg/ml using a 4-parameter curve fitted by the software SoftMaxPro (Molecular Device). TLR2 and TLR9 were determined by in-house developed indirect ELISA. Rabbit anti-porcine TLR2 and TLR9 antibodies (Cosmo Bio Co., Ltd., Japan) were used. In brief, anti-porcine TLR antibody was coated in high binding capacity 96-well microplate (Costar Corning) over night at 4^{o}C. Then, the antibody was washed with PBS containing 0.05% Tween 20 and incubated with blocking buffer for 2 h at 37^{o}C. After washing, serum was incubated for 1 h at 37^{o}C. Again after washing, the plates were incubated at the same temperature with rabbit anti-pig IgG conjugated with HRP (Sigma) for 30 minutes. Finally, chromagen (o-Phenylenediamine dihydrochloride) (SigmaFast® OPD tablet) was added and incubated for 30 minutes at room temperature before adding stop solution. The optical density (OD) was read at 490 nm and the result of TLRs were determined as S/P ratio (Wimmers et al. 2008). In all cases, two replications of each sample were used and the average value was considered as serum level of respective traits.

Statistical analysis

After each vaccination of the Mh, TT and PRRSV, all the five traits (IL2, IL10, IFNg, TLR2 and TLR9) were measured. Measurement of the each trait after each vaccination was considered as a trait. The data were analysed using the software package SAS (version 9.2) for a detailed description of the data structure. Generalized linear models (PROC GLM) were used to identify any possible effect of sire, dam, sex, birth weight, average daily weight gain, litter size, parity and month of birth on the blood level of immune traits. The phenotypic data followed approximately a normal distribution and were used for linkage analysis

Markers analysis

A linkage map with the total length of 2588.7 cM and an average marker interval of 31.57 cM was constructed. P, F_1 and F_2 animals of the DUPI population were genotyped with 82 genetic markers to cover all porcine autosomes for this study. The set of markers included 79 microsatellites and 3 biallelic markers. SNP tested include the *COL10A1* and *MMP3* (the sequences were obtained from GenBank accession no

AF222861 and FJ788664, respectively) and assays were designed to permit genotyping using a multiplex SNP genotyping platform (Beckman coulter).

The order of markers and the genetic distances between them are given in table 3. Most of the markers were selected from the USDA/MARC map (http://www.marc.usda.gov) and were available in the porcine genome build Sscrofa9 (Ensembl). Genotyping, electrophoresis, and allele determination were done using a LI-COR 4200 Automated Sequencer including the software OneDscan (Scanalytics). Allele and genotyping errors were checked using Pedcheck software (version 1.1) (O'Connell and Weeks 1998). The relative positions of the markers were assigned using the build, twopoint and fixed options of CRIMAP software (version 2.4) (Green *et al.* 1990). Recombination units were converted to map distances using the Kosambi mapping function. Marker information content and segregation distortion were tested following Knott et al. (1998).

QTL analysis using QTL Express

F_2 QTL interval mapping was performed using the web-based program QTL Express (Seaton et al. 2002) based on a least square method. The analysis was carried out at chromosome- and genome-wide level with a single and two-QTL model. The basic QTL regression model used in the present study was:

$y_i = \mu + F_i + \beta\ cov_i + c_{ai}a + c_{di}d + \varepsilon_i$

where: y_i = phenotype of the i^{th} offspring; μ = overall mean; F_i = fixed effect (parity, year of birth); β = regression coefficient on the covariate; cov_i = covariate (age at blood collection and litter size for IL2 and TLRs; age at blood collection for IL10 and IFNg); c_{ai} = additive coefficient of the i^{th} individual at a putative QTL in the genome; c_{di} = dominant coefficient of the i^{th} individual at a putative QTL in the genome; a = additive effects of a putative QTL; d = dominant effects of a putative QTL; and ε_i = residual error.

The regression model was fitted at 1-cM interval along each chromosome and the *F*-value for the QTL effect was calculated at each point. Chromosome- (CW) and genome-wide (GW) significance levels were determined by permutation tests (Churchill and Doerge 1994). Chromosome-wide 1% and 5% significance thresholds became genome-wide significance thresholds after Bonferroni correction for 18 autosomes of the haploid porcine genome (de Koning et al. 2001). Methods for mapping a single QTL can be biased by the presence of other QTL (Meuwissen and Goddard 2004; Raadsma

et al. 2009). To address this situation, two-QTL models were also fitted for all traits using QTL Express (Seaton et al. 2002). Multiple QTL were declared on a chromosome if they were separated by at least 30 cM and exceeded 5% CW level significance (Kim et al. 2005, Liu et al. 2008). The empirical 95% confidence intervals and flanking markers for the QTL positions were obtained by applying the bootstrapping approach with 1000 re-sampling steps (Visscher et al. 1996). The phenotype variation that was explained by a QTL was calculated by the following equation.

$$Var\% = \frac{MS_R - MS_F}{MS_R} \text{ x } 100$$

Where, MS_R was the mean of square of the reduced model; MS_F was the mean of square of the full model.

Results

Phenotypes distribution

The level of IFNg and IL10 increased over the time from 52 to 115 days of age (Fig. 1). The IL2 was reduced at 83 days of age after vaccination with TT. Both TLR2 and TLR9 exhibited the same pattern of serum level in response to different vaccines. Lowest values of TLRs were found at 83 days of age (Fig. 1) in response to TT vaccine. The variations for IL2 titres were found to be significant ($P < 0.001$) between Mh and TT vaccination. In case of IFNg titre, the differences between TT and PRRSV, and between Mh and PRRSV vaccination were significant ($P < 0.001$). At all time points, TLR9 responses were higher than that of TLR2. TLRs levels between Mh and TT, and between TT and PRRSV vaccination were significant ($P < 0.001$). Th1 type cytokines (IL2 and IFNg) were 5 - 6 times lower than that of Th2 type cytokine (IL10) in response to vaccines. Moreover, in response to Mh vaccine IFNg titre was15 times lower than that of IL10. Immune traits were found to be affected by parity, litter size, age and sex after different vaccinations (supplementary file 1).

QTL for cytokines and Toll-like receptors in response to vaccines

Under the line-cross model a total of 33 single QTL were detected, of which four QTL were for each of IL2, IFNg and TLR2; eight and 13 for IL10 and TLR9, respectively (Table 1). With regards to the immune triggering vaccine antigens, eight, 12 and 13

QTL were identified for the innate immune traits when vaccinated with the Mh, TT and PRRSV vaccine, respectively.

A total of 16 single QTL were detected for different cytokines in response to different vaccines, of which four QTL were identified for each of IFNg and IL2, and eight for IL10 (Table 1). Among the detected QTL for cytokines, two QTL for IL10 reached to the 1% chromosome-wide (CW) level significance and all remaining QTL were suggestive. Among the QTL identified for IL2, three suggestive ($P < 0.05$, CW) QTL were detected in response to the TT vaccine at 136 cM, 141 cM and 88 cM on SSC5, 6, and 7, respectively. A chromosomal region influencing IL2 following Mh vaccination was mapped at 14 cM on SSC11 (Fig. 2). These QTL explained between 2.7 % to 3.8 % of the phenotypic variation. Among the ten single QTL identified for IL10, five suggestive ($P < 0.05$, CW) QTL were detected in response to PRRSV vaccine on SSC2, 3, 6, 12, and 18. Two suggestive chromosomal regions influencing Mh vaccine induced IL10 were mapped on SSC10 and SSC12 (Fig. 2), whereas a significant ($P < 0.01$, CW) QTL was found on SSC11 at 25 cM affecting IL10 after TT vaccination. The phenotypic variation explained by the QTL identified for IL10 were between 2.3 % to 3.5 %. Four suggestive chromosomal regions were identified for IFNg, of which two QTL regions were affecting IFNg in response to TT vaccine at 81 cM and 35 cM on SSC2 and SSC11 (Fig. 2), respectively. The remaining two QTL for PRRSV vaccine induced IFNg was identified at 53 cM and 50 cM on SSC3 (Fig. 2) and SSC11, respectively. The positive additive genetic effects of the QTL for IFNg indicated the allele forcing higher traits values coming from the Duroc breed.

In this study, 17 line-cross single QTL were identified for TLRs (Table 1), of which four and 13 QTL were affecting TLR2 and TLR9, respectively. The four suggestive ($P < 0.05$, CW) QTL for TLR2 were identified on SSC3, 11, 12 and 15. Among these QTL, three QTL regions were affecting TLR2 induced by Mh vaccine and the remaining QTL was found to influence TT vaccine induced TLR2. Among the 13 single QTL influencing TLR9, a QTL affecting TT vaccine induced TLR9 reached to the 5% genome-wide (GW) level significance mapped at 34 cM on SSC2 (Fig. 2). Two significant QTL ($P < 0.01$, CW) were detected at 8 cM and 31 cM on SSC11 and SSC16, respectively affecting TLR9 induced by PRRSV vaccine. Moreover, three significant QTL ($P < 0.01$, CW) were detected for TLR9 following TT vaccine at 117 cM, 142 cM and 100 cM on SSC11, 14 and 18, respectively. The phenotypic variation

explained by the QTL for TLRs were between 2.2 % to 5.4 % in this study. Negative additive genetic effects indicated that the QTL alleles originated from Piétrain pig associated with higher TLR9 titres in response to different vaccines.

Two-QTL for innate immune response

Significant results for the two-QTL model are presented in Table 2. Evidence for an additional QTL under a two-QTL model was found in three cases, on SSC14 for TLR9_TT, and on SSC18 for TLR9_TT and IL10_PRRSV with a difference of 109 cM, 91 cM, and 94 cM between two loci, respectively. In all cases, a marker (*S0007* on SSC14 and *SW787* on SSC18) was located in between the two QTL regions and one of the two chromosomal regions (QTL B in all cases) was identified in the single QTL approach. Two loci on SSC18 (IL10_PRRSV) were in repulsion phase, whereas the two-QTL on SSC14 (TLR9_TT) and SSC18 (TLR9_TT) were in coupling phase. Two QTL affecting TLR9_TT at 9 cM and 100 cM on SSC18 jointly explained 6.7% of the phenotypic variation.

Discussion

Phenotype distribution

Age, gender and litter-related variation of cytokines (IL2, IFNg, IL4 and IL10) production are reported in pigs (de Groot et al. 2005). Henryon et al., (2006) described the effect of litter as the most important environmental source of variation affecting immunological traits in pigs. (O'Neill et al. 2006) stated that age, year of birth, sex and pre-existing antibodies are significant sources of variation for IgG responses. Both cytokines and TLRs production defects are reported in older human and mice in comparison to young human and mice (van Duin et al. 2007). IFNg concentration is reported to be affected by litter, parity, sex and age of pig (Uddin et al., 2010) which is in good agreement to our results. A recent study by (Fievet et al. 2009) demonstrated that age, but not parity, influences cytokines and TLRs production in human. There is no such report available in pig regarding TLRs. Since innate immune response is quick, it would be better to collect samples for the innate immune traits measurement within few hours or days after vaccination or immune stimulation. On the other hand, sampling immediately or shortly after vaccination could be an additional stress that can influence the innate immune responses. Moreover, several times samplings in a large population

shortly after vaccination are difficult and concern to animal welfare. However, most of the cytokines are pleiotropic and redundant in nature. The functions of individual cytokines may be additive, synergistic or antagonistic, synthesis or release of one cytokine may be controlled by others and cytokines may share receptors or parts of receptors. IL10 level was higher than that of IL2 and IFNg. Because, the production of Th1 type and Th2 type cytokines are inhibited by each other. *IFNg* secreted by Th1 cells, can inhibit the proliferation of Th2 cells. On the contrary, *IL10* secreted from Th2 cells, can suppress Th1 functions by inhibiting cytokine production (Scheerlinck and Yen 2005).

QTL for cytokines and Toll-like receptors

Antibody response and interleukin production are one of the first immune competence traits to be examined by QTL analysis (Edfors-Lilja et al. 1998), but a very limited number of QTL analyses have been devoted to health, disease resistance and immune response traits (Edfors-Lilja et al. 1998, Reiner et al. 2007, Wimmers et al. 2008, Wimmers et al. 2009). The causes for low number of QTL studies for immune related traits are pointed out by (Rothschild et al. 2007) that experiments for traits like disease resistance/susceptibility have limitations such as sample sizes, animal welfare and high expenses. However, breeds are reported to affect the baseline immune response and performance traits (Sutherland et al. 2005). Duroc and Piétrain are reported to be different regarding systemic disease resistance trait such as postweaning diarrhea (Vrtkova et al. 2007) as well as in regards to general immune responses like in response to sheep erythrocytes (Buschmann et al. 1974). In this study, equal number of additive and dominance QTL were identified which might be due to the separate loci too closely linked to be detected separately, one locus affecting male traits and the other affecting female traits (Rowe et al. 2008). Identification of QTL on the most chromosomes indicate that different chromosomal regions are influencing cytokines and TLRs reactions in response to diverge antigens.

Moreover, dominance effects are reported to be important source of variation for complex traits in commercial pigs (Rowe et al. 2008). At the recent past the NCBI released the draft assembly of the porcine genome Sscrofa5 (NCBI) and the ENSEMBL released Sscrofa9 (Ensembl). These databases help to search the immunologically important genes located in peck position of the identified QTL regions.

The *CD14* gene located on SSC2 close to the marker *SW834* (Ensembl), is involved in monocyte activation. A QTL for TT induced IFNg was identified in this study close to the marker *SW834* on SSC2. A QTL for IL10_PRRSV and a highly significant QTL ($P < 0.05$, GW) for TLR9_TT was identified at the proximal region on SSC2. Since cytokines and TLRs work together through complex networks (Takeda and Akira 2004), these QTL indicate pleiotropic effects. Importantly, flanking markers of these QTL incorporated the location of *C3* gene (Complement 3) on SSC2. Complement is an important part of innate immune system and is reported to be associated with TT and PRRSV antibodies in pig (Wimmers et al., 2009). Moreover, these QTL for TT and PRRSV induced immune traits are supported by previously detected line-cross QTL for PRRSV induced C3c in pig (Wimmers et al., 2009). Therefore it could be hypothesized that *CD14* and *C3* genes might play very important roles to stimulate the innate immune response of pig when challenged with vaccine antigens. Moreover, QTL for neutrophil and monocytes number are reported at 11 cM and 64 cM on SSC2 (Reiner et al. 2008). *IL10* is important for disease resistance in livestock secreted from lymphocytes and monocytes (Scheerlinck and Yen 2005). A QTL for IgG titre at 40 cM is also reported on SSC2 (Edfors-Lilja et al. 1998).

We identified QTL for PRRSV induced IL10 and IFNg closely linked on SSC3. This is a novel QTL and does not overlap with QTL reported previously. *IL10* has anti-inflammatory effects and down-regulates the expression of Th1 cytokines such as *IFNg* (Danilowicz et al. 2008). *IL1α* and *IL1β* are mapped on SSC3q11-14 which is within the flanking region of our QTL of interest. *IFNg* enhances *IL1* secretion by priming monocytes to be more sensitive to an *IL1*-inducing stimulus (Kruse et al. 2008). On the other hand, *IL10* reduces the production of *IL1α* and *IL1β*. *IFNg* and *IL2* are Th1 type cytokines, act synergistically and contribute to immune response. QTL for lymphocyte, platelet number, C3c (complement C) and Hp (haptoglobin) are mapped earlier on chromosome 3 in the region of our interest (Reiner et al. 2008, Reiner et al. 2007, Wimmers et al. 2008).

STAT2 is located on SSC5p11-15 (Ensembl), where we identified a QTL for TLR9_PRRSV close to the marker *S0092* (SSC5p11-12). This is a newly identified QTL for immune related traits. Recently it has been reported that *STAT2* is one of those genes whose expression is regulated by *TLR9* (Klaschik et al. 2009) indicates that *STAT2* might be a gene of interest for PRRSV induced innate immune response in pigs.

Moreover, the flanking marker of the identified QTL for IL2_TT on SSC5 did incorporate the position of the porcine *IFNg* gene, which is an important cytokine responsible for disease resistance in pigs (Scheerlinck and Yen 2005, Yao et al. 2008). *TGFβ1* (transforming growth factor beta 1) and *IRF3* (interferon regulatory factor 3) are mapped on SSC6q12-21 (Ensembl) and this region is found to influence IL10 when vaccinated with PRRSV. IL10 and TGFβ are immunomodulatory cytokines (Sipos et al. 2010) that are constitutively expressed. Knockdown of *IRF3* by siRNA resulted in downregulation of *IL10* (Samanta et al. 2008). Moreover, QTL for leukocyte proliferation at 78 cM and for IL2 activity at 133 cM and 127 cM on SSC6 are reported (Edfors-Lilja et al. 2000) in pig which is in good agreement with our result since we detected QTL for TT stimulated IL2 in this region. *IL2* activates eosinophil and QTL for eosinophil number is also reported earlier at 147 cM on SSC6 (Edfors-Lilja et al. 1998). Cytokines and TLRs mapped on SSC8 are *IL21*, *IL8*, *IL2*, *TLR1*, *TLR2*, *TLR6* (Muneta et al. 2003) and *TLR10* (Ensembl). The QTL region for TLR9_TT between marker *S0086* and *S0144* incorporated the location of *TLR2* and *IL2*. *TLR9* and *TLR2* signaling together account for *MyD88*-dependent control of parasitemia (Bafica et al. 2006). QTL for basophil, monocytes, leukocyte numbers (Reiner et al. 2008), polymorphonuclear leukocytes (PMNL) and segmented neutrophil numbers (Edfors-Lilja et al. 1998) are reported previously on SSC8 close to our detected QTL region.

The pleiotropic QTL mapped within 25 cM to 50 cM on SSC11 for IFNg_TT, IFNg_PRRSV, IL10_TT, TLR2_Mh, TLR9_TT are supported by the previously reported QTL for IFNg at 7 cM, 23 cM and 23 cM on SSC11 in response to different vaccines in pig (Uddin et al. 2010). QTL for immunologically important blood cells such as for lymphocyte, monocytes, neutrophil and eosinophil numbers are also reported at 19 cM, 11 cM, 23 cM and 69 cM, respectively on SSC11 (Reiner et al. 2008, Reiner et al. 2007). QTL for monocyte number is reported at the region on SSC12 (Reiner et al. 2008) where QTL for Mh induced TLR9 is identified in this study. Novel QTL for IL10_Mh, TLR2_Mh and TLR9_PRRSV are identified within region very close to the marker *SW605* on SSC12q11-15 where the *MPO* (myeloperoxidase) gene is located. *MPO* is reported to be important for host defense and plays a role in the activation of neutrophils during extravasation (Haegens et al. 2009). *MPO* knockout mice are reported to be unresponsive to CpG DNA stimulation ligand for *TLR9* and *MPO* activity is diminished in cells from $TLR2^{-/-}$ mice (Tessarolli et al. 2009).

Therefore, it could be hypothesized that *MPO* might be an important candidate gene for the innate immune response when triggered with Mh and PRRSV vaccine. However, large confidence regions in this experiment is a common problem in QTL study, which hampered interpretation of QTL results since this region could contain many of potential candidate genes (de Koning et al. 2005).

TLR9 following stimulation with TT vaccine is found to be affected by a chromosomal region very close to the marker *SWC27* on SSC14 where *MBL2* (Mannose binding lectin 2; also called Mannan-binding lectin) is located . *MBL2* is a PRR, cooperates with TLRs within the phagosome to facilate the engulfment of bacteria and amplifies the host response (Ip et al. 2008). Interestingly, *MBL* deficient children are reported to have a lower development of tetanus antibodies (Cedzynski et al. 2004) indicating that *MBL* could be a gene of interest for innate immune response after vaccination with TT antigen. *NRAMP1* (Natural resistance associated macrophage protein 1) is a potential candidate gene in controlling pig's resistance to salmonella infection (Sun et al. 1998). *NRAMP1* is mapped on SSC15q close to the marker *SW1119* (Ensembl) and the location of this gene is incorporated within the flanking markers of the QTL detected for TLR2_TT indicated that *NRAMP1* might be an important candidate gene for TT vaccine induced immune response in pig.

The *C9* (Complement 9) gene is the central component of the complement system located on SSC16 (Ensembl) and a chromosomal region affecting TLR9_PRRSV is detected close to the location of this gene. Many TLR ligands such as LPS, zymosan are also reported to be the activators of complement (Takeda and Akira 2004) and complements are found to regulate TLRs signalling specially in *TLR4* and *TLR9* (Zhang et al. 2007). Immunologically important QTL such as QTL for sarcocystis bradizoit at 15 cM (Reiner et al. 2007) and tetanus antibody at 30 cM (Wimmers et al. 2008) are reported on SSC16. Moreover, complement is an important part of innate immune system and the role of *TLR9* in protozoan infections are reviewed in detail (Gazzinelli and Denkers 2006).

The proximal region of SSC17 was found to affect TLR9_PRRSV, close to the reported QTL for C3c, Hp and TT antibody in pig (Wimmers et al. 2008). The flanking marker of this QTL did incorporate the position of *LBP* (lipopolysaccharide-binding protein), *BPI* (bactericidal/permeability-increasing protein) and *PTLP* (phospholipid transfer protein) genes. These genes are reported to have antimicrobial and bactericidal effects

especially on Gram- bacteria (Shi and Tuggle 2001a, Shi and Tuggle 2001b). Likewise, a chromosomal region for TLR9_TT and IL10_PRRSV is mapped at a region on SSC18, close to the QTL reported earlier for *C3c* and *Hp* (Wimmers et al. 2008) in pigs. Importantly, Wimmers et al. (2009) found the close relation of C3c and Hp with TT and PRRSV antibodies.

In conclusion, it could be found that with regard to number and magnitude of their impact, QTL for innate immune traits behave like those for other quantitative traits. Both genetic and environmental factors contributed to the innate immune response. *MPO*, *MBL2* and *NRAMP1, C3c, C9, LBP, PLTP* and *BPI* might be interesting candidate genes contributing to immune function. Anyhow, it is still a major task to identify causative genes and polymorphism. Moreover, in the complex network of cytokines and TLRs it is difficult to unambiguously assign one or more biological role and response of each immune trait. Furthermore, follow-up research is needed to further characterize these quantitative trait loci in animal population challenged with infection and in other crosses. To identify candidate gene by fine mapping using denser marker sets like large scale SNPs and association of genes and causal mutations could be the further steps to define the genetic basis of immune function. However, this discovery of the QTL regions will facilitate to understand the genetic basis underlying innate immune traits and to identify the candidate genes for immune competence and disease resistance.

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Table 1: Summary of QTL for the innate immune traits using QTL Express.

	Trait[a]	SSC[b]	POS[c] (cM)	*F*-value[d]	Var[e] (%)	A[f]±SE	D[g]±SE	Marker interval
IL2	IL2_TT	5	136	7.1*	3.8	-19.74±5.27	-6.18±8.88	SW1987-IGF1
	IL2_TT	6	141	5.3*	2.7	-11.70±4.65	-16.61±8.09	S0059 -S0003
	IL2_TT	7	88	5.2*	2.7	-11.75±5.19	-22.08±8.18	SW175-S0115
	IL2_Mh	11	14	6.6*	3.5	-28.10±13.82	-100.19±32.28	SW2008-S0071
IL10	IL10_PRRSV	2	22	4.7*	2.3	112.11±37.95	100.34±129.43	SW2443-SW240
	IL10_PRRSV	3	45	5.2*	2.6	42.92±28.92	155.15±49.99	S0164 -SW2570
	IL10_PRRSV	6	78	5.1*	2.5	-67.67±21.16	-15.77±36.98	SW1067-SW193
	IL10_Mh	10	58	4.8*	2.3	-75.50±32.81	116.21±55.55	S0070-S0070
	IL10_TT	11	25	6.5**	3.5	56.24±28.30	181.72±59.92	SW2008 -S0071
	IL10_PRRSV	12	13	5.5*	2.8	-29.08±29.33	-196.69±61.51	SW2490-SW874
	IL10_Mh	12	162	6.5**	3.4	-63.66±48.87	-537.16±158.1	SW605-SW605
	IL10_PRRSV	18	120	5.7*	2.9	31.20±22.17	105.51±32.50	SW787-SWR414
IFNg	IFNg_TT	2	81	5.0*	2.6	-27.06±9.80	20.47±15.94	SW240-SW834
	IFNg_PRRSV	3	53	5.9*	3.0	6.16±12.94	74.58±21.80	S0164-SW2570
	IFNg_TT	11	35	5.7*	3.0	25.44±8.93	28.43±15.80	SW2008-S0071
	IFNg_PRRSV	11	50	5.8*	2.9	23.32±11.05	46.99±18.27	S0071 -S0009
TLR2	TLR2_Mh	3	92	5.8*	3.0	-0.08±0.04	-0.25±0.08	SW2570-S0002
	TLR2_Mh	11	38	6.2*	3.2	0.02±0.03	0.20±0.06	SW2008-S0071
	TLR2_Mh	12	150	5.9*	3.1	-0.14±0.08	0.94±0.32	SW874-SW605
	TLR2_TT	15	126	5.9*	3.1	-0.07±0.04	-0.16±0.06	SW936-SW1119
TLR9	TLR9_TT	2	34	9.6***	5.4	-0.13±0.08	-1.04±0.25	SW2443-SW240
	TLR9_PRRSV	5	76	6.3*	3.1	-0.10±0.07	-0.36±0.11	SWR453-S0092
	TLR9_TT	8	117	5.5*	2.9	0.01±0.06	0.38±0.11	S0086-S0144
	TLR9_PRRSV	11	8	7.3**	3.7	-0.25±0.08	-0.40±0.18	SW2008-S0071
	TLR9_TT	11	28	6.4**	3.5	0.09±0.06	0.43±0.13	SW2008 -S0071
	TLR9_Mh	11	95	5.5*	2.8	-0.39±0.12	0.17±0.18	S0009-SW703
	TLR9_Mh	12	43	5.4*	2.7	-0.53±0.20	-1.17±0.55	SW2490 -SW874
	TLR9_PRRSV	12	175	7.2*	3.7	-0.09±0.10	-0.90±0.25	SW874-SW605
	TLR9_TT	14	142	7.0**	3.8	0.33±0.14	5.14±1.48	S0007-SWC27
	TLR9_PRRSV	16	31	6.9**	3.5	-0.11±0.10	-1.06±0.30	S0111-S0026
	TLR9_PRRSV	17	0	4.8*	2.2	-0.08±0.05	0.22±0.08	SW335-SW840
	TLR9_PRRSV	18	0	5.0*	2.4	0.24±0.08	-0.14±0.15	SW1808-SW1023
	TLR9_TT	18	100	7.9**	4.4	-0.08±0.07	-0.50±0.13	SW787 -SWR414

[a] Trait abbreviations: IFNg_Mh, IFNg_TT and IFNg_PRRSV= IFNg level after vaccination with Mh, TT and PRRSV, respectively; IL2_Mh, IL2_TT and IL2_PRRSV= IL2 level after vaccination with Mh, TT and PRRSV, respectively; IL10_Mh, IL10_TT and IL10_PRRSV= IL10 level after vaccination with Mh, TT and PRRSV, respectively; TLR2_Mh, TLR2_TT and TLR2_PRRSV= TLR2 level after vaccination with Mh, TT and PRRSV, respectively; TLR9_Mh, TLR9_TT and TLR9_PRRSV= TLR9 level after vaccination with Mh, TT and PRRSV, respectively; [b]

Sus scrofa chromosome; [c] Chromosomal position in Kosambi; [d] Significance of the QTL: *, significant on a chromosome-wide level with $P \leq 0.05$; **, significant on a chromosome-wide level with $P \leq 0.01$; ***, significant on a genome-wide level with $P \leq 0.05$; [e] The percentage of phenotypic variance explained by the QTL; [f]Additive effect and standard error. Positive values indicate the Duroc alleles result in higher values than Pietrain alleles; negative values indicate that Duroc alleles result in lower values than Pietrain alleles; [g] Dominance effect and standard error.

Table 2: Summary of significant QTL for innate immune traits using QTL Express under two-QTL model.

SSC[a]	Trait[b]	Pos cM[c]		*F*[d]		Var%[e]	Effect A[f]		Effect B[f]		Sig[g]	
		QTL A	QTL B	2vs1	2vs0		a+se	d+se	a+se	d+se	A	B
14	TLR9_TT	35	142	5.9	4.6	6.1	0.1± 0.1	-0.3± 0.1	0.3± 0.1	5.5± 1.5	*	*
18	TLR9_TT	9	100	6.4	4.7	6.7	-0.1± 0.1	-0.4± 0.2	-0.1± 0.1	-0.6± 0.1	*	*
18	IL10_PRRSV	20	114	5.2	4.6	5.1	-90.1± 39.1	304.1± 108.9	42.8± 24.8	134.9± 41.7	*	*

[a, b, c, d, e] See footnotes for Table 1; [f] the QTL effect and the standard error (SE) of both QTL positions QTL A and QTL B; [g] significant threshold of the *F*-value (significant threshold) determines if the QTL reached the significance level under 2 vs 0 QTL (2 degrees of freedom), or 2 vs 1 QTL (1 degree of freedom); with *chromosome-wide $P \leq 0.05$.

Table 3: Markers used in the QTL analysis and genetic map as established from the DUPI resource (sex average, Kosambi).

Chrom.	Markers and genetic distances[a] (cM)								
SSC1	SW1515 (16.4)	34.8	SW1581	71.7	COL10A1	59.9	S0155	55.4	SW1301 (140.5)
SSC2	SW2443 (0)	58.2	SW240	28.9	SW834	8.3	SW1517	8.3	S0226 (74.8)[b]
SSC3	SW72 (17.8)	33.5	S0164	26	SW2570	36.6	S0002(102.2)		
SSC4	S0227 (4.1)	50	S0001	31	S0214	49.3	S0097(120.0)		
SSC5	ACR (0)	10.9	SW413	31.9	SW1482	20.7	SWR453	14.2	
	S0092	16.9	S0005	41.5	SW1987	24.4	IGF1	48.7	SW967 (145.9)
SSC6	S0035 (7.3)[c]	61.2	S0087	13	SW1067	12.7	SW193	12.5	
	S0300	14.5	S0220	19.4	S0059	16.9	S0003 (102)[c]		
SSC7	S0025 (3.7)	33	S0064	36.6	S0102	16.9	SW175	31.5	
	S0115	38.9	S0101(134.9)						
SSC8	SW2611(2.5)	89.6	S0086	27.6	S0144	12.8	SW61 (112.3)		
SSC9	SW21 (11.1)	52.9	MMP3	50	SW911	23.4	SW54	15.1	
	S0109	25.6	S0295(96.5)						
SSC10	SW830 (0)	70.5	S0070	28.1	SWR67(122)				
SSC11	SW2008 (14.1)	43.3	S0071	24.6	S0009	27.2	SW703 (76.2)		
SSC12	SW2490 (0)	75.5	SW874	100	SW605(108.3)				
SSC13	S0219 (1.6)	44	SW344	37.4	SW398	87	S0289 (112.1)		
SSC14	SW857 (7.4)	42.5	S0007	100	SWC27(111.5)				
SSC15	S0355 (1.3)	37.8	SW1111	47.8	SW936	40.8	SW1119 (107.4)		
SSC16	S0111 (0)	67.2	S0026	89.1	S0061(92.6)				
SSC17	SW335 (0)	40.4	SW840	99.6	SW2431 (94.0)				
SSC18	SW1808(0)	9.5	SW1023	70.9	SW787	40.3	SWR414 (57.6)		

[a] Numbers in the parentheses at the first and last marker are relative positions of those in the USDA-MARC v2 linkage map; [b] S0226 not covered by USDA-MARC v2, but SW14, which is closely linked to S0226 (PigMap v1.5); [c] S0035 at 0 and S0003 at 144.5 in the International Workshop 1 SSC6 integrated map with a total length of 166.0 (Wimmers et al. 2009).

Table 4: (Additional file 1) Phenotypic innate immune traits and effect of different environmental and genetic factors on these traits. Summary of the phenotypic innate immune traits measured after different vaccinations and effect of different environmental and genetic factors on these innate immune traits (proc means, proc univariate and proc glm) by SAS (v9.2).

Traits	Mean±SE	N	Sex	Age at sampling	Date of birth	Year of birth	Parity	Litter size	R^2	Model
IL2_Mh	60.53±4.86	313	ns	ns	ns	***	**	ns	0.11	***
IL2_TT	41.56±2.16	312	ns	***	***	ns	ns	***	0.19	***
IL2_PRRSV	59.94±3.55	328	*	**	ns	ns	ns	***	0.10	***
IL10_Mh	218.03±11.45	322	ns	ns	ns	***	***	ns	0.13	***
IL10_TT	258.19±10.93	312	**	**	ns	ns	ns	ns	0.10	***
IL10_PRRSV	295.99±11.75	328	ns	ns	***	***	***	ns	0.37	***
IFNg_Mh	14.35±1.92	321	ns	ns	ns	***	ns	ns	0.14	***
IFNg_TT	40.15±4.09	308	***	ns	ns	***	ns	ns	0.22	***
IFNg_PRRSV	74.82±5.5	329	ns	ns	ns	***	**	ns	0.13	***
TLR2_Mh	1.04±0.02	323	ns	ns	***	***	**	ns	0.39	***
TLR2_TT	0.95±0.02	312	*	*	**	***	**	ns	0.31	***
TLR2_PRRSV	1.02±0.03	334	*	ns	***	***	*	ns	0.33	***
TLR9_Mh	1.25±0.06	319	ns	ns	*	***	*	ns	0.28	***
TLR9_TT	1.06±0.03	308	ns	ns	**	***	***	ns	0.33	***
TLR9_PRRSV	1.19±0.03	334	ns	*	***	***	***	ns	0.48	***

N = number of animals; ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

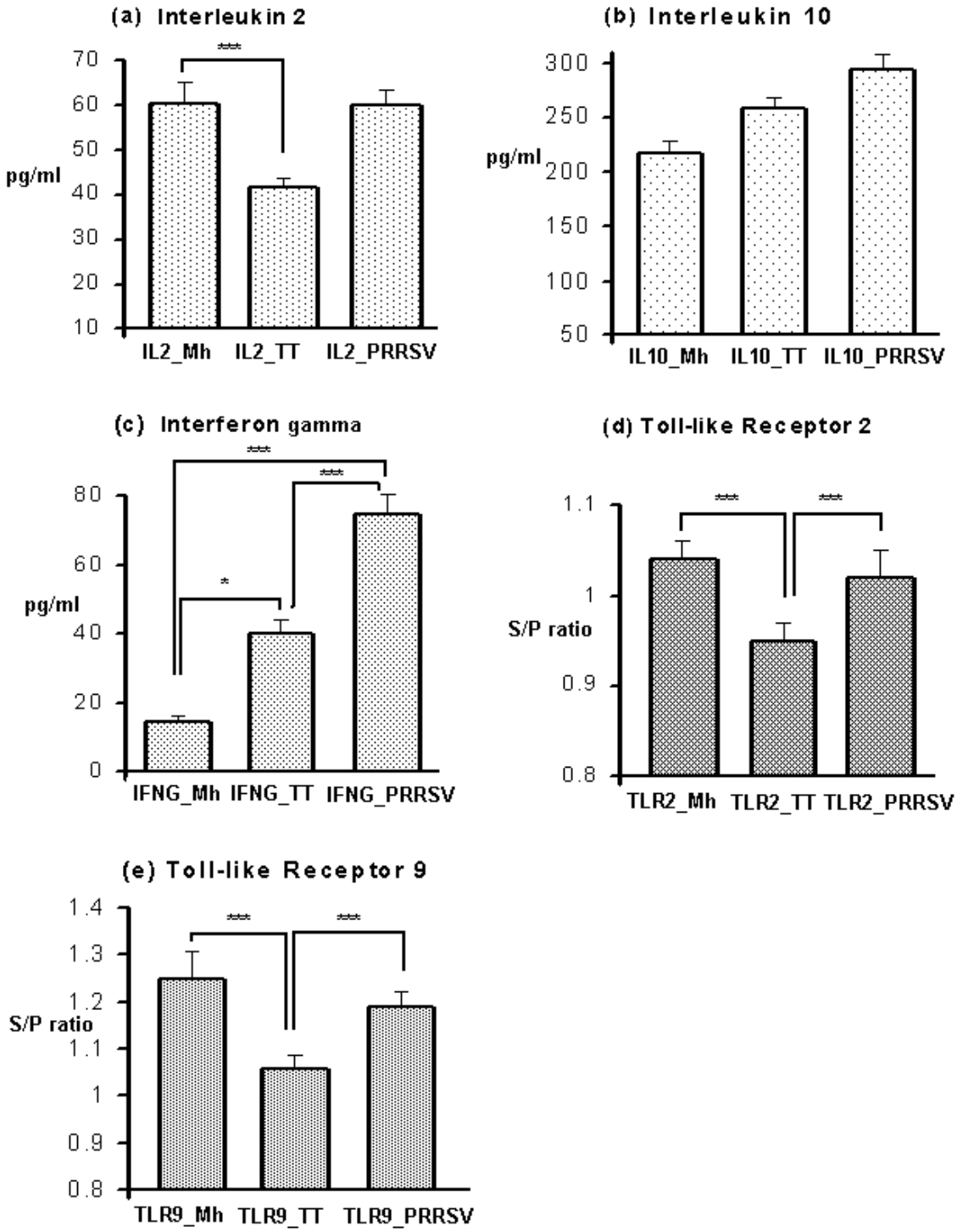

Figure 1. The concentration of cytokines and Toll-like receptors after vaccination with Mh, TT and PRRSV.

* $P \leq 0.05$; *** $P \leq 0.001$

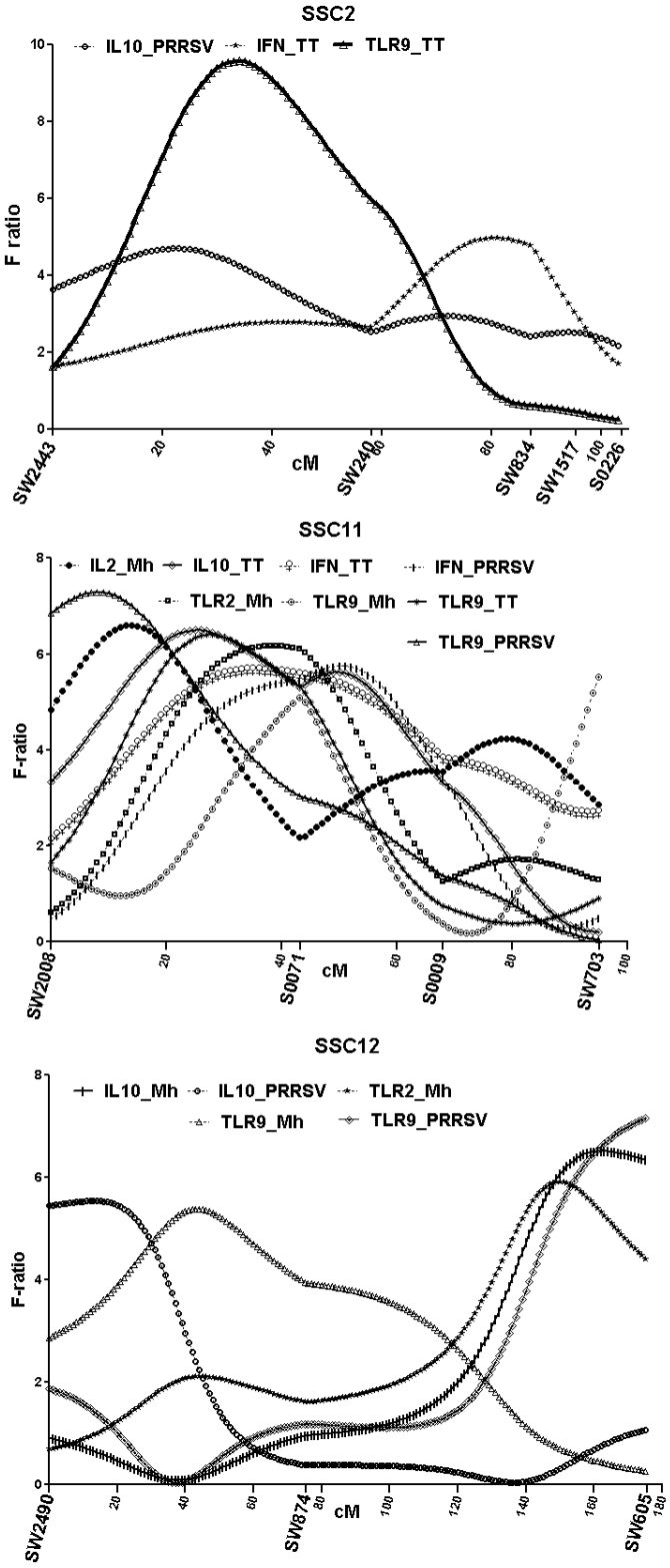

Figure 2: F-ratio test statistics for innate immune traits on SSC2, SSC11 and SSC12. The quantitative trait loci for traits related to innate immune response with chromosome-wide significance at $P < 0.05$ (curve in dotted; suggestive) and $P < 0.01$ (curve in solid; significant); genome-wide level significance at $P < 0.05$ (curve in solid bold; highly significant) on SSC2, SSC11 and SSC12 estimated from data of the DUPI F2 resource population. Positions of the markers are indicated at the x-axis, F-values are at the y-axis.

4. Chapter 3 (Published in: *BMC Research Notes* 2011, 44(1): 441)

Age-related changes in relative expression stability of commonly used housekeeping genes in selected porcine tissues

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Short title: suitable set of housekeeping genes for pigs.

Keywords: candidate reference genes, age, tissues, pigs

Abstract

Background: Gene expression analysis using real-time RT-PCR (qRT-PCR) is increasingly important in biological research due the high-throughput and accuracy of qRT-PCR. For accurate and reliable gene expression analysis, normalization of gene expression data against housekeeping genes or internal control genes is required. The stability of reference genes has a tremendous effect on the results of relative quantification of gene expression by qRT-PCR. The expression stability of reference genes could vary according to tissues, age of individuals and experimental conditions. In the pig however, very little information is available on the expression stability of reference genes. The aim of this research was therefore to develop a new set of reference genes which can be used for normalization of mRNA expression data of genes expressed in varieties of porcine tissues at different ages.

Results: The mRNA expression stability of nine commonly used reference genes (*B2M*, *BLM*, *GAPDH*, *HPRT1*, *PPIA*, *RPL4*, *SDHA*, *TBP* and *YWHAZ*) was determined in varieties of tissues collected from newborn, young and adult pigs. geNorm, NormFinder and BestKeeper software were used to rank the genes according to their stability. geNorm software revealed that *RPL4*, *PPIA* and *YWHAZ* showed high stability in newborn and adult pigs, while *B2M*, *YWHAZ* and *SDHA* showed high stability in young pigs. In all cases, *GAPDH* showed the least stability in geNorm. NormFinder revealed that *TBP* was the most stable gene in newborn and young pigs, while *PPIA* was most stable in adult pigs. Moreover, geNorm software suggested that the geometric mean of three most stable genes would be the suitable combination for accurate normalization of gene expression study.

Conclusions: Although, there was discrepancy in the ranking order of reference genes obtained by different analysing software methods, the geometric mean of the *RPL4*, *PPIA* and *YWHAZ* seem to be the most appropriate combination of housekeeping genes for accurate normalization of gene expression data in different porcine tissues at different ages.

Background

The pig is one of the most studied organism in research community as a food as well as a model animal, and many projects in pigs require the quantification of the genes for many purposes. Real-time quantitative PCR (qRT-PCR) is the most frequently used methods for gene quantification nowadays. qRT-PCR is an efficient method for

quantification of mRNA transcript levels due to its high sensitivity, reproducibility and large dynamic range. Furthermore, it is fast, easy to use and provides simultaneous measurement of gene expression in many different samples for a limited number of genes (Arya et al. 2005, Nolan et al. 2006, Nygard et al. 2007). In case of qRT-PCR, when analyzing data for relative quantification, results are normalized to a reference. The most accepted approach to mRNA quantification is normalization of the expression level of a gene of interest (target gene) to the expression level of an internal stably expressed gene (control gene) (Huggett et al. 2005, Radonic et al. 2004, Vandesompele et al. 2002). The control gene, often termed reference gene, is a stably expressed gene that is experimentally verified in given species and tissues under given experimental conditions (Erkens et al. 2006, Lovdal and Lillo 2009, Maroufi et al. 2010, Nygard et al. 2007). Normalizing to a reference gene is a widely used method because it is simple in theory. The normalization adjusts for differences in the quality or quantity of template RNA or starting material and differences in RNA preparation and cDNA synthesis, since the reference gene is exposed to the same preparation steps as the gene of interest. This allows the direct comparison of normalized transcript expression levels between samples. However, this approach requires the selection of at least one reference gene for validation of a corresponding qRT-PCR method. Normalization is extremely important to allow accurate comparison of the results between different samples and conditions in gene expression studies (Huggett et al. 2005). For instance, the commonly used reference genes such as *GAPDH* and β-actin are unfortunately often used without prior validation of their expression stability under the specific study conditions, but a number of studies have shown that the expression of those genes is significantly altered in some experimental conditions (Barber et al. 2005; Jung et al. 2007; Selvey et al. 2001). It is therefore necessary to validate the expression stability of reference genes prior to their use in an experimental protocol. Recently it has been recommended that a combination of reference genes should be used to obtain a more stable reference (Vandesompele et al. 2002) and the use of a single reference gene is nowadays discouraged by more and more authors (Huggett et al. 2005, Tricarico et al. 2002, Vandesompele et al. 2002). Because, a variability or alteration in the chosen reference gene by the experiment, however, may change the obtained results entirely and could be incorrect. Therefore, the validation of potential reference genes is essential.

An ideal reference gene should be stably expressed and unaffected by experimental protocol or status (Schmittgen and Zakrajsek 2000). But, the recent studies showed that

the housekeeping gene expressions could be changed according to the type of tissues (Maroufi et al. 2010, Nygard et al. 2007, Pierzchala et al. 2011) breeds (Pierzchala et al. 2011), experimental condition (such as treatment or disease) (Beekman et al. 2011, De Boever et al. 2008, Maccoux et al. 2007, Penning et al. 2007) and age (Al-Bader and Al-Sarraf 2005, Pierzchala et al. 2011, Touchberry et al. 2006). A set of reference genes are suggested on the basis of their stability over tissues in pigs (Erkens et al. 2006, Gu et al. 2011, Nygard et al. 2007, Pierzchala et al. 2011, Piorkowska et al. 2010) but studies for expression stability of commonly used housekeeping in varieties of porcine tissue collected from different age of pigs are scare. Therefore, this study was aimed to explore the expressions of nine mostly used house keeping genes in 14 different tissues collected from three different ages of pigs (1 day old piglet, 2 months old young and 5 moths old adult pigs) and to select the suitable set of house keeping genes that could be used as an internal control to normalize gene expression in pigs.

Materials and Methods

Tissues collection

A total of nine clinically healthy pigs of three age group were selected: neonatal (one day old), young (2 months old) and adult (5 months old) for this experiment. Each age group was consisted of three animals of Pietrain, and all the animals were male and from the same batch. All pigs were kept at the Frankenforst experimental research farm at the University of Bonn (Germany). The animals were reared and slaughtered according to the rules of German performance stations (ZDS 2003). The animals were fed same diet *ad libitum* during the whole experimental period. Blood was collected for peripheral blood mononuclear cells (PBMC) isolation. Lymph nodes (cervical and mesenteric), intestinal mucosa from duodenum, jejunum and ileum, tissues from stomach, liver, spleen, thymus, lung, kidney, heart and skin from ear were collected for mRNA isolation after slaughter. For mRNA isolation from tissues, samples were directly put into liquid nitrogen after washing in PBS. PBMC was isolated from whole blood using Ficoll-Histopaque (Sigma) following manufacturer's protocol. All samples were kept in -80^{o}C till used.

RNA isolation and cDNA synthesis

Total RNA was isolated from individual sample by using Tri-Reagent (Sigma-Aldrich, Munich, Germany) according to the standard protocol. In brief, sample was first grinded in a mortar, then mixed and homogenized with 1 ml Tri-Reagent using electric homogenizer. To ensure complete dissociation of nucleoprotein complexes, the sample was allowed to stand for 5 min before adding 0.2 ml of chloroform. The mixture was shaken and left at room temperature for 10 min and centrifuged at 12,000 x g for 15 min at 4^{o}C. The upper aqueous phase was transferred to another fresh centrifuge tube and RNA was precipitated with 0.5 ml of isopropanol. After being incubated at room temperature for 10 min, the sample was centrifuged at 12,000 x g for 10 min at 4^{o}C to get the RNA pellet, which was subsequently washed by 75% (v/v) ethanol. Centrifugation was then performed and the RNA pellet was air-dried and resuspended in 25 µl of DEPC treated water. RNA was isolated from PBMC using Picopure RNA isolation kit (Cat.# KIT0202; Arcturus). All samples were kept at -80^{o}C until cleanup.

In order to remove possible contaminating genomic DNA, the extracted RNA was treated with 5 µl RQ1 DNase buffer, 5 units DNase and 40 units of RNase inhibitor in a 40 µl reaction volume. The mixture was incubated at 37^{o}C for 1h followed by purification with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Concentration of clean-up RNA was determined spectrophotometrically by using the NanoDrop (ND-8000) instrument; the purity of RNA was estimated by the ratio A260/A280 with respect to contaminants that absorb in the UV. Additional examination of integrity was done by denaturing agarose gel electrophoresis and ethidium bromide staining. Finally, the purified RNA was stored at -80^{o}C for further analysis.

Approximately 1.5 µg of total RNA for each sample was transcribed into cDNA. cDNA was synthesised using GoScript (Cat.#A5000) reverse Transcription System (Promega, Germany) combined with OligoDT_{15} Primers, Recombinant RNasin® Ribonuclease Inhibitor and GoScript™ Reverse Transcriptase according to the manufacturer's specification and protocol. cDNA was stored at -80^{o}C until further use.

Selection of reference genes and primer design

There are few previous studies validated selected reference genes across selected tissues in pigs (Erkens et al. 2006, Gu et al. 2011, Nygard et al. 2007, Pierzchala et al. 2011, Piorkowska et al. 2010) with specific purpose but no study was devoted to validate reference genes in the different tissues collected from different ages of pigs. However, 'traditional' reference genes like *GAPDH* and *TBP* have been most often used in pigs (Gu et al. 2011, Kaewmala et al. 2011, Kayan et al. 2011a, Kayan et al. 2011b, Laenoi et al. 2010, Nygard et al. 2007, Oczkowicz et al. 2010, Piorkowska et al. 2010). Regarding porcine organs, *ACTB*, *B2M*, *GAPDH*, *HMBS*, *HPRT1*, *RPL4*, *SDHA*, *TBP* and *YWHAZ* have been previously compared (Nygard et al. 2007). More specifically in recent days, *GAPDH*, *ACTB*, *RPL27*, *RPS29*, *RPS13* are compared in porcine stomach (Oczkowicz et al. 2010); *GAPDH*, *TBP*, *HPRT*, *RPS29*, *ACTB* and *RPL27* are validated in porcine adipose tissues in different breeds of pigs (Piorkowska et al. 2010) and *B2M*, *SDHA*, *ACTB*, *GAPDH*, *HPRT1* and *TBP* expression stability are compared in porcine muscle and liver tissues in pigs (Pierzchala et al. 2011). The genes used in our study were selected based on these previous studies. Information about the nine candidate reference genes used in the present study is shown in Table 1. The following nine commonly used reference genes were selected: *ACTB*, *GAPDH*, *HPRT1*, *B2M*, *SDHA*, *RPL4*, *YWHAZ*, *TBP* and *PPIA*. Primers were designed using the publicly available web-based Primer3 program (Rozen and Skaletsky 2000) and are listed in Table 1. They were tested using a BLAST analysis against the NCBI database (www.ncbi.nlm.nih.gov/tools/primer-blast).

qReal-Time PCR

Nine-fold serial dilution of plasmids DNA were prepared and used as template for the generation of the standard curve. In each run, the 96-well microtiter plate contained each cDNA sample, plasmid standards for the standard curves and no-template control. A no-template control (NTC) was included in each run for each gene to check for contamination. Quantitative real-time RT-PCR (qRT-PCR) was set up using 2 µl first-strand cDNA template, 7.4 µl deionized H_2O, 0.3 µM of upstream and downstream primers and 10 µl 1× Power SYBR Green I master mix with ROX as reference dye (Bio-Rad). The thermal cycling conditions were 3 min at 95 °C followed by 15 s at 95 °C (40 cycles) and 1 min at 60 °C. Experiments were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Based on the Ct values

for all dilution points in a series, a standard curve was generated using linear regression and the slope and the PCR amplification efficiency of each primer pair is calculated from the slope of a standard curve (Maroufi et al. 2010). Melting curve analysis were constructed to verify the presence of gene-specific peak and the absence of primer dimer. Agarose gel electrophoresis was performed to test for the specificity of the amplicons. To ensure repeatability of the experiments, all the reactions were executed in triplicate and the mean was used for further analysis.

Determination of reference gene expression stability

The raw qRT-PCR amplification data was exported from the StepOne® software (Applied Biosystem) to Microsoft® Excel. The averages of the Ct-values for each triplicate were used for stability comparison of candidate reference genes in the NormFinder, GeNorm and BestKeeper.

Ct values of all samples were exported to Excel, ordered for use in geNormPlus software (15 days free trial version qBasePlus; www.biogazelle.com) and transformed to relative quantities using the gene-specific PCR amplification efficiency (Hellemans et al. 2007). These relative quantities were then exported to geNormPlus to analyze gene expression stability (Vandesompele et al. 2002). The approach of reference gene selection implemented in geNorm relies on the principle that the expression ratio of two ideal reference genes should be identical in all samples, independent of the treatment, condition, or tissue type. Increasing variations in the expression ratio between two genes correspond to lower expression stability across samples. GeNorm calculates the stability using a pairwise comparison model (Vandesompele et al. 2002). geNorm determines the level of pairwise variation for each reference gene with all other reference genes as the standard deviation of the logarithmically transformed expression ratios. In this way, the reference gene expression stability measure (*M* value) was calculated as the average pairwise variation of a particular gene with all other control genes included in the analysis (Maroufi et al. 2010, Vandesompele et al. 2002). Lower *M* values represent higher expression stabilities. Sequential elimination of the least stable gene (highest *M* value) generates a ranking of genes according to their *M* values and results in the identification of the genes with the most stable expression in the samples under analysis. geNorm was also used to estimate the normalization factor (NF_n) using *n* multiple reference genes, by calculating the geometric mean of the expression levels of the *n* best reference genes (Vandesompele et al. 2002). The

optimisation of the number of reference genes starts with the inclusion of the two genes with the lowest *M* value, and continues by sequentially adding genes with increasing values of *M*. Thus, geNorm calculates the pairwise variation V_n/V_{n+1} between two sequential normalization factors NF_n and NF_{n+1} containing an increasing number of reference genes (Vandesompele et al. 2002). A large variation means that the added gene has a significant effect on the normalization and should preferably be included for calculation of a reliable normalization factor. Ideally, extra reference genes are included until the variation V_n/V_{n+1} drops below a given threshold. If $V_{n/n+1} < 0.15$ the inclusion of an additional reference gene is not required and the recommended number of reference genes is given by *n* (Vandesompele et al. 2002). Although, the recommended threshold of 0.15 should not be taken as too strict of a cut-off (Vandesompele et al. 2002).

NormFinder uses an ANOVA-based model (Andersen et al. 2004). The software calculates a stability value for all candidate reference genes tested. The stability value is based on the combined estimate of intra- and inter-group expression variations of the genes studied (Andersen et al. 2004). For each gene, the average Ct value of each triplicate reaction was converted to relative quantity data as described for geNorm, to calculate the stability value with NormFinder program (Andersen et al. 2004). The NormFinder reference tool was applied to rank the candidate reference gene expression stability for all samples with no subgroup determination as well as with age as subgroup. A low stability value, indicating a low combined intra- and inter-group variation, indicates high expression stability (Andersen et al. 2004).

The average Ct value of each triplicate reaction was used (without conversion to relative quantity) to analyze the stability value of studied genes (Pfaffl et al. 2004). BestKeeper creates a pairwise correlation coefficient between each gene and the BestKeeper index (BI). This index is the geometric mean of the Ct values of all candidate reference genes grouped together. BestKeeper also calculates standard deviation (S.D) of the Ct values between the whole data set. The gene with the highest coefficient of correlation with the BI indicates the highest stability (Pfaffl et al. 2004).

Results

Purity, quantity of extracted RNA and verification of amplicons

The optical density (OD) ratio A260/A280 nm measured with a Nanodrop spectrophotometer was 1.95 ± 0.16 (OD A260/A280 ratio ± SD). The average RNA concentration after extraction using the Tri-reagent (for tissues) and PicoPure (for PBMC) was 1.65 µg/µl ± 1.03 (µg/µl ± SD). The results of the averaged amplification efficiencies are shown in table 1. The amplification efficiencies for the nine candidate reference genes ranged between 81.88% and 99.59%. The agarose gel electrophoresis (figure 1a) and melting curve analysis (figure 1b and Table 1) revealed that all primer pairs amplified a single PCR product with expected size. Furthermore, sequence analysis of cloned amplicons revealed that all sequenced amplified fragments were identical to sequences used for primer design from GenBank.

Expression levels of candidate reference genes

The cycle threshold (Ct) values obtained throughout the study were low enough to pursue the analysis reliably: Overall (by combining Ct values of all ages for each gene), out of the nine genes studied, *PPIA* (mean Ct 16.91) and *RPL4* (mean Ct 16.92) were expressed at the highest levels, followed by *YWHAZ* (mean Ct 19.97), *B2M* (mean Ct 20.03), *SDHA* (mean Ct 21.17) and *HPRT1* (mean Ct 22.05). *GAPDH* (mean Ct 26.44) was expressed at the lowest level in the porcine tissues used in this study (Additional file S1). Prior to any referencing, when expression values were compared between ages in a tissue, the average Ct values for *B2M*, *SDHA* was stable in 12 tissues and *BLM* was stable in 11 tissues, whereas *GAPDH*, *PPIA*, *TBP* and *YWHAZ* were stable in seven of the tissues out of 14 tissues (figure 2). In case of PBMC and skin, all the candidate reference genes were expressed differentially between ages (figure 2x and 2xi). According to the Ct values for candidate genes, less expression variability could be seen in duodenum followed by kidney, spleen and heart (figure 2). Moreover, the expression of reference genes were found to be influenced by organ, age and age-organ interaction (Additional file S2).

Identification of optimal reference genes

Figure 3a and 3e shows the ranking of the nine candidate reference genes across the tissues without considering ages of individuals based on their stability values calculated using geNorm and NormFinder, respectively. Both softwares showed that *RPL4*, *PPIA* and *YWHAZ* are the most stable genes. Similar stability for candidate genes could also be found in tissues collected from 5 months adult pigs (figure 3d and 3h). However, the expression stability was always not consistent between the used softwares. geNorm showed that *RPL4* was the most stable candidate reference gene followed by *PPIA* and *YWHAZ* in tissues collected from 1 day old piglets (figure 3b), whereas *B2M* was the most stable reference gene followed by *YWHAZ* and *SDHA* in case of 2 months old young pigs (figure 3c). *GAPDH* has the highest stability value in all ages group when expression stability were analyzed using geNorm (figure 3a-d). On the other hand, NormFinder showed that *PPIA* is the most stable gene when all tissues were considered together and in tissues collected from 5 months old adult pigs (figure 3e, h), whereas *TBP* showed highest stability in tissues collected from 1 day old piglet and in 2 months old young pigs (figure 3f, g). Additionally, *BLM* and *RPL4* were recommended as the best combination of two genes with the stability value 0.083, while *PPIA* was recommended as the best gene with stability value 0.091 by NormFinder. Figure 3a-d shows the ranking of the nine candidate reference genes based on their *M* value calculated using GeNorm. In all age groups, the most stable three candidate reference genes started with an *M* value below or equal to 1.5, which is the default limit below which candidate reference genes can be classified as stably expressed.

The results of reference gene evaluation by the BestKeeper tool are shown in Table 2. According to the variability observed, candidate reference genes can be identified as the most stable genes, as they exhibited the lowest coefficient of variance (CV ± SD). In this context, we found that *YWHAZ* is the most stable reference genes in tissues collected from 2 months old young pig (table 2). It is important to note that, genes that show a SD higher than 1 should be considered unacceptable (Pfaffl et al. 2004, Stern-Straeter et al. 2009). A low SD of the cycle threshold (Ct) values should be expected for a useful reference gene. In this study, the estimation of the SD (± Ct) of the CV [%Ct] values for all the genes except *YWHAZ* at 2 months (bold italic letters; Table 3), was higher. This constitutes a reason to exclude these genes from the BestKeeper index calculation, as they are not reliable reference candidate gene in this setting (Pfaffl et al. 2004).

Determination of the optimal number of reference genes for normalization

In addition to the stability results, the GeNorm software can determine the optimal number of reference genes necessary to calculate a normalization factor (NF). The results are shown in figure 4. As shown in figure 4a to 4d, 6 endogenous control genes are necessary to obtain the lowest changing V values in all analyzed samples. However, it is impractical to use excessive numbers of endogenous control genes for normalization, particularly when only a small number of target genes need to be studied or for rare samples that are very difficult to acquire (Gu et al. 2011, Vandesompele et al. 2002). Therefore, the use of the three most stable housekeeping genes for the calculation of the NF was considered acceptable for the majority of experiments (Gu et al. 2011, Vandesompele et al. 2002). To verify that the use of three housekeeping genes simultaneously is adequate for normalization of qRT-PCR, the correlation of NF values between the geometric means of the three most stable genes and the optimal number of genes was calculated for all sample groups. As shown in figure 5, there is a very good correlation between the two NF measures (i.e., the theoretical optimal number and proposed number, three) for all 14 samples in all ages including overall tissues irrespective of age (r = 0.99 to 0.98, Pearson) (Figure 5a to 5d). This result demonstrates that the three most stable housekeeping genes are sufficient for an accurate normalization of our qRT-PCR data (Gu et al. 2011, Vandesompele et al. 2002). In addition, there is a very good agreement between geNorm and NormFinder softwares identifying three out of six most stable genes, namely *RPL4*, *PPIA* and *YWHAZ*. We therefore in general postulate that the combination of *RPL4*, *PPIA* and *YWHAZ* is the most appropriate normalization approach for gene expression studies in different tissues from pigs at different ages.

Discussion

For an exact comparison of mRNA transcription in different samples or tissues it is crucial to choose the appropriate reference gene. The optimal reference gene should be constantly transcribed in all types of cells at any time in cell cycle and differentiation. Moreover the transcription of such a gene should not be regulated by internal or external influences, at least not more than the general variation in RNA synthesis (Nygard et al. 2007). The reference gene used for normalization of gene expression in qRT-PCR studies should also pass through the same steps of analysis as the gene to be

quantified. However, such a perfect reference gene does probably not exist. Recent research has demonstrated that the expression of housekeeping genes may be altered due to differences in tissue types (Gu et al. 2011, Nygard et al. 2007, Pierzchala et al. 2011), breeds (Piorkowska et al. 2010), ages (Piorkowska et al. 2010, Touchberry et al. 2006) and experimental condition or treatment (Beekman et al. 2011, De Boever et al. 2008, Maccoux et al. 2007, Penning et al. 2007, Vandesompele et al. 2002). Such data indicate some housekeeping genes may better serve as a control when making comparisons to other genes of interest. Therefore, it is critical to elucidate the changes, if any, that may exist in housekeeping genes between younger and older adults. As an increasing volume of data continues to be published exploring mRNA expression in cases of age-depended disease, there has been a greater interest in evaluating the commonly used, widely expressed housekeeping genes for comparisons between ages. Without this information, age-dependent comparisons are very difficult to make. Therefore, it is necessary to investigate the validity and reliability of measuring the expression of various housekeeping genes in porcine tissues at different ages using qRT-PCR. To the author's knowledge, this study is the first to report that aging can influence the expression of certain housekeeping genes in pigs.

Numerous studies have been carried out in order to evaluate reference genes in specific tissues in several species. The majority of these studies are directed towards specific tissues in pigs (Erkens et al. 2006, Kuijk et al. 2007, Nygard et al. 2007, Oczkowicz et al. 2010, Svobodova et al. 2008). Taken together, it is very difficult to find a 'universal' reference gene having stable expression in all cell types and tissues, and in particular to find reference genes that remain stable between samples taken at different ages under different experimental conditions. According to the NCBI-PubMed statistics (Gu et al. 2011), *GAPDH* and *ACTB* are the two mostly used porcine housekeeping genes. But they have been shown to vary considerably and are consequently unsuitable as reference genes for normalization of gene expression analysis in some cases (Barber et al. 2005, Jung et al. 2007, Selvey et al. 2001). Also the low expressed reference gene TBP is highly regulated in pigs (Kuijk et al. 2007). The first priority, however, is to identify genes with stable expression preferably across cell types since many qRT-PCR studies are performed on cDNA isolated from tissues with a mixed cell population. Presently, only few major publications describe the stability of housekeeping genes in pig and are based on limited samples of specific categories (Erkens et al. 2006, Kuijk et al. 2007, Nygard et al. 2007, Oczkowicz et al. 2010, Svobodova et al. 2008). Our comprehensive

set of representative tissue samples and selected housekeeping genes provide valuable recommendations for the choice of endogenous control genes for the study of gene expression patterns in normal tissues. Notably, our results coincided with the finding of Gu et al. (2011) reported that *YWHAZ* is one of the most stably expressed reference genes across tissues in healthy pigs. Nygard et al. (2007) reported that *RPL4*, *TBP* and *YWHAZ* have the highest stability across tissues collected from healthy pigs which are in good agreement with our findings. In this study, geNorm showed that *PPIA*, *YWHAZ* and *RPL4* are the most stable housekeeping genes across tissues in case of newborn piglets, adults and in irrespective of ages. Additionally, *TBP*, *PPIA*, *RPL4* and *YWHAZ* are detected to be the most stably expressed gene across the tissues by NormFinder.

geNorm finding is contradictory to the findings of Erkens et al. (2006) who reported that *TBP* is one of the most stable housekeeping gene in porcine backfat and muscle (longissimus dorsi) while *SDHA* is reported as an unstable gene. Similar findings are reported by Kuijk et al. (2007) that *GAPDH* and *B2M* are the most and least stably gene, respectively in porcine oocytes and perimplantation embryo. On the other hand, our findings are in good agreement with Piorkowska et al. (2010) who recently reported that *RPL27* and *ACTB* are the most stable genes, and *GAPDH* and *TBP* are the least stable reference candidate genes in porcine adipose tissues collected from different pig breeds. The findings of this study that commonly used housekeeping genes studied are expressed differentially across porcine tissues is supported by Svobodova et al (2008) in pigs. Moreover, Svobodova et al (2008) found that *HPRT1* has the highest stability while *GAPDH* was the unstable across porcine tissues. Pierzchala et al. (2011) recently reported that *TOP2B*, *HPRT1* and *TBP* are the most stable housekeeping genes in porcine liver and in three different muscle tissues which is partially supporting as well as conflicting to our result. Because we found that *HPRT1* is one of the most stable genes, whereas *TBP* is one of the unstable genes in geNorm analysis, but in good agreement with NormFinder results. *RPL4*, *HPRT1* and *B2M* are reported as stably expressed and suitable candidate genes in intestinal tissues collected from healthy pig and from pigs with enteritis (Schroyen et al. 2008). Reportedly, *GAPDH* is the least stable gene while *RPL27* is most stable housekeeping gene in porcine stomach tissue (Oczkowicz et al. 2010). However, different housekeeping genes are identified between the previous studies and our study, as the samples varied in their cell, tissue, sex and developmental stage specificities, and different catalogues of selected housekeeping genes are chosen.

According to the BestKeeper analysis software, all the studied reference candidate genes, except *YWHAZ* at 2 months old young pigs tissues, are less suitable. Several studies previously reported similar findings for BestKeeper (Maroufi et al. 2010, Oczkowicz et al. 2010, Stern-Straeter et al. 2009) and few studies followed the BestKeeper analysis method compared to geNorm and NormFinder. It is important to note that very similar discrepancies between the different algorithms have been observed in previous studies comparing statistical analysis methods (Beekman et al. 2011, Cappelli et al. 2008, Hosseini et al. 2010, Maroufi et al. 2010, Oczkowicz et al. 2010, Stern-Straeter et al. 2009). However, we found that the first three most stable reference genes in most cases were consistently the same when using GeNorm and NormFinder, even if they were not in the exact same ranking order. Similar findings are reported by previous studies in horse, human and plants (Beekman et al. 2011, Cappelli et al. 2008, Kriegova et al. 2008, Maroufi et al. 2010). Such discrepancy could be explained by genes' co-regulation. Indeed, co-regulated genes may become highly ranked independently of their expression stabilities with GeNorm software (Andersen et al. 2004). Moreover, NormFinder takes into account variation across subgroups, thus avoiding artificial selection of coregulated genes by analyzing the expression stability of candidate genes independently from each other (Vandesompele et al. 2002). However, no studies dealing with porcine reference genes stability used different analysis methods except geNorm (Erkens et al. 2006, Gu et al. 2011, Nygard et al. 2007, Oczkowicz et al. 2010, Piorkowska et al. 2010).

As described above, GeNorm also provides a measure for the best number of reference genes that should be used for optimal normalization. In agreement with several previous studies, we postulate that the use of more than one reference gene allows for a more accurate normalization than the use of only one reference gene (Andersen et al. 2004, Beekman et al. 2011, Gu et al. 2011, Huggett et al. 2005, Vandesompele et al. 2002). Based on a cut-off point for the V value, as described by Vandesompele et al. (2002), a combination of the six most stable reference genes was calculated as being optimal for gene expression studies in different porcine tissues over ages (figure 4). However, as we described above and other studies (Gu et al. 2011, Vandesompele et al. 2002) recommended that the combination of the most three stable genes are appropriate for accurate normalization.

Conclusion

This investigation found evidence that there can be variation in the expression of commonly used housekeeping genes with populations of different ages. Due to the new influx of data suggesting alterations in mRNA expression according to ages, we feel that beside therapy uses or experimental condition, there needs to be special consideration given to the selection of housekeeping genes based upon the age of populations used. This shows again that the choice of reference genes cannot be transposed from on study to the other without validation for the specifics of each experimental protocol. In general, we recommend using the geometric mean of *RPL4*, *PPIA* and *YWHAZ* to guarantee suitable normalization in across the porcine tissues obtained from pigs of different ages.

List of abbreviations

qRT-PCR: quantitative real-time reverse transcriptase polymerase chain reaction; *B2M*: beta-2-microglobulin; *BLM*: Bloom syndrome, RecQ helicase-like; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *HPRT1*: hypoxanthine phosphoribosyltransferase 1; *PPIA*: peptidylprolyl isomerase A (cyclophilin A); *RPL4*: ribosomal protein L4; *SDHA*: succinate dehydrogenase complex subunit A flavoprotein; *TBP*: TATA box binding protein; *YWHAZ*: tyrosine 3/tryptophan 5-monooxygenase activation protein zeta polypeptide; NTC: no-template control; Ct: cycle threshold; S.D: standard deviation; BI: BestKeeper Index.

Author's contributions

MJU performed the experiments, analyzed data and prepared and edited the manuscript. MUC, DT, ET and CL edited the manuscript with MJU. KS criticized the experimental design and edited the manuscript.

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Competing interest

The authors declare that they have no competing financial or other interest in relation to this work.

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Table 1: Selected candidate reference genes, primers, and PCR reactions efficiencies.

Gene name	GeneBank accession number	Primer sequence (forward/reverse)	Ampl-icon length (bp)	Amplifi-cation effici-ency (%)	R^2	Average Ct of cDNA		
						1 Day	2 months	5 months
B2M	NM_213978.1	ACTTTTCACACCGCTCCAGT CGGATGGAACCCAGATACAT	180	86.83	0.999	20.23	19.24	20.63
BLM	NM_001123084.1	TCCTCACCTTCTGCATTTCC GTGGTGGCTGAGAATCCTGT	152	95.94	0.995	25.29	24.12	24.89
GAPDH	AF017079.1	ACCCAGAAGACTGTGGATGG ACGCCTGCTTCACCACCTTC	247	95.95	0.991	26.82	26.22	26.29
HPRT1	NM_001032376.2	AACCTTGCTTTCCTTGGTCA TCAAGGGCATAGCCTACCAC	150	81.88	0.997	22.27	21.28	22.29
PPIA	NM_214353.1	CACAAACGGTTCCCAGTTTT TGTCCACAGTCAGCAATGGT	171	82.96	0.995	16.82	16.31	17.61
RPL4	DQ845176.1	AGGAGGCTGTTCTGCTTCTG TCCAGGGATGTTTCTGAAGG	185	91.07	0.995	16.65	16.80	17.32
SDHA	DQ178128.1	AGAGCCTCAAGTTCGGGAAG CAGGAGATCCAAGGCAAAAT	149	86.41	0.989	20.55	20.64	22.34
TBP	DQ178129.1	ACGTTCGGTTTAGGTTGCAG GCAGCACAGTACGAGCAACT	118	99.59	0.995	24.44	23.92	24.31
YWHAZ	DQ178130.1	ATTGGGTCTGGCCCTTAACT GCGTGCTGTCTTTGTATGACTC	146	93.83	0.997	20.35	19.64	19.92

*R^2, correlation coefficient of the slope of the standard curve.

Table 2: Expression stability of nine candidate reference gens evaluated by BestKeeper software.

	B2M	BLM	GAPDH	HPRT1	PPIA	RPL4	SDHA	TBP	YWHAZ	BK
Irrespective of age										
n*	42	42	42	42	42	42	42	42	42	42
SD [± Ct]	1.91	1.36	1.56	2.12	1.69	1.55	1.90	1.19	1.56	1.49
CV [% Ct]	9.54	5.50	5.90	9.67	9.99	9.16	8.95	4.92	7.81	7.07
1day										
n**	42	42	42	42	42	42	42	42	42	42
SD [± Ct]	1.86	1.70	1.42	2.11	1.70	1.69	1.61	1.30	1.99	1.47
CV [% Ct]	9.17	6.70	5.28	9.45	10.11	10.17	7.82	5.30	9.76	6.95
2momths										
n**	42	42	42	42	42	42	42	42	42	42
SD [± Ct]	1.37	1.10	1.19	1.73	1.35	1.05	1.49	1.04	***0.96***	1.11
CV [% Ct]	7.13	4.55	4.54	8.02	8.30	6.24	7.23	4.36	4.89	5.38
5 months										
n**	42	42	42	42	42	42	42	42	42	42
SD [± Ct]	2.49	1.47	2.01	2.86	2.04	2.00	2.21	1.31	1.92	1.92
CV [% Ct]	12.07	5.92	7.65	13.12	11.56	11.57	9.89	5.39	9.64	8.97

Descriptive statistics of nine candidate reference genes based on their cycle threshold (Ct) values. In the last column the BestKeeper (BK) index is computed together with the same descriptive parameters for nine genes. Abbreviations: CV [%Ct]: the coefficient of variance expressed as a percentage on the Ct level; SD [± Ct]: the standard deviation of the Ct; Results from overall tissues irrespective of age and in different ages (1 day, 2 months and 5 months) are shown. * indicated the number of samples (since BestKeeper tool has limitation for 100 samples, the average Ct for three individuals was used for analysis); ** indicated the average for triplicate run was used for analysis.

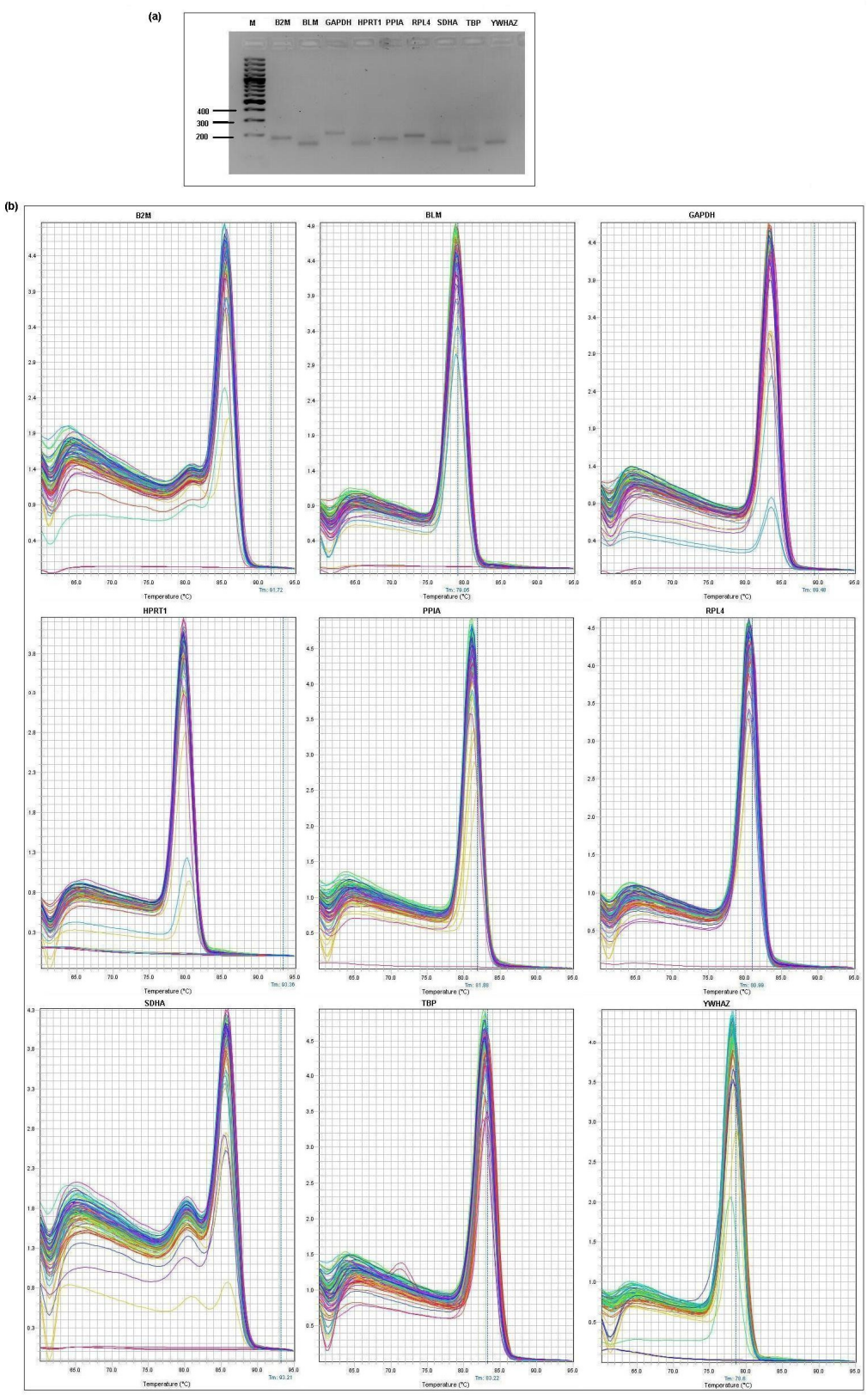

Figure 1: Confirmation of amplicon size and primer specificity of studied genes. (b) Agarose gel electrophoresis showing specific reverse transcription PCR products of the expected size for each gene, M represents DNA size marker. (a) Melting curves generated for all genes.

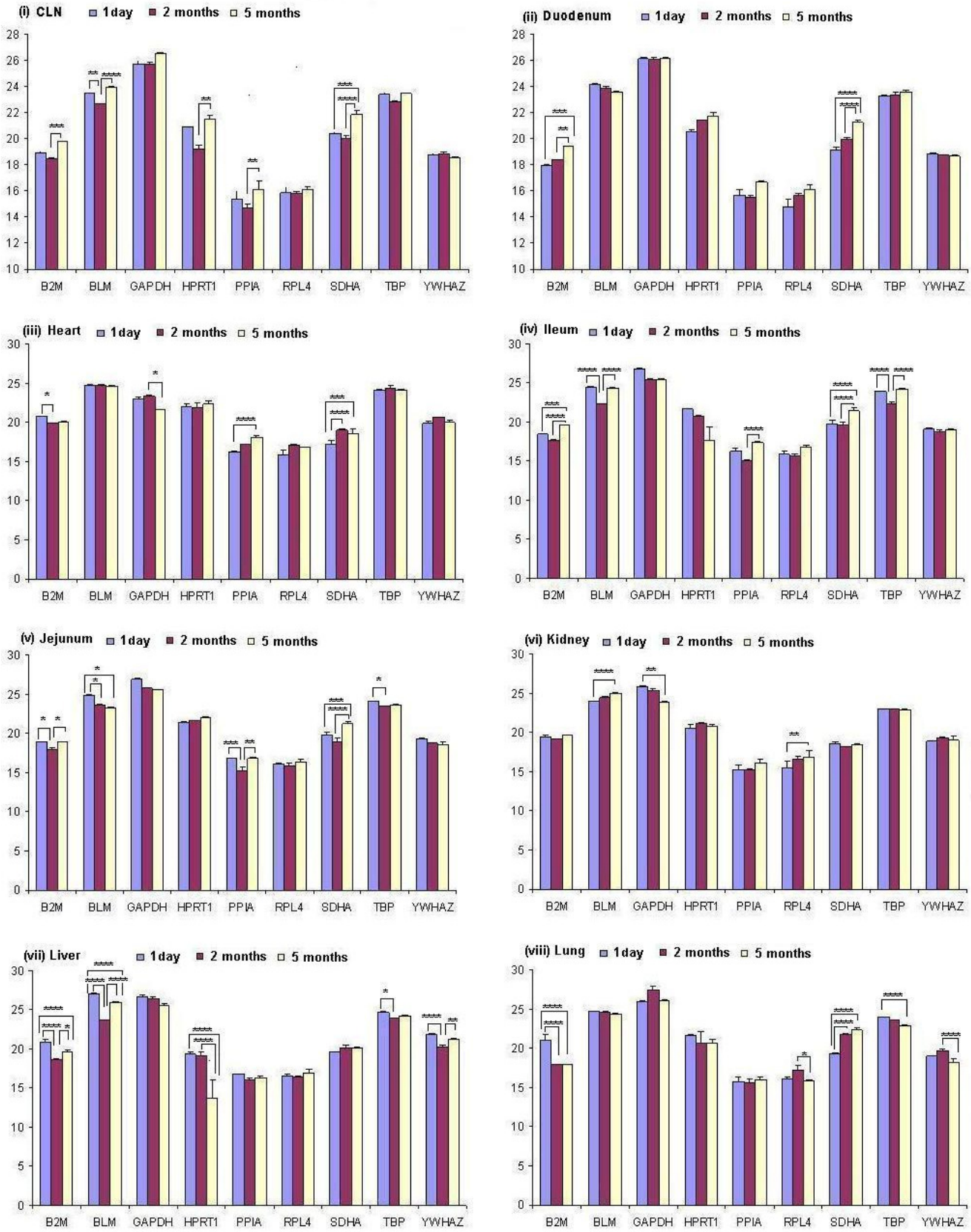

Figure 2: Average cycle threshold (Ct) values of candidate reference genes tested in porcine tissues at different ages. (Continue)

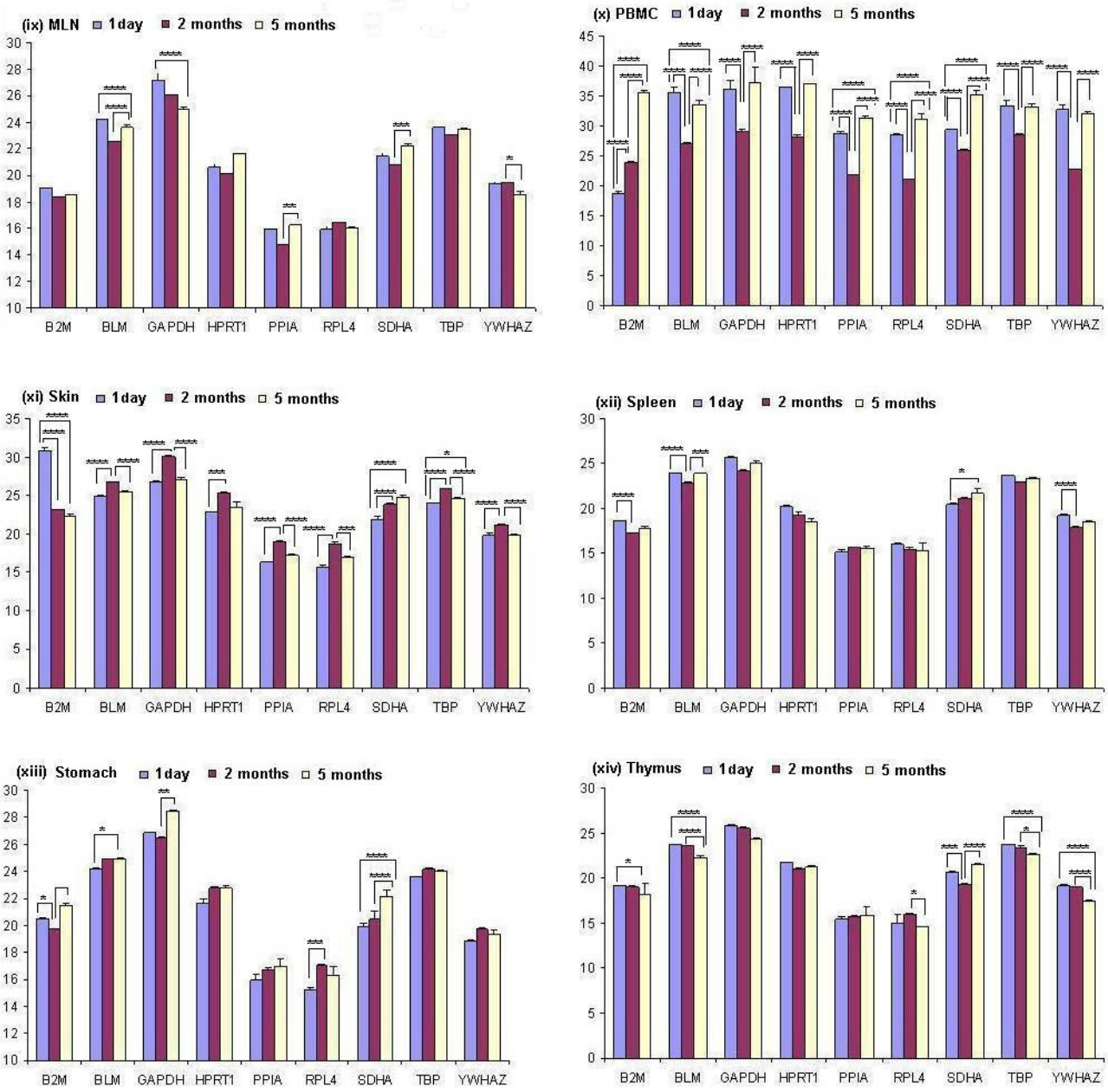

Figure 2: Average cycle threshold (Ct) values of candidate reference genes tested in porcine tissues at different ages. The values are the average qRT-PCR cycle threshold numbers (Ct values). The bars indicate standard deviation.

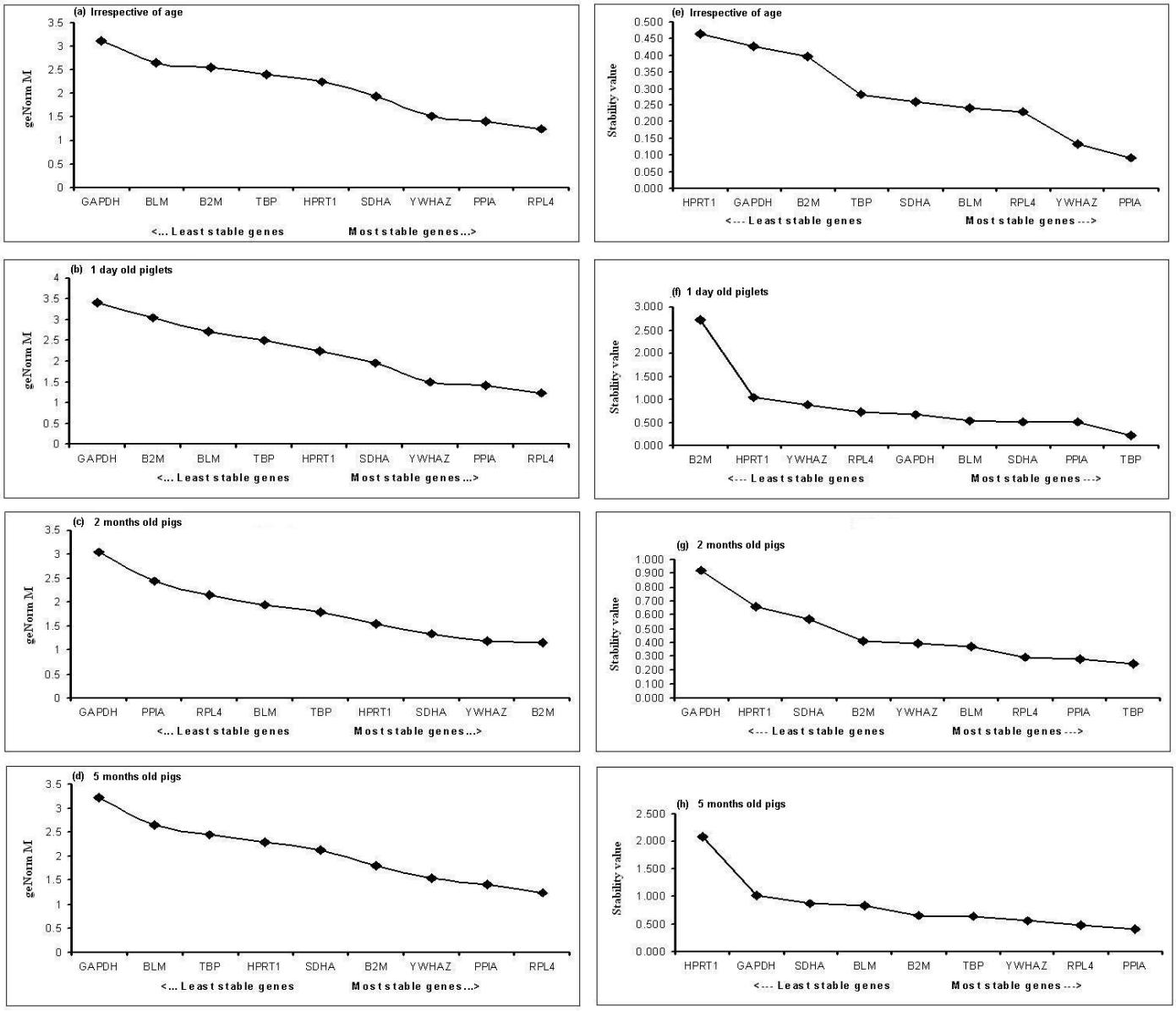

Figure 3: Ranking of nine candidate reference genes using GeNorm and NormFinder softwares. (a-d) GeNorm ranks the candidate reference genes based on their stability parameter *M*. The lower the *M* value, the higher the expression stability. (e-h) NormFinder ranks the genes based on a calculated stability value. The lower the stability value, the higher the expression stability.

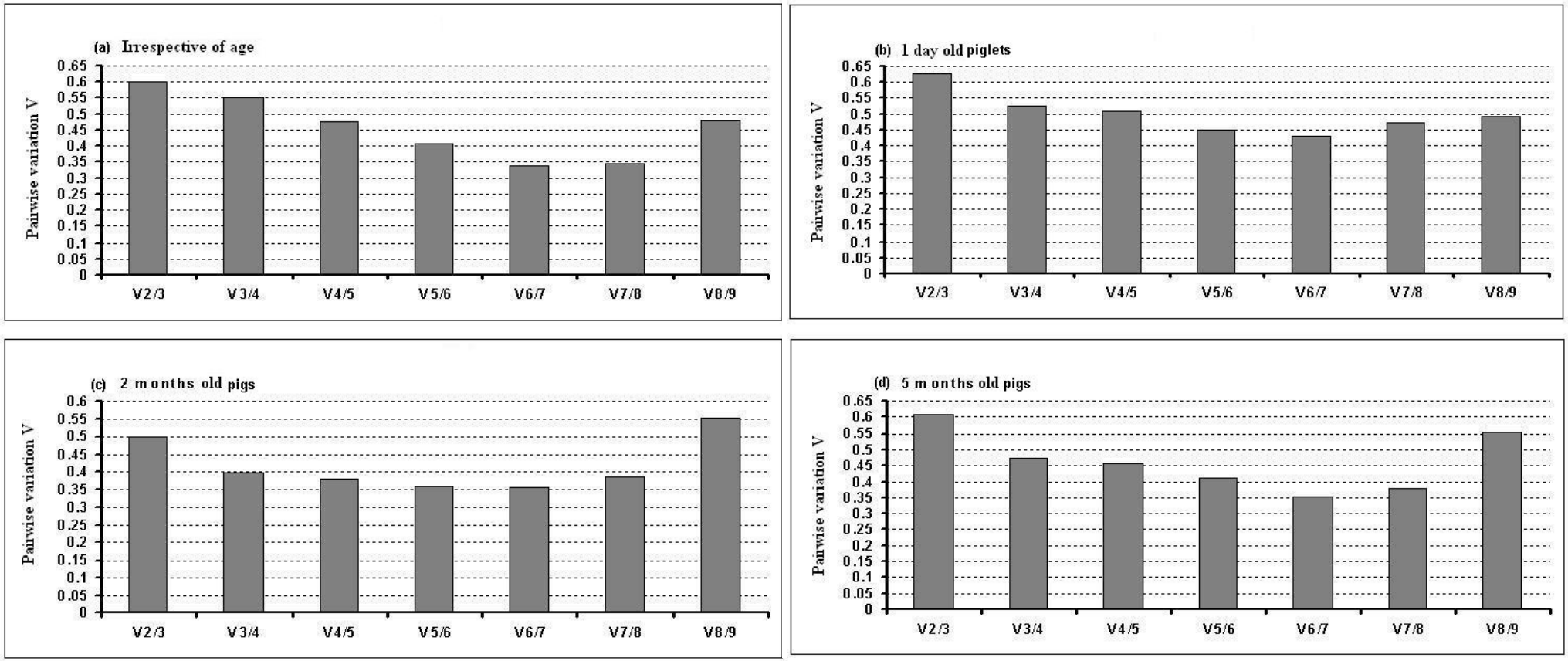

Figure 4: Determination of the optimal number of reference genes for normalization. The GeNorm software calculates the normalization factor from an increasing number of genes (starting with at least two) for which the variable V defines the pairwise variation between two sequential normalization factors. The lower the pairwise variation, the better is the combination of genes for reference. V5/6 for example, shows the variation between the normalization factors of five genes in relation to six genes and shows that six genes is the combination providing the lowest pairwise variation.

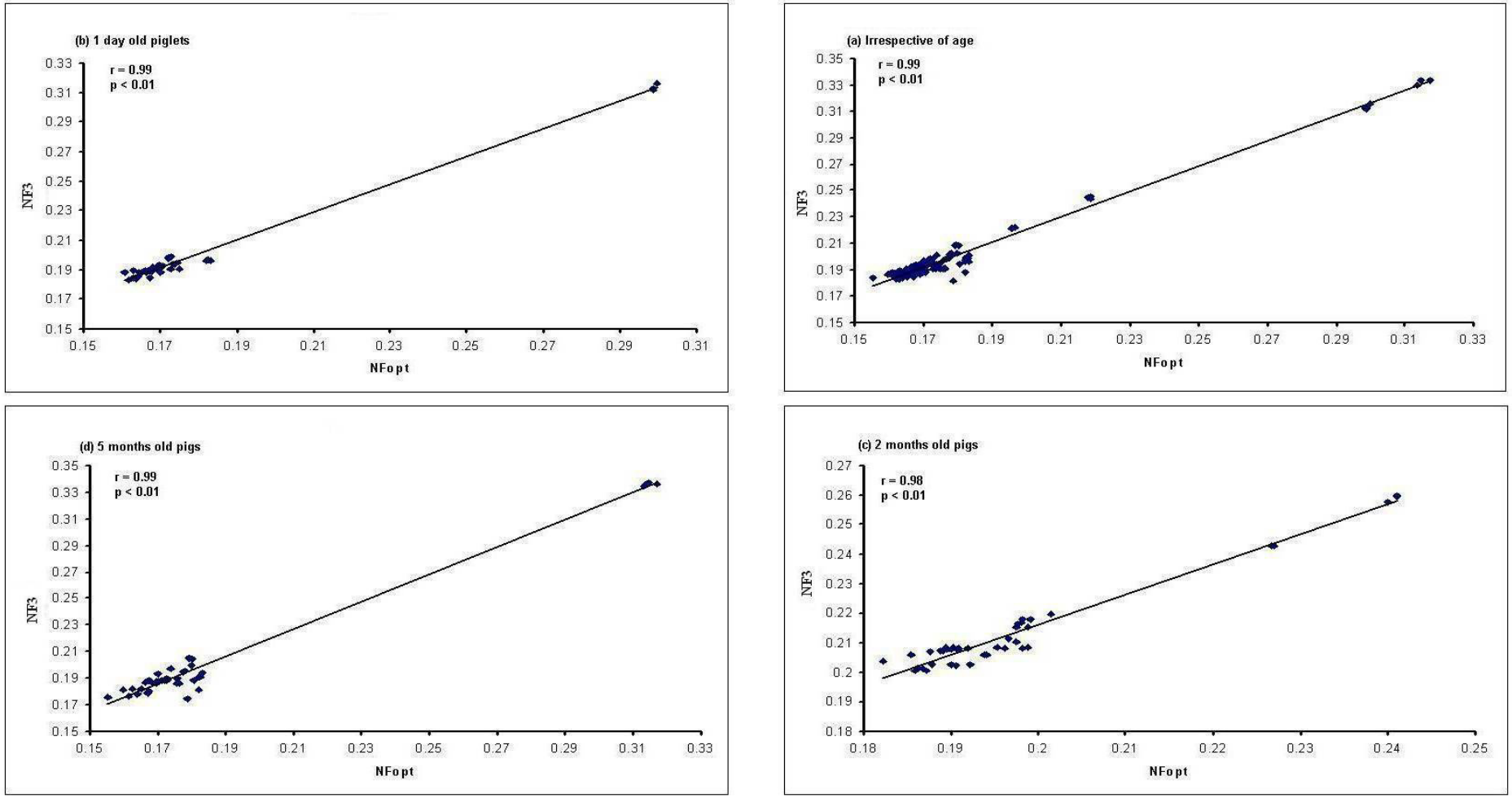

Figure 5: Correlation between the NF of most three stable and optimal number endogenous control. Pearson's correlations between the NFs of three endogenous control genes (NF3) and optimal number (six) of endogenous control genes (NFopt) for (a) all samples irrespective of age, (b) all tissues collected from 1 day old piglets, (c) all tissues collected from 2 months old young pigs, and (d) all tissues collected from 5 months old adult pigs.

Table 3: (Table S1) Relative expression levels (average ± S. D. of Ct) of candidate genes at different tissues according to age. The average and S.D. of the Ct values for different candidate reference genes studied in different tissues collected from 1 day old piglets, 2 months old young and 5 months old adult pigs.

Age	HKG	CLN (SD)	Duodenum (SD)	Heart (SD)	Ileum (SD)	Jejunum (SD)	Kidney (SD)	Liver (SD)	Lung (SD)	MLN (SD)	PBMC (SD)	Skin (SD)	Spleen (SD)	Stomach (SD)	Thymus (SD)
1 day	B2M	18.91(0.08)	17.96(0.04)	20.75(0.08)	18.42(0.11)	18.85(0.05)	19.48(0.15)	20.92(0.25)	21.01(0.80)	19.05(0.06)	18.62(0.32)	30.92(0.34)	18.61(0.09)	20.54(0.03)	19.18(0.09)
	BLM	23.48(0.06)	24.18(0.09)	24.81(0.10)	24.46(0.06)	24.94(0.07)	23.96(0.02)	26.99(0.07)	24.80(0.02)	24.24(0.07)	35.46(0.96)	24.92(0.07)	23.93(0.04)	24.18(0.03)	23.75(0.07)
	GAPDH	25.74(0.24)	26.16(0.03)	22.99(0.25)	26.74(0.12)	26.90(0.06)	25.85(0.17)	26.68(0.17)	25.99(0.17)	27.13(0.50)	36.09(1.50)	26.77(0.24)	25.67(0.14)	26.86(0.06)	25.89(0.06)
	HPRT1	20.91(0.08)	20.56(0.08)	22.00(0.30)	21.68(0.01)	21.44(0.14)	20.58(0.43)	19.37(0.22)	21.70(0.05)	20.62(0.27)	36.46(0.00)	22.81(0.13)	20.24(0.08)	21.66(0.29)	21.75(0.08)
	PPIA	15.40(0.62)	15.65(0.51)	16.26(0.02)	16.18(0.49)	16.80(0.12)	15.24(0.63)	16.77(0.02)	15.77(0.62)	15.93(0.03)	28.69(0.36)	16.35(0.09)	15.08(0.32)	15.94(0.42)	15.40(0.23)
	RPL4	15.87(0.45)	14.80(0.53)	15.86(0.55)	15.86(0.46)	16.16(0.10)	15.44(0.86)	16.59(0.15)	16.06(0.29)	15.97(0.17)	28.47(0.21)	15.73(0.18)	16.01(0.07)	15.28(0.13)	14.97(1.02)
	SDHA	20.39(0.12)	19.15(0.16)	17.19(0.52)	19.82(0.41)	19.82(0.33)	18.51(0.26)	19.68(0.02)	19.29(0.10)	21.43(0.24)	29.39(0.13)	21.93(0.31)	20.49(0.08)	19.90(0.27)	20.68(0.08)
	TBP	23.43(0.13)	23.27(0.06)	24.13(0.04)	23.85(0.05)	24.13(0.01)	22.96(0.05)	24.63(0.12)	23.96(0.02)	23.60(0.05)	33.32(1.04)	23.95(0.04)	23.61(0.07)	23.59(0.04)	23.75(0.03)
	YWHAZ	18.82(0.08)	18.88(0.04)	19.93(0.21)	19.09(0.10)	19.36(0.05)	18.85(0.07)	21.87(0.13)	18.97(0.01)	19.36(0.09)	32.74(0.69)	19.87(0.19)	19.25(0.06)	18.84(0.09)	19.12(0.17)
2 months	B2M	18.44(0.08)	18.38(0.05)	19.85(0.06)	17.70(0.10)	17.88(0.33)	19.20(0.04)	18.71(0.04)	17.92(0.10)	18.36(0.13)	23.80(0.14)	23.19(0.02)	17.17(0.03)	19.70(0.05)	19.00(0.12)
	BLM	22.65(0.03)	23.89(0.08)	24.64(0.27)	22.30(0.07)	23.67(0.08)	24.50(0.09)	23.67(0.05)	24.58(0.06)	22.57(0.03)	27.05(0.08)	26.78(0.06)	22.94(0.02)	24.86(0.03)	23.60(0.07)
	GAPDH	25.75(0.11)	26.03(0.16)	23.37(0.05)	25.50(0.09)	25.79(0.15)	25.31(0.20)	26.47(0.22)	27.42(0.45)	26.08(0.22)	29.05(0.38)	30.02(0.23)	24.24(0.05)	26.44(0.16)	25.57(0.08)
	HPRT1	19.19(0.34)	21.37(0.05)	21.91(0.52)	20.66(0.21)	21.61(0.06)	21.07(0.17)	19.09(0.50)	20.65(1.45)	20.13(0.02)	28.10(0.37)	25.31(0.08)	19.23(0.30)	22.77(0.12)	21.04(0.09)
	PPIA	14.72(0.25)	15.52(0.17)	17.18(0.04)	15.03(0.12)	15.25(0.56)	15.21(0.11)	16.01(0.21)	15.58(0.58)	14.82(0.24)	21.77(0.06)	19.04(0.11)	15.65(0.01)	16.70(0.18)	15.78(0.13)
	RPL4	15.77(0.13)	15.69(0.09)	17.14(0.06)	15.69(0.15)	15.89(0.33)	16.50(0.49)	16.43(0.14)	17.27(0.46)	16.45(0.61)	21.09(0.06)	18.70(0.22)	15.48(0.14)	17.00(0.15)	16.05(0.09)
	SDHA	19.97(0.33)	19.92(0.23)	18.95(0.13)	19.56(0.39)	18.93(0.52)	18.25(0.03)	20.11(0.31)	21.78(0.09)	20.84(0.42)	25.85(0.08)	23.81(0.17)	21.13(0.14)	20.47(0.56)	19.35(0.13)
	TBP	22.87(0.06)	23.36(0.22)	24.39(0.23)	22.36(0.20)	23.41(0.09)	22.98(0.05)	23.89(0.05)	23.58(0.13)	23.07(0.07)	28.45(0.18)	25.95(0.00)	22.96(0.08)	24.16(0.09)	23.37(0.17)
	YWHAZ	18.89(0.12)	18.71(0.06)	20.69(0.03)	18.78(0.17)	18.75(0.02)	19.30(0.10)	20.23(0.21)	19.64(0.19)	19.50(1.15)	22.66(0.04)	21.15(0.13)	17.93(0.12)	19.71(0.15)	19.00(0.04)
5 months	B2M	19.78(0.01)	19.39(0.06)	20.02(0.04)	19.59(0.12)	18.86(0.03)	19.67(0.03)	19.61(0.23)	17.89(0.05)	18.56(0.03)	35.47(0.60)	22.35(0.24)	17.83(0.13)	21.49(0.15)	18.27(1.13)
	BLM	23.90(0.09)	23.62(0.05)	24.59(0.04)	24.31(0.10)	23.25(0.07)	25.00(0.08)	25.85(0.11)	24.33(0.09)	23.58(0.29)	33.48(0.76)	25.55(0.11)	23.90(0.02)	24.90(0.07)	22.28(0.17)
	GAPDH	26.50(0.12)	26.16(0.04)	21.67(0.05)	25.49(0.04)	25.51(0.05)	23.86(0.15)	25.59(0.15)	26.08(0.09)	24.97(0.23)	37.23(2.62)	27.04(0.29)	25.10(0.26)	28.45(0.09)	24.36(0.07)
	HPRT1	21.50(0.30)	21.73(0.29)	22.31(0.44)	17.70(1.66)	22.05(0.10)	20.78(0.24)	13.62(3.06)	20.67(0.45)	21.62(0.06)	37.05(0.01)	23.48(0.60)	18.57(0.30)	22.78(0.16)	21.24(0.24)
	PPIA	16.15(0.68)	16.66(0.13)	18.14(0.18)	17.37(0.17)	16.73(0.28)	16.08(0.44)	16.38(0.17)	15.95(0.42)	16.26(0.07)	31.33(0.34)	17.22(0.13)	15.50(0.25)	16.93(0.57)	15.84(0.99)
	RPL4	16.11(0.23)	16.11(0.33)	16.80(0.14)	16.83(0.14)	16.36(0.26)	16.94(0.76)	16.91(0.44)	15.85(0.12)	15.98(0.14)	31.25(0.68)	16.97(0.12)	15.37(0.70)	16.29(0.64)	14.64(0.08)
	SDHA	21.85(0.32)	21.28(0.09)	18.56(0.56)	21.39(0.52)	21.21(0.34)	18.48(0.04)	20.09(0.14)	22.37(0.25)	22.28(0.13)	35.18(0.62)	24.72(0.28)	21.62(0.55)	22.16(0.46)	21.52(0.17)
	TBP	23.44(0.09)	23.61(0.14)	24.14(0.06)	24.19(0.11)	23.66(0.16)	22.90(0.06)	24.28(0.05)	22.94(0.04)	23.48(0.08)	33.13(0.54)	24.67(0.09)	23.34(0.10)	23.99(0.06)	22.63(0.12)
	YWHAZ	18.51(0.11)	18.65(0.16)	19.97(0.20)	19.00(0.12)	18.58(0.28)	19.05(0.46)	21.21(0.08)	18.24(0.45)	18.59(0.21)	31.97(0.28)	19.80(0.10)	18.58(0.14)	19.32(0.29)	17.40(0.16)

Table 4: (Table S2) Relative expression of candidate genes and effect of age and organ on expression level (calculated by PROC GLM). Description of dataset**:** Overall expression data of reference candidate genes. Summary of the Proc GLM (ver.9.2; SAS, SAS Institute Inc., Cary, NC, USA) analysis detecting effect of age, organs and age-organ interaction on the expression of reference candidate genes.

Gene	Mean±SD	Tissue	Age	Tissue*Age	R^2	Model
B2M	20.03±3.32	<0.001	<0.001	<0.001	0.996	<0.001
BLM	24.77±2.44	<0.001	<0.001	<0.001	0.995	<0.001
GAPDH	26.44±2.75	<0.001	<0.001	<0.001	0.977	<0.001
HPRT1	22.05±3.82	<0.001	<0.001	<0.001	0.981	<0.001
PPIA	16.91±3.21	<0.001	<0.001	<0.001	0.991	<0.001
RPL4	16.92±3.12	<0.001	<0.001	<0.001	0.989	<0.001
SDHA	21.17±3.1	<0.001	<0.001	<0.001	0.993	<0.001
TBP	24.22±2.25	<0.001	<0.001	<0.001	0.994	<0.001
YWHAZ	19.97±2.96	<0.001	<0.001	<0.001	0.995	<0.001

5. Chapter 4 (under review in: *Veterinary Immunology and Immunopathology*)

Expression patterns of porcine Toll-like receptors family set of genes (TLR1-10) in gut-associated lymphoid tissues alter with age

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Short title: Expressions patterns of porcine TLRs in GALT

Key words: Newborn; Young; Adult; TLRs; GALT;

Abstract

Toll-like receptors (TLRs) function as the pathogen recognition receptors in mammals and play essential roles in the recognition of microbial components. In addition to the intestinal epithelium, the mechanical and chemical barrier, gut-associated lymphoid tissues (GALT) include lymphoid cells in lamina propria, Peyer's patches in intestinal mucosa and mesenteric lymph node which are important to defend the host from commensal pathogens. TLR expressions may alter with age and may not be restricted to cell types. Only individual expression studies of some TLRs have been performed especially in GALT in pigs. Therefore, the aim of this research was to study the expression pattern of the TLR family (TLR1-10) genes in GALT in pigs of varying ages. A total of nine clinically healthy pigs of three age group were selected (1 day, 2 months and 5 months old) for this experiment. Each age group consisted of three animals. Tissues from intestinal mucosa in stomach, duodenum, jejunum and ileum and mesenteric lymph node (MLN) were collected for both total RNA and protein isolation and for protein localization. GenomeLab Genetic Analysis System (GeXP) was used for multiplex mRNA expression measurement of TLRs (1-10), and western blot and immunofluorescence was performed for protein expression and localization of selected TLRs (TLR2, 3 and 9). mRNA expression showed that *TLR1* and *TLR2* were highly expressed in MLN. *TLR3* showed the highest mRNA abundance among all TLRs in this study, it was expressed especially highly in the intestine. In the case of MLN, *TLR1* and *TLR6* mRNA expressions were higher ($P < 0.05$) in 5 months old pigs than that of 1 day old pigs. The western blot results of TLR2, 3 and 9 appeared to be consistent with the mRNA expression results. The protein localization of TLR2, 3 and 9 showed that TLR expressing cells were abundant in the lamina propria, Peyer's patches in intestine and around and within the lymphoid follicles in the MLN. Variance analysis showed that both age and organs have an effect on all TLRs expressions ($P < 0.001$). This expressions study sheds the first light on the expression patterns of all TLR genes in GALT at different ages of pigs.

Introduction

The induction of the immunological defense system begins with the recognition of pathogens and is mediated by a set of germline-encoded receptors that are referred to as pattern-recognition receptors (PRRs). These receptors recognize conserved molecular

patterns (pathogen-associated molecular patterns; PAMPs), which are shared by a large group of microorganisms (Akira and Takeda 2004). PRRs are described as the key molecules to unlock the door to animal diseases (Werling and Coffey 2007). Toll-like receptors (TLRs) function as the PRRs in mammals and play an essential role in the recognition of PAMPs (Akira and Takeda 2004). TLRs are considered as critical proteins linking innate and acquired immunity (Werling and Coffey 2007). Eleven TLRs have been identified in humans and 13 in mice, whereas 10 members have been identified in other mammals. Studies in mice and humans show that most tissues and cells express at least a subset of TLRs (Applequist et al. 2002, Hornung et al. 2002, Ignacio et al. 2005). Porcine TLRs are considered as the front line of pathogen monitoring and their PAMPs are used as vaccine adjuvants (Uenishi and Shinkai 2009). The tissue, cellular and subcellular localization and distribution of TLRs influence the type of immune response elicited. Since porcine intestinal tissues are heavily populated with dendritic cells and T cells, for the development of mucosal vaccines pigs are considered as appropriate model (Stokes and Bailey 2000). Thus, the first step in understanding the role of TLRs in health and disease is to determine which TLRs are expressed in tissues and by specific cell types. However, altered immune responsiveness is reported to depend on the variation of TLRs expression level (Jaekal et al. 2007). In order to gain an understanding of how responsive tissues and cells are likely to be at detecting pathogens, TLR mRNA expression patterns have been determined in different species. Such expression studies of the complete TLR family (TLR1-10) genes have been done in human (Hornung et al. 2002), bovine (Menzies and Ingham 2006), ovine (Chang et al. 2009; Menzies and Ingham 2006, Nalubamba et al. 2007, Taylor et al. 2008), chicken (Iqbal et al. 2005) and fish (Meijer et al. 2004). Individual expression studies of some TLRs have been performed in pig such as TLR1, TLR6, and TLR10 (Shinkai et al. 2006); TLR2 (Alvarez et al. 2008, Tohno et al. 2005) ; TLR3 and TLR7 (Sang et al. 2008); TLR9 (Shimosato et al. 2005) and TLR4 (Thomas et al. 2006). A complete study, considering all members of the Toll-like receptor family (TLR1-10), has not yet been done in the porcine gut-associated lymphoid tissues (GALT). GALT consists of scattered effector lymphocytes in the lamina propria of the gastrointestinal tract (GIT) and organized inductive sites, namely the Peyer patches (Tyrer et al. 2006) and mesenteric lymph nodes (MLN).

GALT provides specific host defense and encompasses the largest collection of immune cells in the body (Mowat 2003). GALT protects hosts from various commensal and pathogenic microorganisms entering through the oral route and may be equally important to achieve a homeostatic balance between immune tolerance and immune responsiveness (Artis 2008). Therefore, the GALT, especially the mucosal immunity of the gastrointestinal tract, has been the subject of great interest for the past several years in humans and mice (Brandtzaeg and Pabst 2004, Par 2000) as well as in pigs (Burkey et al. 2009b, Stokes 2001, Stokes et al. 1994).

The epithelium of the gut recognizes the immunobiotic foods and / or pathogenic organism through a crosstalk via TLRs (Kitazawa et al. 2008, Tohno et al. 2006, Uenishi and Shinkai 2009). The GALT including intestinal epithelia (IEC), Peyer's patches (Tyrer et al. 2006) and mesenteric lymph nodes (MLN) works together synergistically to fight against pathogens that shapes the intestinal ecosystem (Burkey et al. 2009b). Central to the protective nature of the intestinal barrier is its need to sense and respond to proinflammatory bacterial and viral products and to recognize them. Although TLRs are thought to have predominantly beneficial effects in pathogen recognition and bacterial clearance by leukocytes, emerging evidences suggest that the innate immune system, comprised of Toll-like receptors and their associated molecules, play pivotal roles in the regulation of intestinal inflammation in response to invading pathogens (Burkey et al. 2009a, Burkey et al. 2009b, Gribar et al. 2008, Tohno et al. 2006, Uenishi and Shinkai 2009). Importantly, the immune responsiveness of GALT varies depending on the development and maturation of gut with age (Barman et al. 1997, Blecha 2001, Tohno et al. 2006). Notably, the immune responsiveness to antigens or vaccine varies according to the age of the individuals (Panda et al. 2010, van Duin and Shaw 2007) which are thought to be associated with TLRs expression (Dunston and Griffiths 2010, Renshaw et al. 2002, van Duin and Shaw 2007). Therefore, the aim of this research was to study the expression pattern of TLR family genes in GALT in newborn, young and adult pigs.

Materials and methods

Animals and tissue collection

A total of nine clinically healthy male pigs of three age groups (newborn: one day old, young: 2 months old and adult: 5 months old) were selected for this experiment. Each age group consisted of three animals of the Pietrain breed. All pigs were kept at the Frankenforst experimental research farm at the University of Bonn (Germany) according to the rules of German performance stations (Zentralverband der Deutschen Schweineproduktion (ZDS): Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein, 10.12.2003). The animals were fed the same diet *ad libitum* during the whole experimental period. After slaughter, intestinal mucosa from the duodenum, jejunum and ileum, stomach and tissues from mesenteric lymph node (MLN) were collected for both mRNA and protein isolation and protein localization. For mRNA and protein isolation, samples were directly put into liquid nitrogen after washing in PBS. For immunofluorescence studies, samples were collected in RNA*later* (Invitrogen) for transportation to the laboratory and were put into Tissue-Tek O.C.T. using cryomold (Sakura). All samples were kept at -80 ^{o}C until required.

RNA isolation

Total RNA was isolated from individual samples by using Tri-Reagent (Sigma-Aldrich) according to the standard protocol. All samples were kept at -80 ^{o}C until cleanup. In order to remove possible contamination of genomic DNA, the extracted RNA was treated with 5 µl RQ1 DNase buffer, 5 units DNase and 40 units of RNase inhibitor in a 40 µl reaction volume. The mixture was incubated at 37 ^{o}C for 1h followed by purification with the RNeasy Mini Kit (Qiagen). Concentration of clean-up RNA was determined spectrophotometrically by using the NanoDrop (ND-8000) instrument and the purity of RNA was estimated by the ratio A260/A280 with respect to contaminants that absorb in the UV. Additional examination of integrity was done by denaturing agarose gel electrophoresis and ethidium bromide staining. Finally, the purified RNA was stored at -80 ^{o}C for further analysis.

TLRs mRNA expression analysis

Total RNA, measured by using the NanoDrop ND-8000 spectrophotometer (Thermo Scientific) was diluted to 50 ng/µl. In this study, the GeXP expression profiling method was used, as explained in Rai et al. (2009). The mRNA expression of ten TLRs and three housekeeping genes (*ACTB*, *GAPDH* and *TBP*) were detected in a GeXP (GenomeLab Expression Analysis) multiplex system described earlier by our research group (Gandolfi et al. 2011). Briefly, an amount of 250 ng RNA was used as a template for reverse transcriptase (RT) reaction, performed using GenomeLab GeXP Start Kit (Beckman Coulter, Fullerton, CA, USA), in a total volume of 20 µl. In the RT reaction, a pool of all reverse primers (Table 1) at a final concentration of 50 nM was used. Primers were designed using proprietary software provided by Beckman-Coulter. Each of these primers is chimeric, having a 3′ gene-specific end and a 5′ end containing a quasi-T7 universal sequence, which serves as a template in subsequent amplification steps. The RT reaction was performed under the following conditions: 1 min at 48 °C, 60 min at 42 °C, 5 min at 95 °C, hold at 4 °C, in a thermal cycler (Bio-Rad). After RT reaction, 9.3 µl of the products were used as template for a PCR with 20 nM of each forward primer and 1 U Beckman Coulter Thermo-StartR DNA Polymerase (Beckman Coulter). Each of the forward primers contains an SP6 universal sequence at the 5′ end and a gene-specific sequence at the 3′ end (Table 1). The PCR reaction was performed in a thermal cycler under the conditions: 10 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 70 °C; with a hold at 4 °C. PCR products were electrophoretically separated by the fragment analysis method (Frag-3) on the GenomeLab GeXP (Beckman Coulter), by diluting 1 µl PCR reaction with 28.5 µl SLS buffer and 0.50 µl size standard-400. Kanamycin RNA internal positive control was included and produced a peak at 326 bp when samples were separated via electrophoresis. All experiments include “no template” (i.e. without RNA) and “no enzyme” (i.e. no reverse transcriptase) negative controls to confirm the absence of peaks at the expected target sizes. The “no template” sample produces a single peak at 326 bp, corresponding to the externally spiked-in kanamycin RNA. Electrophoretic separation was done by the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Fullerton, USA). The GenomeLab GeXP software matches each fragment peak with the appropriate gene, and reports peak height and area under curve (AUC) for all peaks in the electropherogram. The data were exported from the expression analysis

module of the GenomeLab GeXP software as expression data for subsequent analyses. The expression of TLR1-10 genes was normalized by dividing for the geometric mean of the expression of three house keeping genes (*ACTB*, *GAPDH* and *TBP*). These normalized expression values were used for further statistical analysis using SAS ver9.2 (SAS Institute Inc., Cary, NC, USA). The average expression value of TLR1-10 genes in three animals in each group was considered for expression study.

Western blot analysis of TLR2, TLR3 and TLR9

Whole cell protein was extracted from tissues following standard protocol using Nonidet-P40 buffer along with protease inhibitor 1 mM (final concentration) PMSF (phenylmethylsulfonyl fluride). The protein from each sample of three animals in each age group was pooled together for western blot. The protein was separated by 4-18% gradient SDS-PAGE. Subsequently the proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). After blocking in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% Polyvinylpyrolidone) at room temperature for 1 h, the membrane was incubated with the primary antibody anti-TLR2 and anti-TLR9 antibody purified from rabbit polyclonal antibody (THU-A-TLR2 and THU-A-TLR9, CosmoBio Co Ltd.) in the blocking medium (diluted 1:700) at 4 ^{o}C overnight. Anti-TLR3 antibody (SC-8691; Santa Cruz) purified from goat (diluted 1:500) was used as primary antibody for TLR3. Non-specific binding of antibody was washed off with six changes of 0.1% PBST (10 min per time). The membrane was incubated for 1 h at room temperature with the secondary antibody, followed by washing with six changes of 0.1% PBST (10 min per time). As a secondary antibody, the horseradish peroxidase conjugated donkey anti-goat IgG antibody (SC2020; Santa Cruz) was used (diluted 1:50000) for TLR3 and the horseradish peroxidase conjugated goat anti-rabbit IgG antibody (SC2004; Santa Cruz) was used (diluted 1:50000) for TLR2 and TLR9. The chemiluminesce was detected by using the SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific) and was visualized by using Kodak BioMax XAR film (Kodak). GAPDH (SC20357; Santa Cruz) was used as a loading control and for normalization. The membrane was stripped by washing 3 times (5 min per time) in 20 ml of glycin (0.1 mol/L; pH 2.5) and then washed with 20 ml (1x) PBS, 3 times (5 min per time) and re-probed.

Immunoflurescence localization of TLR2, TLR3 and TLR9

Tissues were selected for the localization of TLR2, 3 and 9 protein on the basis of the mRNA expression of respective TLRs. The tissues having mRNA expression value close to the mean value for corresponding TLR mRNA among all nine individuals was selected for protein localization. For each of the TLR2, 3 and 9, immunofluorescence staining was performed on 8 μm cryostat sections of tissues. All sections were kept in -80 ºC for further analysis. To block unspecific staining, sections were incubated for 60 min at room temperature with 3% bovine serum albumin in PBS (50 nM sodium phosphate, pH 7.4; 0.9% NaCl). Sections were incubated overnight at 4 ºC with the same primary antibodies used in western blot (section 3.4) diluted at 1:50 in blocking solution followed by six (10 min per time) washings with PBS. Sections for TLR3 were incubated with the TLR3 goat polyclonal primary antibody (SC8691; Santa Cruz) (dilution 1:50 in blocking solution), whereas the sections for TLR2 and TLR9 were incubated with the rabbit anti-porcine TLR2 and TLR9 polyclonal primary antibody (THU-A-TLR2 and THU-A-TLR9, CosmoBio Co Ltd.) (dilution 1:50 in blocking solution), overnight at 4 ºC and subsequently the sections were washed six times (10 min per time) with PBS. The donkey anti-goat IgG-B conjugated with rhodamine (TRITC) reactive water-soluble fluorescent dye (SC2094; Santa Cruz) (dilution 1:200) was used for TLR3 and the biotinylated donkey anti-rabbit IgG-B conjugated with fluorescein isothiocyanate (FITC) reactive water-soluble fluorescent dye (SC2090; Santa Cruz) (dilution 1:200) was used for TLR2 and TLR9 as a secondary antibody, respectively. Finally, the samples were counterstained with vectashield mounting medium (Vector Laboratories) containing 40,6-diamidino-2-phenyl indole (DAPI) and covered with a cover glass slip. The staining was observed by confocal laser scanning microscope (Carl Zeiss). In the case of negative controls, PBS was used instead of the primary antibody.

Statistical analysis

The PROC GLM (ver.9.2; SAS, SAS Institute Inc., Cary, NC, USA) analysis was performed to detect the effect of age and organs on the expression of TLRs genes. Differences in gene expression levels between groups were determined using t-test in SAS. $p<0.05$ was considered statistically significant.

Results

Expression patterns of TLRs mRNA

All TLRs did not show the same pattern of expressions in gut associated lymphoid tissues (GALT) and mesenteric lymph node (MLN) tissues in this study (Fig. 1). Most of the TLRs increased with age most tissues, except *TLR5*, which showed highest expression at young ages (2 months) in all tissues (Fig. 2). All TLRs except *TLR5* expression increased with age in stomach tissue (Fig. 2a). TLR expressions were lowest in one day old piglets, except *TLR2* in ileum tissue. In the case of the ileum, *TLR2* expression was found to be reduced when age increased (Fig. 2d). In the case of the duodenum, TLR2, 3, 4, 5 and 9 were found to be expressed highest at young ages (2 months), whereas all other TLRs were expressed highest in adult pigs (5 months) (Fig. 2b). In the case of the jejunum, all TLRs were highest in abundance in adult pigs and were lowest in abundance in newborn piglets, except *TLR3* and *TLR5*. *TLR3* was found to be expressed higher in newborn piglets than that of adult pigs in jejunum (Fig. 2c). The ileum of the adult pigs showed highest expression of TLR3, 4, 6 and 8, whereas TLR1, 5, 7, 9 and 10 were expressed highest in young animals (Fig. 2d). In the case of the mesenteric lymph node (MLN), all TLRs were expressed highest in adult pigs, except *TLR5* and *TLR10*, which were expressed higher in young pigs (Fig. 2e). Importantly, the *TLR1* and *TLR6* showed higher expression ($P < 0.05$) in adult pigs than in newborn piglets.

Expression patterns of TLR2, TLR3 and TLR9 proteins

TLR2 protein expression was most remarkable in the tissues collected from adult pigs (Fig 3a). However, the TLR2 protein was also detected in different tissues in newborn and young pigs (Fig. 3a), especially in the stomach and ileum in newborn and in all GALT in young pigs (Fig. 3a). TLR3 protein expression was detectable in newborns, particularly in the stomach, jejunum and ileum (Fig. 3b). Higher expression of TLR3 protein was found in most tissues collected from young and adult pigs (Fig 3b). TLR9 protein was expressed in the stomach collected from all ages of pigs, whereas in case of duodenal tissue, the protein expression was higher in newborn compared to adult pigs (Fig 2c). On the other hand, the TLR9 protein expression was higher in the jejunum and ileum tissue collected from young and adult pigs compared to newborn piglets (Fig.3c).

Notably, GAPDH protein was not expressed similarly in all tissues at different ages (Fig. 3).

Localization of TLR2, TLR3 and TLR9 proteins

In case the of stomach tissue, TLR2, 3 and 9 were localized in the lining cells of the gastric glands and glandular mucosa (Fig 4, 5 and 6). These proteins were expressed in the epithelium in the stomach (Fig. 4b, 5b and 6b). With duodenal tissue, all three proteins were expressed in intestinal epithelium cells (IEC) as well as in the lymphoid cells in lamina propria, in the lining of villi, ducts and crypts (Fig. 4f, 5f and 6f). Besides mucosa, TLR2 and TLR3 were found to be expressed in the submucosal regions in the duodenum. In the case of the jejunum and ileum, TLR2, 3 and 9 proteins were localized in the epithelial lining of crypts and villi and lymphoid cells in the lamina propria (Fig. 4j, 5j, 6j, 4n, 5n and 6n). All the proteins were expressed highly in the lymphoid accumulations (Peyer's patches) in mucosa (Fig. 4j, 5j, 6j, 4n, 5n and 6n). TLR2 and 9 strongly localized in the tissues and cells surrounding the Peyer's patches, whereas TLR3 expressed in the lymphoid cells within the Payer's patches as well as tissues and cells surrounding the Peyer's patches (Fig. 4j, 5j, 6j, 4n, 5n and 6n). Additionally, TLR3 and TLR9 proteins were localized in the lining cells of duodenal glands in mucosal regions (Fig. 5f and 6f). All TLRs localized in this study were expressed in the lymphoid cells throughout the section of the mesenteric lymph node (MLN) (Fig. 4r, 5r, 6r). Importantly, TLR3 was localized surrounding the lymph follicle (Fig. 5r). TLR3 and TLR9 proteins were highly localized in the lymphoid cells surrounding the sinuses in the lymph node (Fig. 5r, 6r), whereas TLR2 was localized in the trabeculae of the lymph node (Fig. 4r).

Discussion

Expression patterns of TLRs

TLRs are of great interest to the research community due to their ability to recognize pathogens and initiate development of an immune response. But a complete TLR family gene expression study in porcine gut-associated lymphoid tissues (GALT) has not yet been reported. GALT is a highly organized immune compartment, is intimately associated with the gut epithelium and constitutes the largest mass of immune cells in

the body. The gut immune system protects swine against infectious and non-infectious environmental insults and discriminates ingested nutrients, food, and commensal microflora from pathogenic agents (reviewed by Artis 2008, Burkey et al. 2009b, Dvorak et al. 2006, Neutra et al. 2001). In addition to the physical barrier that the epithelia provide, the mucosal immune system also uses other gut-associated lymphoid tissues (GALT) to protect the organism and to mediate subsequent innate and adaptive immune responses. Transcript expression of each of the 10 TLRs was confirmed in the stomach and in all parts of small intestine including mesenteric lymph node (MLN) (Fig. 1 and 2). Beside the expression study, TLR2, 3 and 9 protein was detected in the GALT as well as in MLN. In general, all TLRs were more abundant in the small intestine and MLN followed by the stomach. It is interesting that *TLR3* and *TLR6* are relatively more abundant in the GALT suggesting gut epithelial cells and lymphoid cells in lamina propria, Peyer's patches and MLN possibly express these molecules. However, the expression of TLRs relative to three house keeping genes (*GAPDH*, *ACTB* and *TBP*) is low, suggesting only a small subset of cells express the TLRs. Similar expression patterns of *TLR3* have been previously shown in sheep (Menzies and Ingham 2006) and in human small intestine (Zarember and Godowski 2002) and intestinal epithelial cells (Cario and Podolsky 2000). If the abundance of these TLRs is indicative of an increased ability to respond to the appropriate PAMP, it seems likely that the GALT or gut epithelium is very sensitive to potential viral and gram-positive bacterial and fungal infections, through the ability of *TLR3* to recognize dsRNA and *TLR6* to recognize lipoteichoic acid and zymonsan (Akira and Takeda 2004). Higher expression of *TLR3* has been reported in porcine small intestines especially in the duodenum, whereas *TLR7* is moderately expressed in GALT in pigs (Sang et al. 2008). *TLR4* is reported to be detectable in the stomach of pigs (Thomas et al. 2006). *TLR4* recognizes the lipopolyscharides of gram-negative bacteria (Akira and Takeda 2004) and *TLR4* is reported to be activated in case of intestinal injury (Gribar et al. 2008). These findings may explain our result of lower *TLR4* expression since the animals used in this study were clinically healthy.

TLR2 and *TLR9* mRNA are reported to be expressed in duodenum, jejunum, ileum, ileal Peyer's patches (Tyrer et al. 2006) and MLN in healthy newborn piglets (Tohno et al. 2006) and in ileal Pps, MLN and GALT of adult pigs (Shimosato et al. 2005, Tohno et al. 2005). Additionally, it is reported that the mRNA expression levels of both *TLR2* and

TLR9 are higher in adult pigs than in newborn piglets (Shimosato et al. 2005, Tohno et al. 2005), which is in good agreement with our findings. *TLR9* was expressed intermediately in all tissues and increased with age, except in ileal tissue, in this study (Fig. 2d). The different expression levels of TLRs in newborn and adult swine GALT provide support for the idea that the intestinal microflora may promote the expression of *TLR2* and *TLR9* in the ileal Pps and MLN during postnatal development of the GALT, resulting in the high expression of *TLR2* and *TLR9* in adult GALT. Kitazawa et al. (2006) suggested that stimulation with intestinal microbes is critical for regulating the expression of *TLR2* and *TLR9* after birth and, thus, the development of a system for recognizing intestinal microorganisms. Immunoregulatory effects of probiotics or functional foods are exerted via TLR in swine (Kitazawa et al. 2008). However, since Tohno et al. (2006)did not include other TLRs, it is difficult to compare all TLRs except *TLR2* and *TLR9*.

Expression of *TLR1* and *TLR6* was higher ($P < 0.05$) in adult pigs compared to newborn piglets in mesenteric lymph node (MLN) (Fig. 2e). *TLR1* recognizes the triacyl lipopeptides of bacteria and mycobacteria whereas *TLR6* recognizes diacyllipopeptides of mycoplasma, the lipoteichoic acid of gram-positive bacteria and zymonsan of fungi (Akira and Takeda 2004). Expressions of all TLRs (TLR1-10) are reported in the feline in which *TLR5* and *TLR9* are abundant in small intestinal epithelial cells and TLR2, 4, 5 and 7 are abundant in intraepithelial lymphocytes, lamina propria lymphocytes and Peyer's patches in mucosa (Ignacio et al. 2005). The highest level of TLR2, 4, 5, 7, 8 and 9 expressions are reported in the feline MLN (Ignacio et al. 2005). Expression of TLR2, 3, 8, 9 and 10 was most remarkable in the mesenteric lymph node in this study (Fig. 2e). The mesenteric lymph node is the draining node of the intestinal tract and the inductive site from the gastrointestinal tract. It is unique in the variety of microbial antigens to which it is exposed and as such, is also uniquely armed with TLRs to recognize microbial structural patterns and initiate an immune response (Ignacio et al. 2005). Menzies and Ingham (2006) reported that *TLR6*, *TLR7* and *TLR9* mRNA were highly expressed in the ovine MLN, whereas Chang et al. (2009) found that *TLR2*, *TLR7* and *TLR10* were highly expressed in the ovine MLN. Notably, Chang et al. (2009) reported higher expression of all TLRs in the MLN when compared to the oviduct, prefemoral and prescapular lymph nodes. Thus, it is likely that the lymphoid

cells in the MLN in adult pigs have been exposed to a wide variety of pathogens as compared to newborns.

TLR6, *TLR7* and *TLR9* are reportedly abundant in ovine Peyer's patches (Menzies and Ingham 2006). *TLR5* recognizes the flagellin of motile bacteria, whereas *TLR7* and *TLR8* recognize single-stranded viral RNA (Akira and Takeda 2004). The mRNA of TLR4, 5, 7 and 8 expression was lower in all tissues compared to other TLRs in this study, suggesting a limitation of porcine GALT in response to lipopolysaccharides of gram-negative bacteria (recognized by *TLR4*), flagellin of bacteria (recognized by *TLR5*) and single-stranded RNA of viruses (ligand of *TLR7*) (Akira and Takeda 2004). This may underlie the homeostatic pathogen recognition system that biases against reactivity to gram-positive commensal bacteria, fungus and dsRNA virus within the gastrointestinal tract. Overall, our results determined in the gut tissues are in agreement with those determined elsewhere and reflect the relative abundance of TLRs determined in human intestinal tissue (Zarember and Godowski 2002) and chicken jejunum (Iqbal et al. 2005). More specifically the low relative abundance of TLR1, 4, 5, 7 and 8 determined in the small intestine matches previous reports that *TLR2* and *TLR4* are barely detectable in the human gut (Cario and Podolsky 2000) and *TLR1* is in low abundance in porcine intestinal tissue (Zarember and Godowski 2002). All tissues used in this study demonstrated expression of *TLR10* which has been previously detected in porcine stomach, small intestines and MLN (Shinkai et al. 2006), in ovine GALT (Chang et al. 2009, Menzies and Ingham 2006) and in bovine lymph nodes (Opsal et al. 2006).

TLR1, *TLR6* and *TLR10* mRNA abundance was reported in the small intestine and stomach in 1 month old pig by Shinkai et al. (2006) who found that *TLR1* and *TLR6* expressions were higher than that of *TLR10*. *TLR6* was expressed higher in all most all tissues compared to *TLR1* and *TLR2* in this study (Fig. 2). It is interesting that the expression pattern of *TLR1* and *TLR6* was similar among different ages in all tissues analyzed in this study (Fig. 2). It has been reported that in an evolutionary perspective *TLR1*, *TLR6* and *TLR10* split from the same precursor (Opsal et al. 2006). *TLR5* and *TLR8* mRNA has been detected in ovine (Chang et al. 2009, Menzies and Ingham 2006) and feline (Ignacio et al. 2005) lymphoid tissues and in dendritic cells in human (Hornung et al. 2002). Our study confirmed for the first time *TLR5* and *TLR8* mRNA expressions in all porcine gut-associated tissues.

The transcript abundance was influenced by age and organs in this study (Fig. 2 and Table 2). In our previous study we have found that in response to antigens, there are age-dependent variations in the serum level of TLR2 and TLR9 proteins in pigs (Uddin et al. 2011). In general, most of the TLRs were expressed higher in GALT of adults (5 months old) than that of newborns (1 day old) in this study. To study TLR expression over different time points is important because development of the mucosal immunity varies with age of pigs. The neonatal pig is immunologically incompetent until about 4 week of age (Blecha 2001). Thus, the period from birth through weaning represents a critical time for pigs. A positive correlation between age and heterogeneity of gene expression is reported in humans (Somel et al. 2006). Moreover, TLRs transcript abundance variation among organs is supported by most reviews since the organs structure and function is not similar in pigs and even varies with ages (Barman et al. 1997, Burkey et al. 2009b, Stokes and Bailey 2000, Stokes et al. 1994, Tohno et al. 2006). In pigs, the Peyer's patches distribution, number and size vary with age at different intestinal segments such as in jejunum and ileum. For example, the follicles of the jejunal Pps grow with age but in case of ileal Pps, these follicles are comparable in size at different time points in pigs (Barman et al. 1997). Differential transcripts profiling have been reported in jejunal Pps collected from juvenile and adult pigs (Machado et al. 2005). Therefore, the developmental and morphological differences may influence the function and transcript abundance of TLRs in GALT in pigs.

This study also found that there was a variation in the expression of some TLR genes in samples from different animals (Fig. 2). These individual differences in TLR expression may reflect differences in the recent nosocomial environmental pathogen experienced by different animals or differences in the composition of the tissue samples obtained (adult pig lymph nodes are much larger than those of newborn piglets and the use of dissected samples rather than the entire lymph node might contribute to sample-specific variation). The variation of mRNA expression in tissues among animals observed in this study may also reflect changes in TLRs gene regulation occurring in lymphoid organs. This may also explain the inconsistent results reported by different research groups in pigs (Shimosato et al. 2005, Shinkai et al. 2006, Tohno et al. 2005, Tohno et al. 2006) or in sheep (Chang et al. 2009, Menzies and Ingham 2006, Nalubamba et al. 2007). Differences in relative abundance may also correlate to the sensitivity with which each TLR recognizes its target PAMP. Moreover, the differences in results from different

research groups might be influenced by the used methods. Recently it has been reported that different quantitative real-time PCR systems may yield different gene expression values (Lu et al. 2010). The analytical specificity for mRNA analysis is reported to be better using the GeXP because the combination of oligonucleotide primer-based PCR amplification and capillary electrophoretic separation minimizes false-positive reactions by adding two layers of specificity i.e. each of the intended targets is interrogated at both the hybridization and electrophoretic separation steps to confirm their identity (Rai et al. 2009).

Immunostaining distribution of TLR2, TLR3 and TLR9 proteins

Visualization of the immunohistochemical staining by confocal microscopy (Fig. 4, 5 and 6) revealed that TLR2, TLR3 and TLR9 were localized in the Peyers patches, lymphoid follicles and around the lymphoid follicles within the lamina propria (Fig. 4, 5 and 6). Similar findings are reported by Tohno et al. (2006) that TLR2 and TLR9 positive cells are distributed in the lymphoid follicles and around the lymphoid follicles in ileal Pps of presuckling pigs. TLR2 is reported to localize in the enterocytes in jejunum in pigs (Alvarez et al. 2008). The gut associated lymphoid tissue is comprised of cells organised within the lymphoid follicles of the Peyer's patches as well as those distributed throughout the lamina propria and intestinal epithelium. The gastrointestinal lamina propria contains macrophages, dendritic cells, neutrophils, mast cells and lymphocytes that participate in lamina propria effector functions (Hunyady et al. 2000, Stokes and Bailey 2000). The intestinal lamina propria of pigs is heavily populated with plasma cells and B cells predominate around the crypts and T cells in the villi (Stokes and Bailey 2000) which may show signals for TLRs.

TLR2, 3 and 9 were localized in epithelial tissues (Fig. 4, 5 and 6) that form the interface between host and pathogen for numerous pathogens. The mucosal immunity is shouldered by intestinal epithelial cells (IEC). The IEC monolayer provides anatomical and physiological barriers designed to maintain homeostasis within the GIT. IEC recognizes and differentiates between commensals and pathogens via the Toll-like receptor family (Burkey et al. 2009b, Uenishi and Shinkai 2009). With respect to immunosurveillance, the synthesis and secretion of cytokines, chemokines and antimicrobial peptides by IEC is largely accomplished via Toll-like receptors (Akira and Takeda 2004).

TLR2, 3 and 9 proteins were highly localized in the follicular cells and in the M-cells in lymphoid follicles in Peyer's patches in the small intestine (Fig. 4j, 5j, 6j, 4n, 5n, and 6n). Similar results for TLR2 and TLR9 localization are reported in pigs (Shimosato et al. 2005, Tohno et al. 2005, Tohno et al. 2006). Pps are the islands of discrete, organized lymphoid tissue with areas populated by B and T lymphocytes located in the small intestine (Makala et al. 2001). Peyer's patches are sites of antigen sampling and have a role in the induction of mucosal immune responses. After induction in the Pps, mature T and B cells travel to the mesenteric lymph nodes via the lymphatic circulation before homing to the lamina propria, where T cells can directly eliminate pathogens and T and B cells can participate in the production of cytokines and immunoglobulins (e.g., IgA) (Butler et al. 2006). Tyrer et al. (2006) has provided evidence that TLRs are important for M-cell recognition of gram-negative bacteria and to induce an appropriate mucosal immune response. Shimosato et al. (2005) and Tohno et al. (2005) showed that TLRs are localized on porcine M-cells and contribute to ligand-specific transcytosis and thereby induce the immune responses. Since TLRs are highly distributed in the intestinal mucosa, this postulates that porcine intestinal mucosa is highly armed with TLRs for its immune responsiveness.

TLR2, 3 and 9 proteins were localized in the lymphoid cells in the lymphoid follicle and interfolicular cells in mesenteric lymph node (MLN) (Fig. 4r, 5r and 6r). TLR2 and TLR9 positive cells are reported to exist in and between the lymphoid follicles in MLN in pigs (Shimosato et al. 2005, Tohno et al. 2005, Tohno et al. 2006). TLR2 was previously localized in cells in the germinal centre, lymphoid follicles and medullary cord in MLNs in pigs (Tohno et al. 2005). On the other hand, this is the first study to localize TLR3 in GALT in pigs. It has been reported that TLR2, TLR3 and TLR9 and innate cytokines are induced in response to the lactobacilli and human rotavirus in gnobiotic pigs (Wen et al. 2009). Therefore, it might be postulated that lymphoid cells in lamina propria respond to the pathogens via TLRs.

In conclusion, we have confirmed the presence of TLR (1-10) mRNA as well as detected and localized TLR2, 3 and 9 proteins in GALT including mesenteric lymph node collected from pigs with different ages. This study revealed that TLR expression is tissue and age dependent. Higher expression of TLRs with age may indicate that gut-associated lymphoid tissues are continuously exposed to both potential pathogens and beneficial commensal microorganism and this creates a requirement for a homeostatic

balance between tolerance and immunity. This expression-based approach will be helpful to improving our knowledge of immunity in the porcine gut. However, the functional importance of TLR expression must also be put into context by determining the TLR expression profile of different cell types in response to TLR ligands or specific antigens.

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Table 1: Multiplex primer sequences and descriptive information regarding porcine TLR1-10 genes.

Gene	GenBank Accession Number	[a]Length (bp)	[b]Primer sequence 5'→3'
TLR1	NM_001031775	277	F: AGATTTCGTGCCACCCTATG
			R: CCTGGGGGATAAACAATGTG
TLR2	NM_213761	163	F: TGCTATGACGCTTTCGTGTC
			R: CGATGGAGTCGATGATGTTG
TLR3	NM_001097444	149	F: GAGCAGGAGTTTGCCTTGTC
			R: GGAGGTCATCGGGTATTTGA
TLR4	NM_001113039	234	F: TCATCCAGGAAGGTTTCCAC
			R: TGTCCTCCCACTCCAGGTAG
TLR5	NM_001123202	114	F: GGTCCCTGCCTCAGTATCAA
			R: TGTTGAGAAACCAGCTGACG
TLR6	NM_213760.1	170	F: TCAAGCATTTGGACCTCTCA
			R: TTCCAAATCCAGAAGGATGC
TLR7	NM_001097434	317	F: TCTGCCCTGTGATGTCAGTC
			R: GCTGGTTTCCATCCAGGTAA
TLR8	NM_214187	241	F: CTGGGATGCTTGGTTCATCT
			R: CATGAGGTTGTCGATGATGG
TLR9	NM_213958	205	F: AGGGAGACCTCTATCTCCGC
			R: AAGTCCAGGGTTTCCAGCTT
TLR10	NM_001030534	128	F: GCCCAAGGATAGGCGTAAAT
			R: CTCGAGACCCTTCATTCAGC
ACTB	DQ178122	107	F: CTGGCACCACACCTTCTACA
			R: GGGTCATCTTCTCACGGTTG
GADH	DQ178124	100	F: ACTCACTCTTCTACCTTTGATGCTG
			R: TGTTGCTGTAGCCAAATTCA
TBP	DQ178129	121	F: TGGACGTTCGGTTTAGGTTG
			R: GCAGCACAGTACGAGCAACT

[a]Length of the expected amplicons

[b]The primers used for expression analysis in GeXP were chimeric, with the following universal sequence at the 5': Forward primers: AGGTGACACTATAGAATA; Reverse primers: GTACGACTCACTATAGGGA .

Table 2: Effect of age and organ on the relative expression of porcine TLR1-10 genes analysed by Proc GLM (SAS). Summary of the Proc GLM (v.9.2; SAS, SAS Institute Inc., Cary, NC, USA) analysis detecting effect of age, organs and age-organ interaction on the expression of reference candidate genes.

Gene	Mean±S.D.	Organ	Age	R^2	Model
TLR1	0.105±0.08	NS	***	0.45	***
TLR2	0.181±0.12	NS	*	0.35	*
TLR3	0.743±0.26	***	***	0.58	**
TLR4	0.101±0.06	NS	***	0.32	*
TLR5	0.128±0.07	*	***	0.56	***
TLR6	0.294±0.15	NS	***	0.48	**
TLR7	0.057±0.05	**	***	0.51	***
TLR8	0.099±0.08	**	***	0.62	***
TLR9	0.117±0.09	NS	***	0.39	**
TLR10	0.122±0.09	NS	***	0.42	**

$p< 0.05$; ** $p< 0.01$; *** $p< 0.001$; NS non-significant

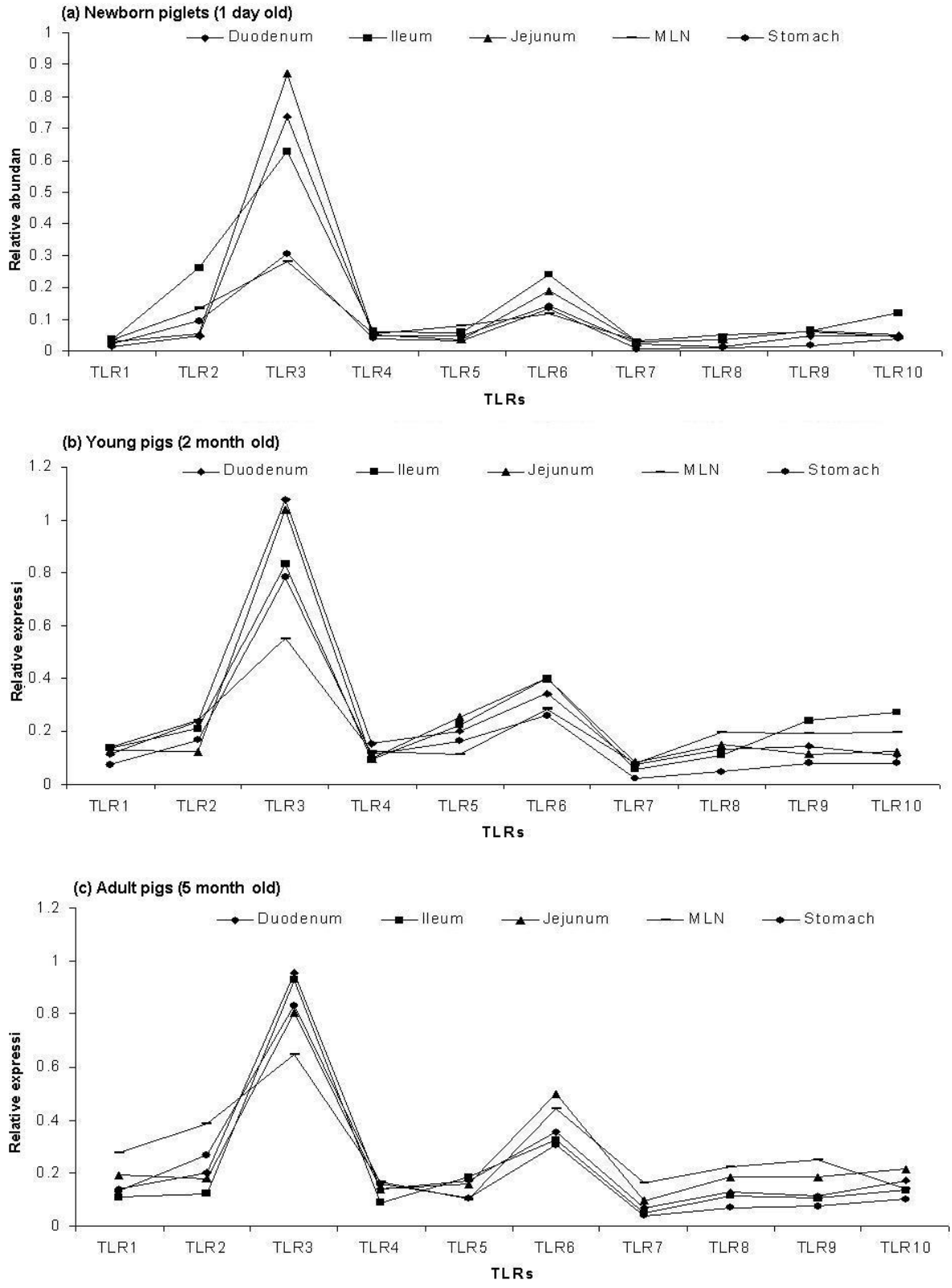

Figure 1: mRNA expression patterns of TLRs in porcine gut-associated lymphoid tissues and mesenteric lymph node. The average expression of Toll-like receptors mRNA in stomach, duodenum, jejunum, ileum and mesenteric lymph node tissues collected from (a) newborn (1 day old) piglets, (b) young (2 month old) pigs and (c) adult (5 month old) pigs.

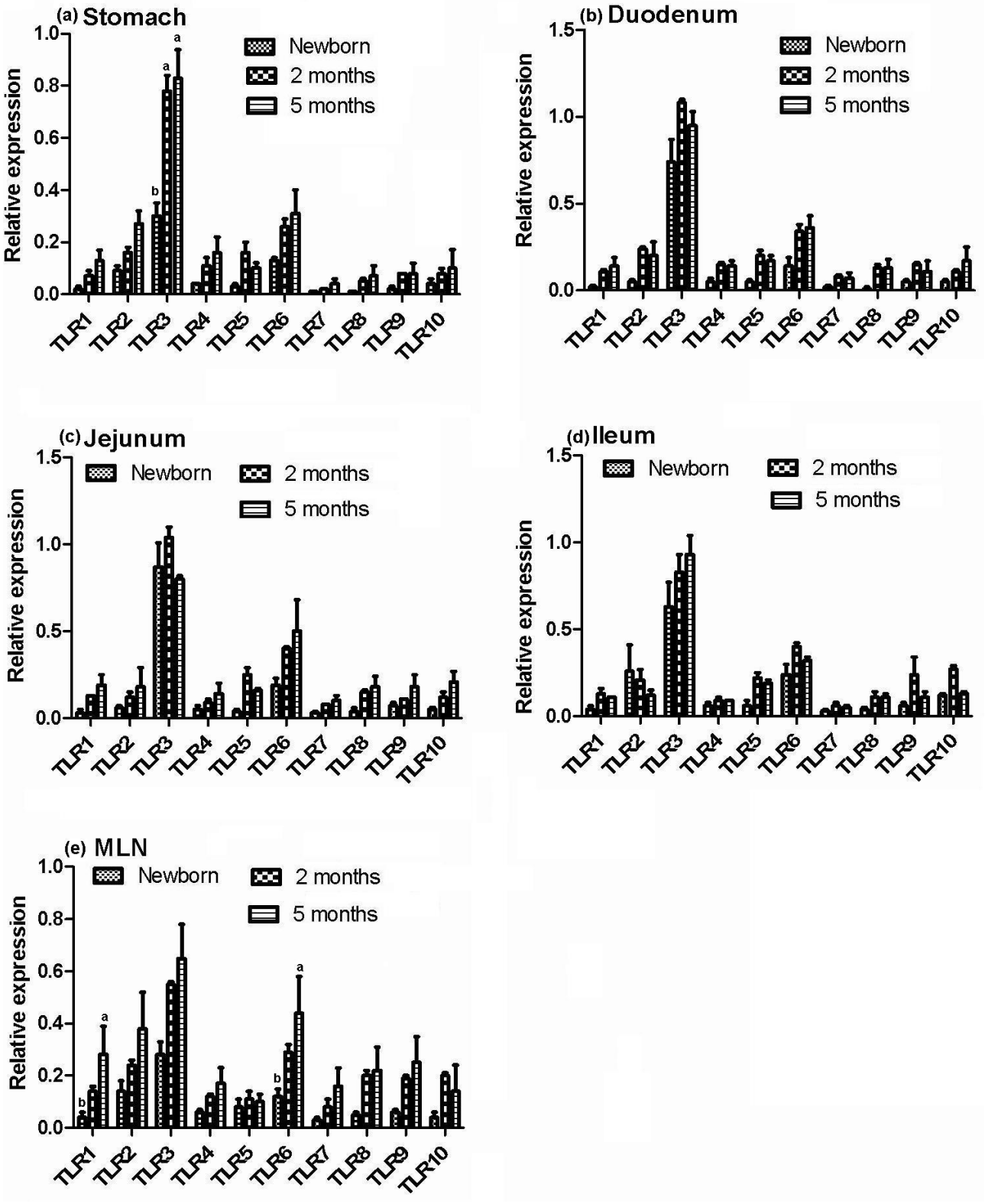

Figure 2: Relative mRNA abundance of TLRs in porcine gut-associated lymphoid tissues and lymph node at different ages. The average expression of TLRs (1-10) mRNA (the bar indicate standard deviation) at 1 day old newborn piglets, 2 month old young pigs and 5 month old adult pigs in a) stomach b) duodenum c) jejunum d) ileum e) mesenteric lymph node (MLN). a,b $P< 0.05$.

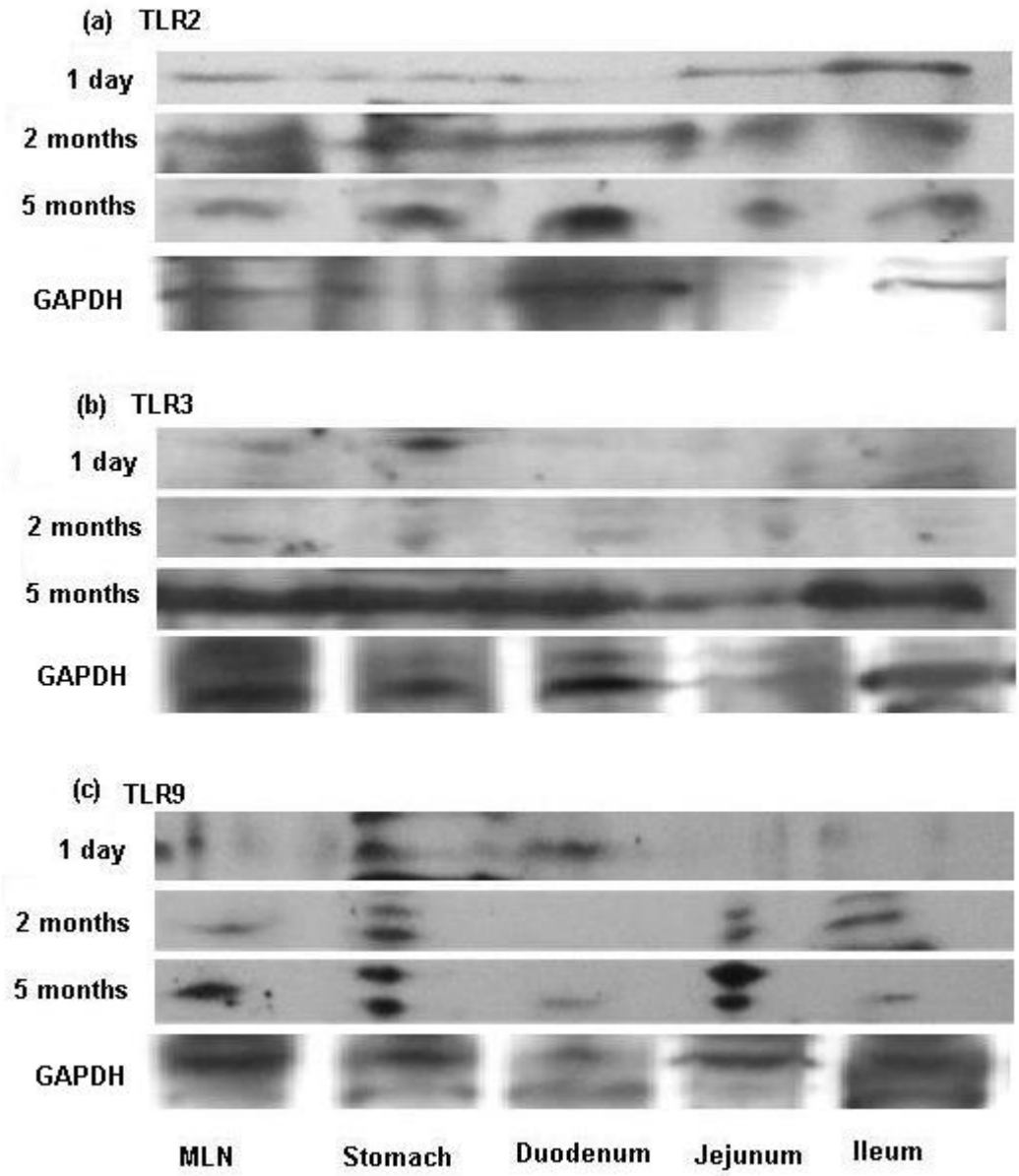

Figure 3: Expression of TLR2, TLR3 and TLR9 protein in gut-associated lymphoid tissues and lymph node. The expression of a) TLR2 b) TLR3 and c) TLR9 protein in mesenteric lymph node (MLN), stomach, duodenum, jejunum and ileum tissues collected from 1 day old newborn piglets, 2 month old young pigs and 5 month old adult pigs.

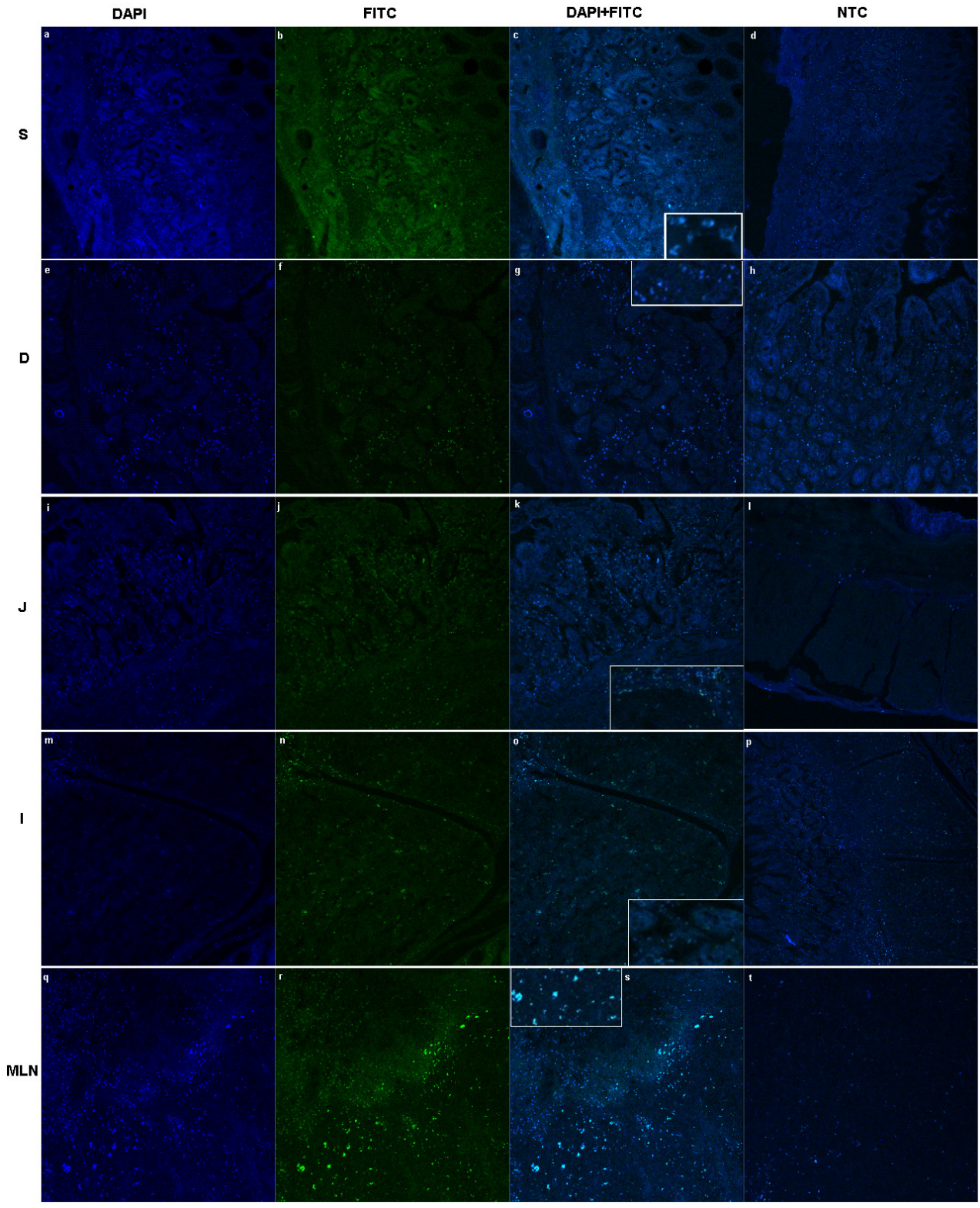

Figure 4: Localization of TLR2 protein in porcine gut-associated lymphoid tissues and lymph node. (4b) Immunofluorescence detection of TLR2 in epithelium cells and gastric gland in the stomach. (4f) TLR2 protein localization in intestinal epithelium

cells, in lymphoid cells in lamina propria, cells in villi and crypts in the duodenum. (4j) TLR2 protein localization in intestinal epithelium cells, in lymphoid cells in lamina propria, cells in villi and crypts in jejunum and around the jejunal Peyer's patches (shown in rectangle). (4n) TLR2 protein localization in the lymphoid cells in lamina propria in ileum and lymphoid cells within and around the ileal Peyer's patches. (4r) TLR2 protein localization in the lymphoid cells in white pulp, trabeculae and in the lymphoid cells in germinal centre in mesenteric lymph node. (4a, e, i, m and q) The cell nuclei were counterstained with DAPI. (4c, g, k, o and s) Merged images. (4d, h, l, p and t) Negative control. Magnification 10X (S: Stomach, D: Duodenum, J: Jejunum, I: Ileum, MLN: Mesenteric lymph node).

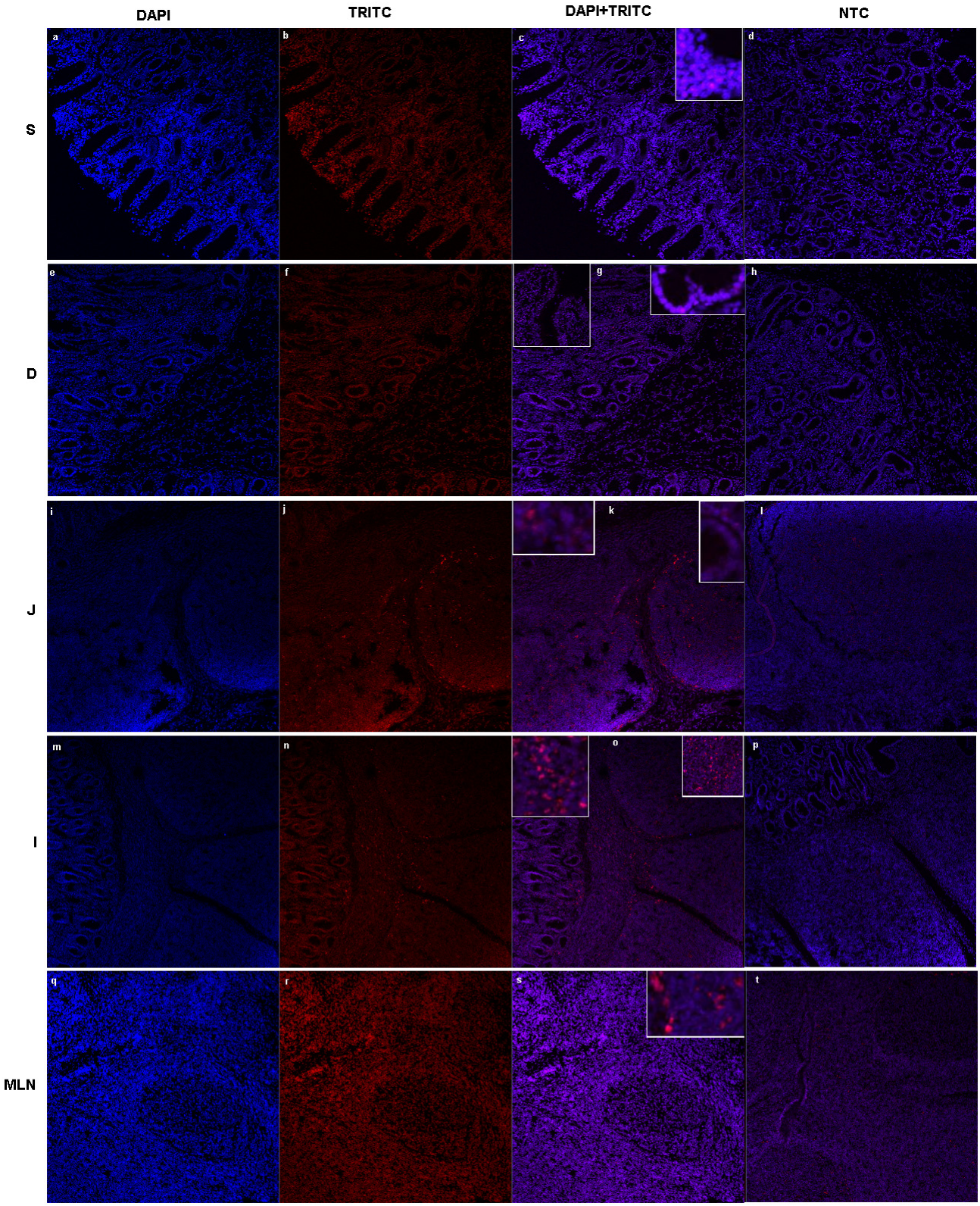

Figure 5: Localization of TLR3 protein in porcine gut-associated lymphoid tissues and lymph node. (5b) Immunofluorescence detection of TLR3 in epithelium cells and gastric gland in the stomach. (5f) TLR3 protein localization in the lymphoid cells in

lamina propria, cells in villi and crypts in the duodenum. (5j) TLR3 protein localization in the lymphoid cells in lamina propria, within and around the jejunal Peyer's patches. (5n) TLR3 protein localization in the lymphoid cells in lamina propria in ileum and lymphoid cells within and around the ileal Peyer's patches. (5r) TLR3 protein localization in the lymphoid cells in white pulp, around the sinus, and in the lymphoid cells in germinal centre in mesenteric lymph node. (5a, e, i, m and q) The cell nuclei were counterstained with DAPI. (5c, g, k, o and s) Merged images. (5d, h, l, p and t) Negative control. Magnification 10X (S: Stomach, D: Duodenum, J: Jejunum, I: Ileum, MLN: Mesenteric lymph node).

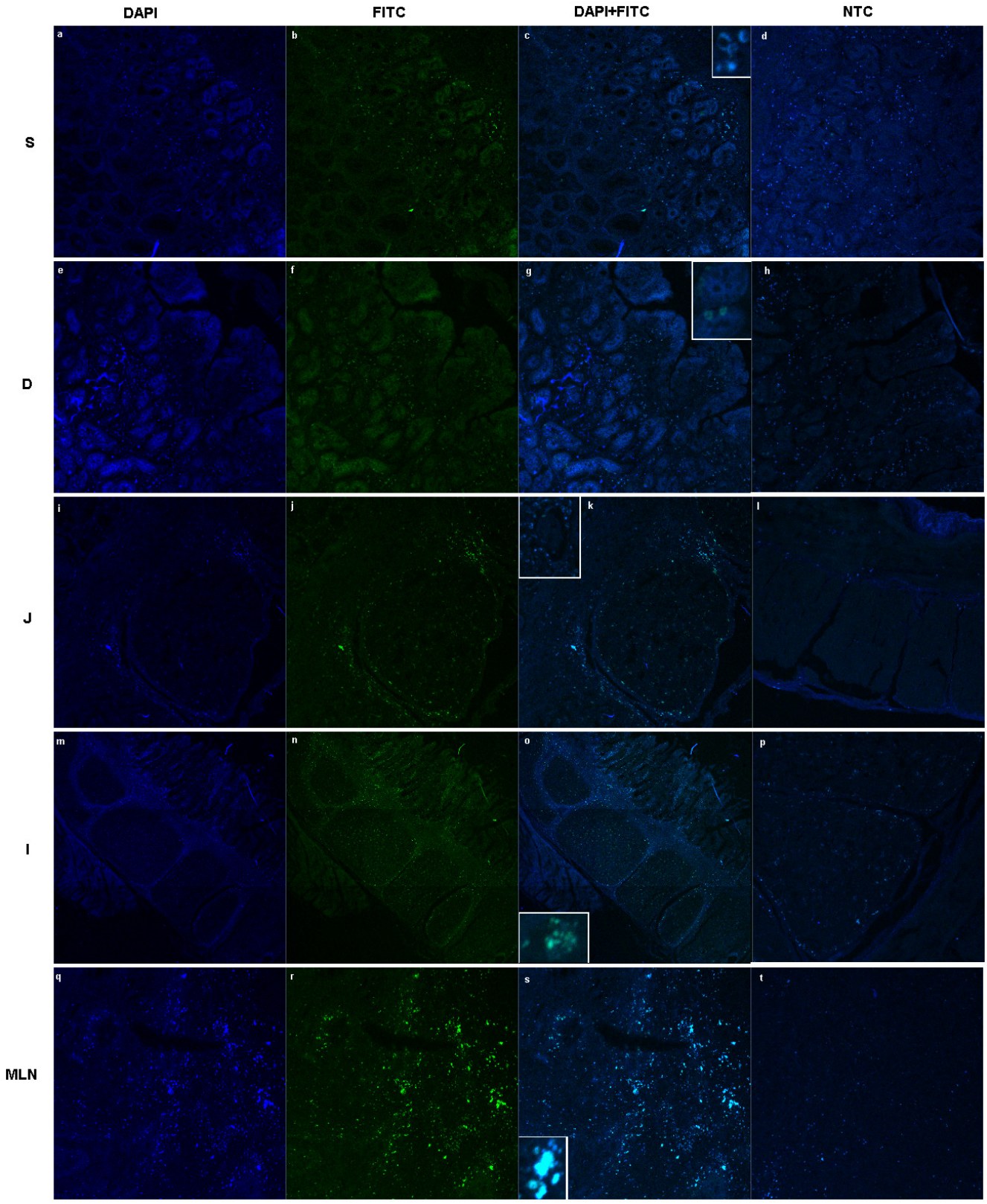

Figure 6: Localization of TLR9 protein in porcine gut-associated lymphoid tissues and lymph node. (6b) Immunofluorescence detection of TLR9 in epithelium cells and gastric gland in the stomach. (6f) TLR9 protein localization in intestinal epithelium

cells, in lymphoid cells in lamina propria and lining cells of duodenal glands. (6j) TLR9 protein localization in lymphoid cells in lamina propria, cells in villi and crypts in jejunum, lymphoid cells within and around the jejunal Peyer's patches. (6n) TLR9 protein localization in epithelial cells in villi and crypts, in the lymphoid cells in lamina propria in ileum and lymphoid cells within and around the ileal Peyer's patches. (6r) TLR9 protein localization in the lymphoid cells in white pulp, around the sinus, and in the germinal centre in mesenteric lymph node. (6a, e, i, m and q) The cell nuclei were counterstained with DAPI. (6c, g, k, o and s) Merged images. (6d, h, l, p and t) Negative control. Magnification 10X (S: Stomach, D: Duodenum, J: Jejunum, I: Ileum, MLN: Mesenteric lymph node).

6. Chapter 5 (under review in: *Veterinary Immunology and Immunopathology*)

Heterogeneous expression of Toll-like receptors 1-10 genes in lymphoid tissues in different ages of pigs

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Short title: Porcine TLRs expression in lymphoid organs

Key words: pigs, innate immunity, lymphoid organ, mRNA, Protein

Abstract

Toll-like receptors (TLRs) function as the pathogen recognition receptors in vertebrate for recognition of microbial components. But the expression patterns of all TLRs have not yet been studied in pigs. Therefore, the aim of this research was to study the expression pattern of the TLR family (TLR1-10) in different lymphoid tissues collected from pigs of different ages. A total of nine clinically healthy pigs of three age groups (1 day, 2 months and 5 months) were selected for this experiment. Each age group consisted of three animals. Cervical lymph node (CLN), thymus, liver, spleen, lung, heart, skin tissue and peripheral blood mononuclear cells (PBMC) were collected for both mRNA and protein isolation. The GenomeLab Genetic Analysis System (GeXP) was used for quantification of mRNA expression of TLRs (1-10) in all tissues, and western blot and immunofluorescence was performed for protein expression and localization of selected TLRs (TLR2, 3 and 9) in selected tissues (CLN, spleen and lungs). mRNA expression showed that TLR1 was highly expressed in the CLN and spleen and moderately in the liver and lungs, whereas TLR2 expression was higher in the liver, lung and spleen. In this study, TLR3 mRNA was the most abundant in all tissues. It was expressed highly in thymus, kidney, lungs and liver. In the case of the spleen, all TLRs (except TLR5) expressions were higher ($p < 0.01$) in 2 month old pigs compared to one day old pigs. In the thymus, TLR3 expression was significantly higher in 2 month old pigs than that of one day and 5 month old pigs. The western blot results of TLR2, 3 and 9 in selected tissues appeared to be consistent with the mRNA expression results. Cells in lungs, spleen and CLN were positively immunostained for TLR2, 3 and 9. Variance analysis showed that both age and organs have an effect on all TLRs expressions ($p < 0.001$). This study sheds light on the expression patterns of TLR (1-10) genes in important lymphoid tissues in pigs of different ages.

Introduction

Toll-like receptors (TLRs) function as pathogen recognition receptors (PRRs) to recognize conserved molecular patterns (pathogen-associated molecular patterns; PAMPs), which are shared by large groups of microorganisms (Akira and Takeda 2004). At least 11 TLRs have been identified in humans and 13 in mice. In other mammals there are at least 10 members of the Toll-like receptor family that recognize specific components conserved among microorganisms. Activation of the TLR leads not

only to the induction of inflammatory responses but also to the development of antigen-specific adaptive immunity (Akira and Takeda 2004). So TLRs are considered as critical proteins linking innate and adaptive immunity. Porcine TLRs are considered as the front line of pathogen monitoring and their PAMPs are used as vaccine adjuvants (Uenishi and Shinkai 2009). Polymorphisms of TLRs are reported to be associated with diseases in pigs (Keirstead et al. 2011, Shinkai et al. 2006b). The immune responsiveness of individuals is reported to depend on the variation of TLR expression level (Jaekal et al. 2007). The tissue, cellular, and sub cellular localization and distribution of TLRs influence the type of immune response elicited. Thus, the first step in understanding the role of TLRs is to determine which TLRs are expressed by specific tissues, organs and cells of interest. In order to gain an understanding of how responsive tissues and cells are likely to be involved at detecting pathogens, TLR mRNA expression patterns have been determined in different species. Expression studies of the complete TLR family (1-10) have been reported in human (Garrafa et al. 2010, Hornung et al. 2002, Siednienko and Miggin 2009), bovine (Menzies and Ingham 2006), ovine (Chang et al. 2009, Nalubamba et al. 2007, Taylor et al. 2008) and chicken (Iqbal et al. 2005). But there is no such complete study of TLRs1-10 expression reported in pigs. Individual expression studies of some TLRs have been performed in pigs such as TLR1, TLR6, and TLR10 (Shinkai et al. 2006a), TLR2 (Alvarez et al. 2008, Tohno et al. 2005), TLR3 and TLR7 (Sang et al. 2008b), TLR4 (Thomas et al. 2006) and TLR9 (Shimosato et al. 2005). Most of these studies have been performed in intestinal tissues especially in gut-associated lymphoid tissue (GALT). TLRs are expressed predominantly in antigen processing and presentation cells such as macrophages, neutrophils, and dendritic cells but TLRs expression is not restricted to these cell types. Since TLRs are vital immune components, it is important to study their expression pattern in tissues or organs related to immune functions. Cervical lymph node (CLN), thymus, liver, spleen, lung, heart, skin tissues and peripheral blood mononuclear cells (PBMC) are vital lymphoid organs or tissues in pigs, protecting the host from pathogens.

The importance of the cervical lymph node in defence against respiratory virus in pigs is reported (Bailey et al. 2000). T-lymphocytes developing within the thymus and thymic B cells produce immunoglobulin (Cukrowska et al. 1996). The spleen is the largest secondary immune organ in the body and is responsible for initiating immune

reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells and aids in the development of white blood cells (reviewed by (Cesta 2006). Liver is the residence for macrophages (Kupffer cells), dendritic cells and liver natural killer (NK) cells that respond to different pathogens in pigs (Skovgaard et al. 2009). The lung is an important immune organ consisting of numerous lymphocytes and macrophages (alveolar macrophages) and fights against most respiratory pathogens in pigs (reviewed by Pabst and Binns 1994). The skin is the interface between the internal milieu and the external environment and acts as a mechanical, physical and biological protective organ (reviewed by Schmitt 1995). Recent studies suggest that the heart possesses an innate immune system that is intended to delimit tissue injury, involved in the pathogenesis of atherosclerosis, acute coronary syndromes, stroke, viral myocarditis, sepsis, ischemia/reperfusion injury, and heart failure as well as orchestrate homoeostatic responses, within the heart. This intrinsic stress response system of heart is mediated by TLRs (reviewed by Mann 2011). Peripheral blood mononuclear cells (PBMC) include different cells (such as lymphocytes, monocytes and macrophages) playing important immune functions in mammals, essential for subsequent sensing in immune monitoring. They are also used as cell lines to study the effect of different antigens, mutagens or vaccines (Hornung et al. 2002, Siednienko and Miggin 2009, Yancy et al. 2001). Notably, the immune responsiveness to antigens or vaccine varies according to the age of the individuals (Panda et al. 2010, van Duin and Shaw 2007) which are thought to be associated with TLRs expression (Dunston and Griffiths 2010, Renshaw et al. 2002, van Duin and Shaw 2007). Age-associated changes of the adaptive immune system are documented in pigs (Dickie et al. 2009, Hoskinson et al. 1990, Uddin et al. 2010); however, studies on the effect of age on innate immune system especially on the TLR expression pattern in pigs are rare. Therefore, the aim of this research was to study the expression patterns of all porcine TLR (1-10) genes in selected immunologically important lymphoid organs or tissues collected from pigs of three different ages.

Materials and methods

Animal and tissue collection

A total of nine clinically healthy male Pietrain pigs of three age groups (neonatal: one day old, young: 2 months old and adult: 5 months old) were selected for this experiment. Each age group consisted of three animals. All pigs were kept at the Frankenforst experimental research farm at the University of Bonn (Germany), according to the rules of German performance stations (Zentralverband der Deutschen Schweineproduktion (ZDS): Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein, 10.12.2003). The animals were fed the same diet *ad libitum* during the whole experimental period. After slaughter, the blood was collected in a heparinized tube and tissues from the cervical lymph nodes (CLN), liver, spleen, thymus, lung, heart and skin from the ear were collected for both mRNA and protein isolation, and for immunohistochemistry. For mRNA and protein isolation, samples were directly put into liquid nitrogen after washing in PBS. For immunofluorescence studies, samples were collected in RNA*later* (Invitrogen) for transportation to the laboratory and were put into Tissue-Tek O.C.T. using cryomold (Sakura). PBMC was isolated from whole blood using Ficoll-Histopaque (Sigma) following the manufacturer's protocol. All samples were kept at -80 ^{o}C until required.

RNA isolation

Total RNA was isolated from individual samples by using Tri-Reagent (Sigma-Aldrich) according to the standard protocol. All samples were kept at -80 ^{o}C until cleanup. In order to remove possible contamination of genomic DNA, the extracted RNA was treated with 5 µl RQ1 DNase buffer, 5 units DNase and 40 units of RNase inhibitor in a 40 µl reaction volume. The mixture was incubated at 37 ^{o}C for 1h followed by purification with the RNeasy Mini Kit (Qiagen). RNA was isolated from PBMC using Picopure RNA isolation kit (cat. KIT0202, Arcturus). Concentration of clean-up RNA was determined spectrophotometrically by using the NanoDrop (ND-8000) instrument and the purity of RNA was estimated by the ratio A260/A280 with respect to contaminants that absorb in the UV. Additional examination of integrity was done by denaturing agarose gel electrophoresis and ethidium bromide staining. Finally, the purified RNA was stored at -80 ^{o}C for further analysis.

TLR mRNA expression analysis

Total RNA, measured by using the NanoDrop ND-8000 spectrophotometer (Thermo Scientific) was diluted to 50 ng/µl. In this study, the GeXP expression profiling method was used, as explained in Rai et al. (2009). mRNA expression of TLR1-10 and three housekeeping genes (*ACTB*, *GAPDH* and *TBP*) were detected in this multiplex system as described earlier (Gandolfi et al. 2011). Briefly, an amount of 250 ng RNA was used as template for reverse transcriptase (RT) reaction using the GenomeLab GeXP Start Kit (Beckman Coulter), in a total volume of 20 µl. In the RT reaction, a pool of all reverse primers (Table 1) at a final concentration of 50 nM was used. Primers were designed using proprietary software provided by Beckman-Coulter. Each of these primers is chimeric, having a 3′ gene-specific end and a 5′ end containing a quasi-T7 universal sequence, which serves as a template in subsequent amplification steps. The RT reaction was performed under the following conditions: 1 min at 48 °C, 60 min at 42 °C, 5 min at 95 °C, hold at 4 °C, in a thermal cycler (Bio-Rad). After RT reaction, 9.3 µl of the products were used as template for a PCR with 20 nM of each forward primer and 1 U Beckman Coulter Thermo-StartR DNA Polymerase (Beckman Coulter). Each of the forward primers contains an SP6 universal sequence at the 5′ end and a gene-specific sequence at the 3′ end (Table 1). The PCR reaction was performed in a thermal cycler under the conditions: 10 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 70 °C; hold at 4 °C. PCR products were electrophoretically separated by the fragment analysis method (Frag-3) on the GenomeLab GeXP (Beckman Coulter), by diluting 1 µl PCR reaction with 28.5 µl SLS buffer and 0.50 µl size standard-400 (Beckman Coulter). Kanamycin RNA internal positive control was included and produced a peak at 326 bp when samples were separated via electrophoresis. All experiments include “no template” (i.e. without RNA) and “no enzyme” (i.e. no reverse transcriptase) negative controls to confirm the absence of peaks at the expected target sizes. The “no template” sample produces a single peak at 326 bp, corresponding to the externally spiked-in kanamycin RNA. Electrophoretic separation was done by GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Fullerton, USA). The GenomeLab GeXP software matches each fragment peak with the appropriate gene, and reports peak height and area under curve (AUC) for all peaks in the electropherogram. These data were exported from the expression analysis module of the GenomeLab GeXP software as expression data for subsequent analyses.

The expression of TLR1-10 genes was normalized by dividing for the geometric mean of the expression of three house keeping genes (*ACTB*, *GAPDH* and *TBP*). These normalized expression values were used for further statistical analysis using SAS ver9.2 (SAS Institute Inc., Cary, NC, USA). The average of the TLR expression value of three animals was considered for further analysis.

Western blot analysis of TLR2, TLR3 and TLR9 proteins

Three tissues (spleen, lung and cervical lymph node) were selected in each age group for the western blot study. Whole cell protein was extracted from tissues following standard protocol using Nonidet-P40 buffer along with protease inhibitor 1 mM (final concentration) PMSF (phenylmethylsulfonyl fluride). The protein from each sample of three animals in each age group was pooled together according to tissue for western blot. The protein was separated by 4-18% gradient SDS-PAGE. Subsequently the proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). After blocking in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% Polyvinylpyrolidone) at room temperature for 1 h, the membrane was incubated with the primary antibody anti-TLR2 and anti-TLR9 purified from rabbit polyclonal antibody (THU-A-TLR2 and THU-A-TLR9, CosmoBio Co Ltd.) in the blocking medium (diluted 1:700) at 4 ^{o}C overnight. Anti-TLR3 antibody (SC-8691; Santa Cruz) purified from goat (diluted 1:500) was used as primary antibody for TLR3. Non-specific binding of antibody was washed off with six changes of 0.1% PBST (10 min per time). As a secondary antibody, the horseradish peroxidase conjugated donkey anti-goat IgG antibody (SC2020; Santa Cruz) was used (diluted 1:50000) for TLR3 and the horseradish peroxidase conjugated goat anti-rabbit IgG antibody (SC2004; Santa Cruz) was used (diluted 1:50000) for TLR2 and TLR9. The membrane was incubated for 1 h at room temperature with the secondary antibody, followed by washing with six changes of 0.1 % PBST (10 min per time). The chemiluminesce was detected by using the SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific) and was visualized by using Kodak BioMax XAR film (Kodak). GAPDH (SC20357; Santa Cruz) was used as a loading control and for normalization. The membrane was stripped by washing 3 times (5 min per time) in 20 ml of glycin (0.1 mol/L; pH 2.5) and then washed with 20 ml (1x) PBS, 3 times (5 min per time) and re-probed.

Immunofluorescence localization of TLR2, TLR3 and TLR9 proteins

Three tissues (spleen, lung and cervical lymph node) were selected for the localization of TLR2, TLR3 and TLR9 proteins. For each of the TLR2, 3 and 9, immunofluorescence staining was performed on 8 μm cryostat sections. All sections were kept at -80 ºC for further analysis. To block unspecific staining, sections were incubated for 60 min at room temperature with 3 % bovine serum albumin in PBS (50 nM sodium phosphate, pH 7.4; 0.9 % NaCl). Sections were incubated overnight at 4 ºC with the same primary antibodies as used in western blot diluted at 1:50 in blocking solution followed by six (10 min per time) washing with PBS. Sections for TLR3 were incubated with the TLR3 goat polyclonal primary antibody (SC8691; Santa Cruz) (dilution 1:50 in blocking solution), whereas the sections for TLR2 and TLR9 were incubated with the rabbit anti-porcine TLR2 and TLR9 polyclonal primary antibody (THU-A-TLR2 and THU-A-TLR9, CosmoBio Co Ltd.) (dilution 1:50 in blocking solution), overnight at 4 ºC and subsequently the sections were washed six times (10 min per time) with PBS. The donkey anti-goat IgG-B conjugated with rhodamine (TRITC) reactive water-soluble fluorescent dye (SC2094; Santa Cruz) (dilution 1:200) was used for TLR3 and the biotinylated donkey anti-rabbit IgG-B conjugated with fluorescein isothiocyanate (FITC) reactive water-soluble fluorescent dye (SC2090; Santa Cruz) (dilution 1:200) was used for TLR2 and TLR9 as a secondary antibody, respectively. Finally, the samples were counterstained with vectashield mounting medium (Vector Laboratories) containing 40,6-diamidino-2-phenyl indole (DAPI) and covered with a cover glass slip. The staining was observed by confocal laser scanning microscope (Carl Zeiss). In the case of negative controls, PBS was used instead of the primary antibody.

Statistical analysis

The Proc GLM (ver9.2; SAS, SAS Institute Inc., Cary, NC, USA) analysis was performed to detect the effect of age and organs on the expression of TLR genes. Differences in gene expression levels between groups were determined using t-test in SAS. $p<0.05$ was considered statistically significant.

Results

TLRs mRNA expressions

The mRNA expression study showed that the TLR expressions were higher in young and adult pigs than the newborn piglets. In this study, *TLR3* showed comparatively higher expression than the other TLRs in the different tissues (Fig. 1). In the case of the cervical lymph node (CLN), the highest expression of TLRs was detected in young pigs, moderate expression was detected in adult pigs and comparatively lower expression was detected in newborn piglets (Fig. 2a). Moreover, the *TLR6* expression was higher ($p < 0.05$) in CLN in young pigs compared to newborn piglets. TLRs expression pattern in the lungs showed that mRNA increased with age (Fig. 2b). *TLR4* was expressed higher ($p < 0.001$) in the lungs in adult pigs than that in newborn piglets (Fig. 2b). The TLRs expression pattern in the spleen showed that the highest expression was in young pigs (Fig. 2c), followed by adult and newborn pigs (Fig. 2c). All TLR expression, except *TLR6*, was significantly higher in young pigs than in the newborn piglets in the spleenic tissue (Fig. 2c). In the case of the thymus, it could be shown that TLR expression was higher in young than adult pigs and newborn piglets (Fig. 2d). *TLR3* expression was higher ($p < 0.01$) in the thymus in young pigs than in the adult and newborn animals (Fig. 2d). Except *TLR1*, *TLR5* and *TLR10*, all TLRs were expressed higher in young animals in the liver (Fig. 2e). *TLR1* and *TLR5* expressions increased with age in liver tissue, especially *TLR5* which was expressed significantly ($p < 0.001$) higher in adult pigs compared to newborn piglets (Fig. 2e). In the case of the kidney, except *TLR3* and *TLR10*, TLRs showed common pattern of expression which implied that the highest expression was detected in young pigs and lowest expression was found in newborn piglets (Fig. 2f). In skin tissue, *TLR5* expression was higher *(*$p < 0.05$) in young pigs compared to the newborn piglets (Fig. 2g). TLR expression in the heart showed that TLR mRNA expression was higher in young than the adult pigs and newborn piglets (Fig. 2h). The TLR expression was more heterogeneous in PBMC than in other lymphoid tissues (Fig. 2i). TLR3, 4, 5, 7 and 8 expressions were higher in newborn, whereas TLR1, 2, 6, 9 and 10 expressions were higher in young pigs (Fig. 2i).

Expression of TLR2, TLR3 and TLR9 proteins in selected tissues

TLR2 protein was expressed in the lungs, spleen and cervical lymph node (CLN) collected from pigs of different ages (Fig. 3a). TLR2 protein was scarcely detectable in lung tissue collected from newborn piglets. Higher expression of TLR2 protein was detected in tissues collected from young animals (Fig. 3a). TLR3 protein expression was higher in the lungs, spleen and CLN collected from young pigs followed by adult pigs (Fig. 3b). TLR3 protein was also detectable in selected tissues collected from newborn piglets, but the expression was low compared to the young pigs (Fig. 3b). TLR9 protein was detected in lungs, spleen and CLN tissues collected from all age groups in this study (Fig. 3c). TLR9 protein expression was in lungs and spleen tissues collected from all pigs, but in the case of the CLN, TLR9 expression was higher in young compared to adult pigs and newborn piglets (Fig. 3c).

Localization of TLR2, TLR3 and TLR9 proteins in selected tissues

TLR2 and TLR9 proteins were localized in the alveolus, lining cells of bronchioles and in the smooth muscle layer surrounding the bronchioles (Fig. 4a, 6a). TRL3 was localized in the squamous cells of the alveolus (Fig. 5a). TLR2, TLR3 and TLR9 proteins were found to be expressed in the spleen (Fig. 4b, 5b and 6b). TLR2 protein was highly localized in the lymphoid cells in the white pulp and in the cells within the follicles in the spleen (Fig. 4b). TLR3 protein was stained homogenously in the cells in red pulp and white pulp in spleen tissue (Fig. 5b). Though TLR9 protein was localized in cells across the spleen, higher staining could be found around the artery and in the trabecules in the spleen (Fig. 6b). TLR2, TLR3 and TLR9 proteins were stained in the lymphocytes and macrophages in the lymph node, especially in the lymphoid follicle in the cortex (Fig. 4c, 5c and 6c). TLR3 protein was expressed in the germinal centre as well as in the lymphoid follicle in the lymph node (Fig, 4c).

Discussion

Toll-like receptors (TLRs) are of great interest to the research community due to their ability to recognize pathogens and initiate development of an immune response. TLR expression is not restricted to cell types or organs but only few studies were devoted to investigate the expression patterns of selected TLRs in pigs (Dvorak et al. 2006, Shimosato et al. 2005, Tohno et al. 2005, Tohno et al. 2006). In the present study, we

investigated the expression patterns of TLRs (1-10) in various porcine lymphoid tissues related to immune functions in order to understand how distinct immune system receptors may vary at different ages. This work has confirmed that each of the 10 TLR genes were expressed in an age-dependant manner (Fig. 1). Heterogeneous expression of genes according to age have been described in humans (Somel et al. 2006). TLR1-10 were expressed heterogeneously across the selected tissues (Fig. 1 and Fig. 2) which coincided with the study of (Garrafa et al. 2010) who reported that TLR1-10 expressions were heterogeneous in lymphatic endothelial cells derived from different tissues in humans. It is important to note that genetic, environmental, demographic and technical factors are reported to have substantial effects on gene expression levels (Leek and Storey 2007). In our previous study we have found that in response to antigens, there are age-dependent variations in the serum level of TLR2 and TLR9 proteins in pigs (Uddin et al. 2011). TLR expression profiles are suggestive of an individual's ability to respond to challenge (Menzies and Ingham 2006) and species-specific differences in recognition of TLR ligands have been observed between man and mouse (Roberts et al. 2005). These differences presumably reflect the distinct selective pressure on each host to adapt to new environments and pathogens (Chang et al. 2009). However, the first step in understanding the role of TLRs is to determine which TLRs are expressed by tissues and cells of interest.

In this study, at least the mRNA of all 10 porcine TLRs were detectable in all lymphoid tissues collected from pigs of different ages. As a lymphoid organ, the lymph node plays crucial immune functions but TLR expression studies rarely included this tissue. Most of the studies analyzing TLR expression in pigs included the mesenteric lymph node (MLN) (Sang et al. 2008b, Shimosato et al. 2005, Tohno et al. 2005, Tohno et al. 2006). Such as *TLR2* (Tohno et al. 2005, Tohno et al. 2006), *TLR3* and *TLR7* (Sang et al. 2008b), and *TLR9* (Shimosato et al. 2005) are reported to be expressed in porcine MLN. Thomas et al. (2006) detected *TLR4* mRNA expression in the lymph node, which was lower than that found in the spleen of the pigs. Heterogeneous TLR1-10 expression is reported in the prefemoral lymph node in sheep (Chang et al. 2009) and in the lymphocytes isolated from the lymph node in cats (Ignacio et al. 2005). In this study, *TLR6* expression was significantly higher in young pigs compared to newborn piglets (Fig. 2a). TLR6 is important in the recognition of *Mycoplasma hypopneumoniae* in pigs (Muneta et al. 2003). The importance of cervical lymph node (CLN) to protect

respiratory infections has been reported in pigs (Bailey et al. 2000) indicating that CLN might play an important role in the recognition of respiratory pathogens. This study identified the distribution of TLR2, 3 and 9 proteins in the lymphoid cells within the lymph node for the first time in pigs. The lymph node contains numerous T and B lymphocytes which are the important components of the immune system. Lymphocytes play an important and integral role in the body's defenses while offering protection against varieties of pathogens including bacteria and viruses. Bacterial lipoprotein, dsRNA and unmethylated CpG DNA of virus are the ligands for TLR2, 3 and 9, respectively (Akira and Takeda 2004) suggesting that CLN might have roles in recognizing of pathogenic bacteria and viruses. The lungs are armed with specialized cells known as alveolar macrophages and fight against most pathogens invading though the respiratory route. We found heterogeneous expression of TLRs in porcine lungs where all TLR expression increased with age (Fig. 2b). Recently, similar findings were reported that during postnatal life the porcine alveolar macrophage function changes in an age-dependent manner (Dickie et al. 2009). *TLR1*, *TLR6* and *TLR10* (Shinkai et al. 2006a), *TLR2* (Alvarez et al. 2008, Tohno et al. 2005), *TLR3* and *TLR7* (Sang et al. 2008b), *TLR4* (Thomas et al. 2006, Wassef et al. 2004) and *TLR9* (Schneberger et al. 2010, Shimosato et al. 2005) mRNA were previously detected in porcine lungs. Shimosato et al. (2005) reported that *TLR9* mRNA expression was higher in lung tissues collected from one year old adult pigs compared to neonatal pigs. *TLR3* was expressed higher than other TLRs in lung tissue indicating that it may have a role in the lungs. Recently, Sacco et al. (2011) reported that TLR3 plays a key role in porcine lungs to recognize influenza virus infection. It has been reported that PRRSV (porcine reproductive and respiratory syndrome virus) persists in the host's body by suppressing *TLR3* (Sang et al. 2008a). TLR2 and TLR6 are reported to be involved in the recognition of *M. hypopneumoniae* by the porcine alveolar macrophages (Muneta et al. 2003). TLR2 is reported to cooperate with TLR6 in response to mycoplasma lipopeptide in mice (Takeda et al. 2002). In this study the expression of *TLR2* and *TLR6* showed a trend to increase with age. Dickie et al. (2009) reported that functional maturation of alveolar macrophage occurs mainly during the first week of postnatal life in pigs. Shinkai et al. (2006a) reported that *TLR10* mRNA expression was higher in porcine lungs than the *TLR1* and *TLR6* but we found similar pattern of expression for *TLR1*, *TLR6* and *TLR10*. Transcripts of all TLRs, except *TLR7*, were detected in ovine alveolar

macrophage (Chang et al. 2009) using qRT-PCR. TLR2, 3 and 9 proteins were localized in the lungs in this study. TLR9 protein was previously localized in porcine lungs especially in the alveolar endothelium and alveolar macrophages by Schneberger et al. (2010) which is in agreement to our results (Fig. 6a).

All porcine TLRs were expressed most remarkably in the spleen. Importantly, this study first detected the *TLR5* and *TLR8* mRNA expressions in porcine spleen. The spleen is the largest secondary lymphoid organ containing about one-fourth of the body's lymphocytes and initiates immune responses to varieties of blood-borne antigens (reviewed by Cesta 2006). The spleen expressed all TLRs which indicate that it may need to recognize varieties of blood-born pathogens. All TLRs except TLR5 expression were significantly higher in young pigs compared to newborn piglets. Age-related changes in spleenic functions and cellular contents are reviewed by Cesta (2006). It has been reviewed that by 2 days of age first T-cells appear, by day 5 dendritic precursors appear, after which B-cell follicles begin to develop, and immunologic function begins at 14 days of age in rats (Cesta 2006). The spleen reaches peak development at puberty in rats, followed by involution; whereas lymphocyte numbers decrease with age in dog and rodents (reviewed by Cesta 2006). However, such type of data is not available in pigs. In comparison, *TLR5* mRNA expression was low in the spleen suggesting a limitation of the spleen to respond to flagella-associated pathogenic bacteria (recognized by *TLR5*). TLR2, 3 and 9 immunostaining was detected in spleenic cells, especially TLR2 and TLR9 which were expressed strongly in the white pulp and around the arteriole, respectively, in this study. It is important to note that, the immune functions of the spleen is charged to the white pulp which surrounds the central arterioles and the white pulp is composed of lymphocytes, macrophages, dendritic cells and plasma cells (Cesta 2006).

Heterogeneous expression of TLRs were detected in the thymus where *TLR3* increased significantly in 2 month old young pigs compared to 1 day old piglets or 5 month old adult pigs. Age-related changes in the cellular composition of thymus in 8 day to 8 year old children are reported (Weerkamp et al. 2005) where thymi of children 3 to 6 month old appeared to be the most active. Shimosato et al. (2005) compared *TLR9* mRNA expression between two age groups of pigs, where *TLR9* expression was higher in the thymus from one year old adult compared to neonatal piglets. Zhang et al. (2008) detected *TLR7* mRNA in different lymphoid tissues like spleen, lymph node, tonsils and

lungs in pigs but could not detect any in the thymus, heart, liver, kidney or skin. Reportedly, expression of *TLR7* and *TLR9* has been detected in feline thymus (Ignacio et al. 2005).

Heterogeneous TLR expression was detected in porcine liver in this study. Since TLRs first recognize the pathogens and initiate the inflammatory response (Akira and Takeda 2004), this data is in agreement with the recent study reported that ageing modulates liver immune responses to infection in mice (Speziali et al. 2010) where T cells, B cells and NK cells of young mice are more immune reactive to infection than that of older mice. Moreover, age-dependent changes in acute phase proteins (APPs) levels are reported in calves (Orro et al. 2008) in which APPs are decreased in 2 month old calves compared to 3 week old calves. In comparison, *TLR5* expression was abundant in the liver, suggesting a predisposition for the recognition of flagella associated pathogenic bacteria by the liver. Motile microorganisms cause liver sepsis and flagellin is the main flagellar protein and is the ligand of TLR5 (Akira and Takeda 2004). It is interesting to note that in all tissues, expression patterns of *TLR1* and *TLR6* were the same which coincides with previous findings in bovine tissues (Opsal et al. 2006). *TLR1* and *TLR6* are closely clustered genes, co-regulated and they are ubiquitously expressed (Opsal et al. 2006). The kidney is a tertiary immune organ. Intrarenal B cells enhance the immunological response by functioning as antigen presenting cells, and act as a source for cytokines promoting T cell proliferation and lymphatic neoangiogenesis (reviewed by (Segerer and Schlondorff 2008). This study detected mRNA expression of all ten porcine TLRs in the kidney. In normal skin, the keratinocytes constitutively expressed *TLR1*, *TLR2* and *TLR5*, but barely expressed *TLR3* and *TLR4* in human skin tissue (Baker et al. 2003). But Chang et al. (2009) and Fitzner et al. (2008) detected all ten TLRs in sheep and human skin, respectively. TLR4 protein was previously localized (Wassef et al. 2004) and *TLR3* and *TLR7* mRNA were detected (Sang et al. 2008b) in normal porcine skin. This study detected the mRNA of all TLRs in pig skin. This might explain the wide ranges of defense mechanism of skin that fight against a variety of pathogens including bacteria, viruses, fungus, parasites, allergens and environmental antigens.

The mRNA of *TLR2* (Tohno et al. 2005) and *TLR9* (Shimosato et al. 2005, Tohno et al. 2006) were previously detected in porcine cardiac tissues. Shimosato et al. (2005) reported that the *TLR9* gene was expressed higher in heart tissue collected from adult

compared to neonatal pigs. Although cardiac TLR expression has been reported in other species, this study is the first to present evidence that all these ten innate immune receptors are expressed in the porcine heart. Knowledge of the expression pattern of these receptors in the normal porcine heart is, however, of immediate importance for further mechanistic studies pertaining to innate immunity and the heart. *TLR2* and *TLR4* gene expression are detected in healthy canine heart tissue (Linde et al. 2007), whereas mRNA expression of ten TLRs were identified in the heart in human (Nishimura and Naito 2005). It is important to note that the heart is the first location reached by blood from the systemic circulation. This may explain the heterogeneous expression of TLRs in this organ to allow early detection of noxae especially the blood borne pathogens. Since TLRs are essential signaling molecules governing an innate immune response, and because mediators of inflammation and innate immunity with increasing certainty are proven to play key roles in different types of cardiovascular diseases, it could be suggested that the normal porcine heart expresses TLRs as a natural part of its intrinsic defense system (Linde et al. 2007). Alterations in the TLR expression levels in peripheral blood mononuclear cells (PBMCs) have been reported in various infections and have been directly correlated with plasma viral load or associated with the severity of disease outcomes (de Kruif et al. 2008, Lester et al. 2008). PBMCs are immunocompetent cells and the mRNA expression of all members of TLR family (TLR1-10) have been detected in PBMCs (Hornung et al. 2002, Siednienko and Miggin 2009) as well as in all subsets of PBMCs such as in monocytes and dendritic cells in humans (Kokkinopoulos et al. 2005). Zhang et al. (2008) detected the *TLR7* mRNA expression in porcine PBMCs as well as in macrophages, B- and T-cells. Alvarez et al. (2008) identified porcine TLR2 protein expression on monocytes and macrophages but could not detect it in peripheral blood lymphocytes by flow cytometry. All TLR transcripts are detected in RNA from ovine PBMCs (Chang et al. 2009) and in feline T cells (Ignacio et al. 2005). However, this is the first study detecting all porcine TLR transcripts in PBMCs which suggest that PBMCs may represent a useful TLR-responsive model cell line for examining TLR1-10 signaling events.

In summary, we have confirmed or established the presence of TLR1–10 in various porcine lymphoid tissues. TLRs exhibit marked differential tissue activity and their levels within a discrete cell type can be highly dynamic. TLR expression is extremely variable among individuals. This quantitative assessment of TLR expression in

immunocompetent tissues or cells in pigs of different ages will open new avenues in the field of porcine TLR research. Assays such as these will help to improve our understanding of the early events controlling immunological development in livestock.

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Table 1: Multi-plex primer sequences and descriptive information regarding genes used for the experiment

Gene	GenBank Accession Number	[a]Length (bp)	[b]Primer sequence (5'→3')
ACTB	DQ178122	107	F: CTGGCACCACACCTTCTACA R: GGGTCATCTTCTCACGGTTG
GADH	DQ178124	100	F: ACTCACTCTTCTACCTTTGATGCTG R: TGTTGCTGTAGCCAAATTCA
TBP	DQ178129	121	F: TGGACGTTCGGTTTAGGTTG R: GCAGCACAGTACGAGCAACT
TLR1	NM_001031775	277	F: AGATTTCGTGCCACCCTATG R: CCTGGGGGATAAACAATGTG
TLR2	NM_213761	163	F: TGCTATGACGCTTTCGTGTC R: CGATGGAGTCGATGATGTTG
TLR3	NM_001097444	149	F: GAGCAGGAGTTTGCCTTGTC R: GGAGGTCATCGGGTATTTGA
TLR4	NM_001113039	234	F: TCATCCAGGAAGGTTTCCAC R: TGTCCTCCCACTCCAGGTAG
TLR5	NM_001123202	114	F: GGTCCCTGCCTCAGTATCAA R: TGTTGAGAAACCAGCTGACG
TLR6	NM_213760.1	170	F: TCAAGCATTTGGACCTCTCA R: TTCCAAATCCAGAAGGATGC
TLR7	NM_001097434	317	F: TCTGCCCTGTGATGTCAGTC R: GCTGGTTTCCATCCAGGTAA
TLR8	NM_214187	241	F: CTGGGATGCTTGGTTCATCT R: CATGAGGTTGTCGATGATGG
TLR9	NM_213958	205	F: AGGGAGACCTCTATCTCCGC R: AAGTCCAGGGTTTCCAGCTT
TLR10	NM_001030534	128	F: GCCCAAGGATAGGCGTAAAT R: CTCGAGACCCTTCATTCAGC

[a]Length of the expected amplicons

[b]The primers used for expression analysis in GeXP were chimeric, with the following universal sequence at the 5': Forward primers: AGGTGACACTATAGAATA; Reverse primers: GTACGACTCACTATAGGGA .

Table 2: Effect of age and organ on the relative expression of porcine TLR1-10 genes analyzed by Proc GLM (SAS). Summary of the Proc GLM (ver.9.2; SAS, SAS Institute Inc., Cary, NC, USA) analysis detecting effect of age, organs and age-organ interaction on the expression of reference candidate genes.

Gene	mean±SD	Organ	Age	Organ*Age	Model	R^2
TLR1	0.081±0.08	***	***	*	***	0.713
TLR2	0.201±0.14	***	***	**	***	0.782
TLR3	0.570±0.35	***	***	**	***	0.856
TLR4	0.127±0.11	***	***	***	***	0.805
TLR5	0.109±0.12	***	***	**	***	0.737
TLR6	0.182±0.13	***	***	**	***	0.801
TLR7	0.045±0.53	***	***	***	***	0.789
TLR8	0.114±0.14	***	***	***	***	0.837
TLR9	0.106±0.11	***	***	**	***	0.745
TLR10	0.091±0.09	***	***	***	***	0.771

* $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$

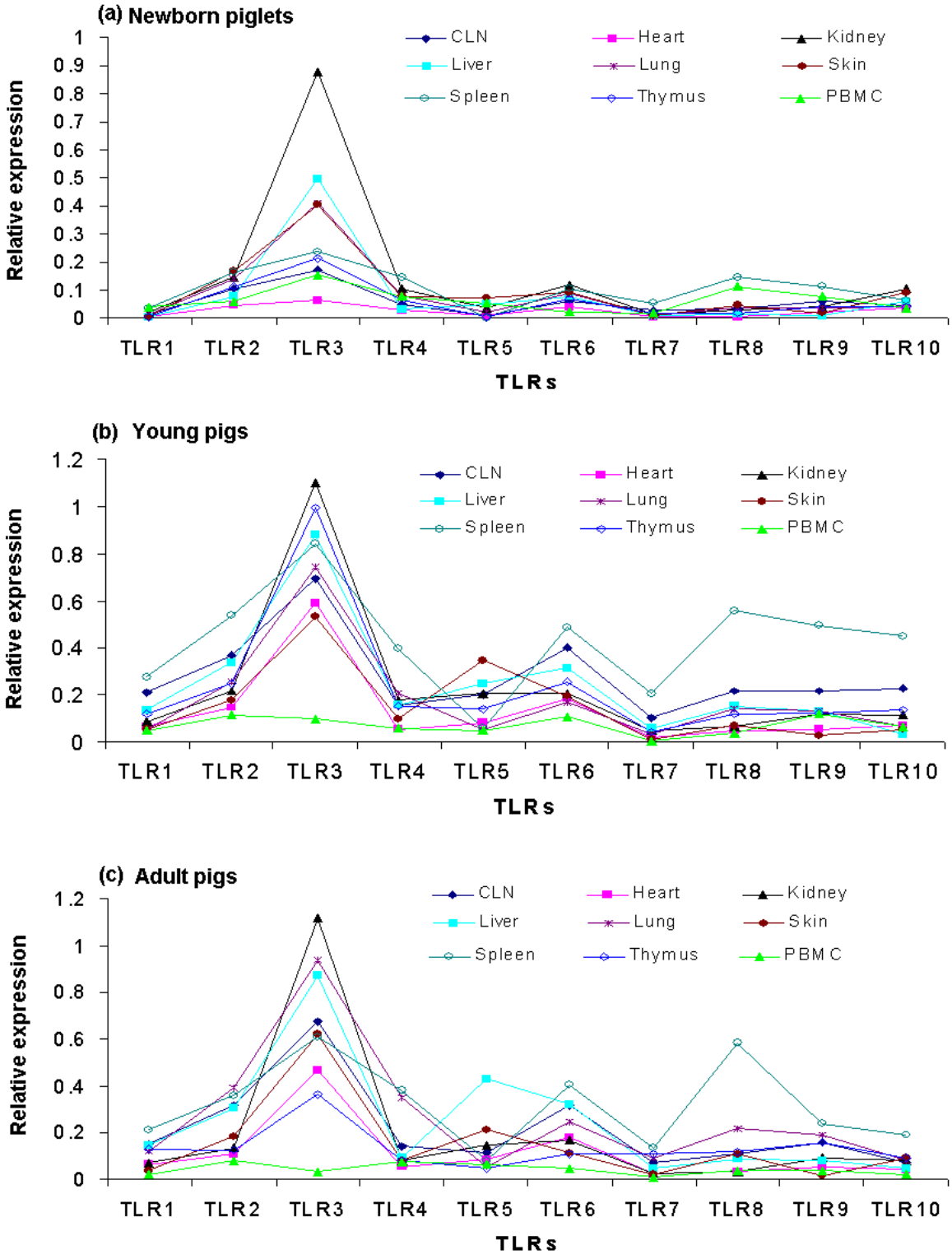

Figure 1: mRNA expression patterns of TLRs in porcine lymphoid tissues. The average expression of Toll-like receptors mRNA in CLN (cervical lymph node), lung, spleen, thymus, liver, kidney, skin, heart and PBMC (peripheral blood mononuclear cells) collected from (a) newborn (1 day old) piglets, (b) young (2 month old) pigs and (c) adult (5 month old) pigs.

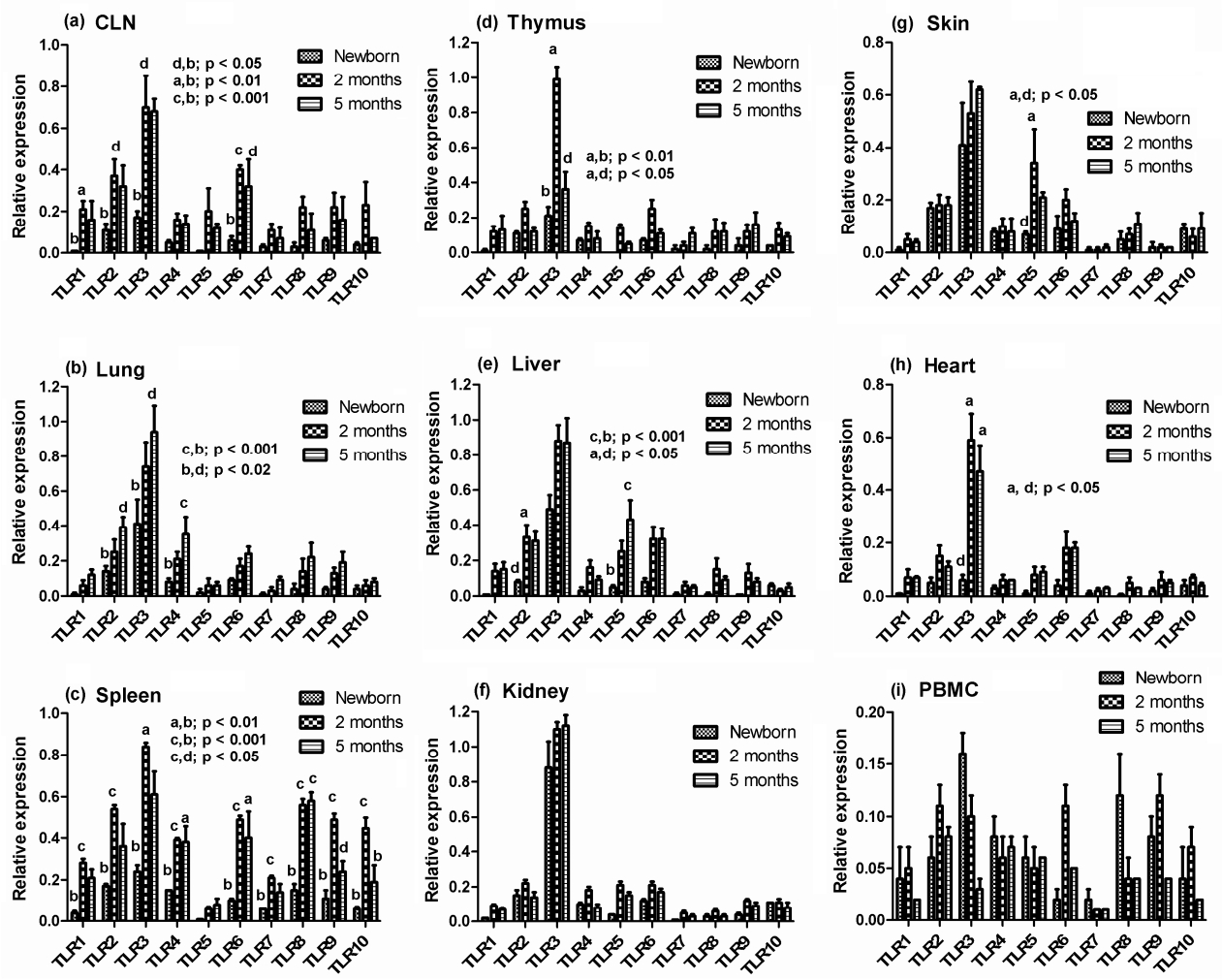

Figure 2: Relative mRNA abundance of TLRs in porcine lymphoid tissues in different ages. The average expression of TLRs (1-10) mRNA (the bar indicate standard deviation) in 1 day old newborn piglets, 2 month old young pigs and 5 month old adult pigs in (a) CLN (b) lung (c) spleen (d) thymus (e) liver (f) kidney (g) skin (h) heart and (i) PBMC

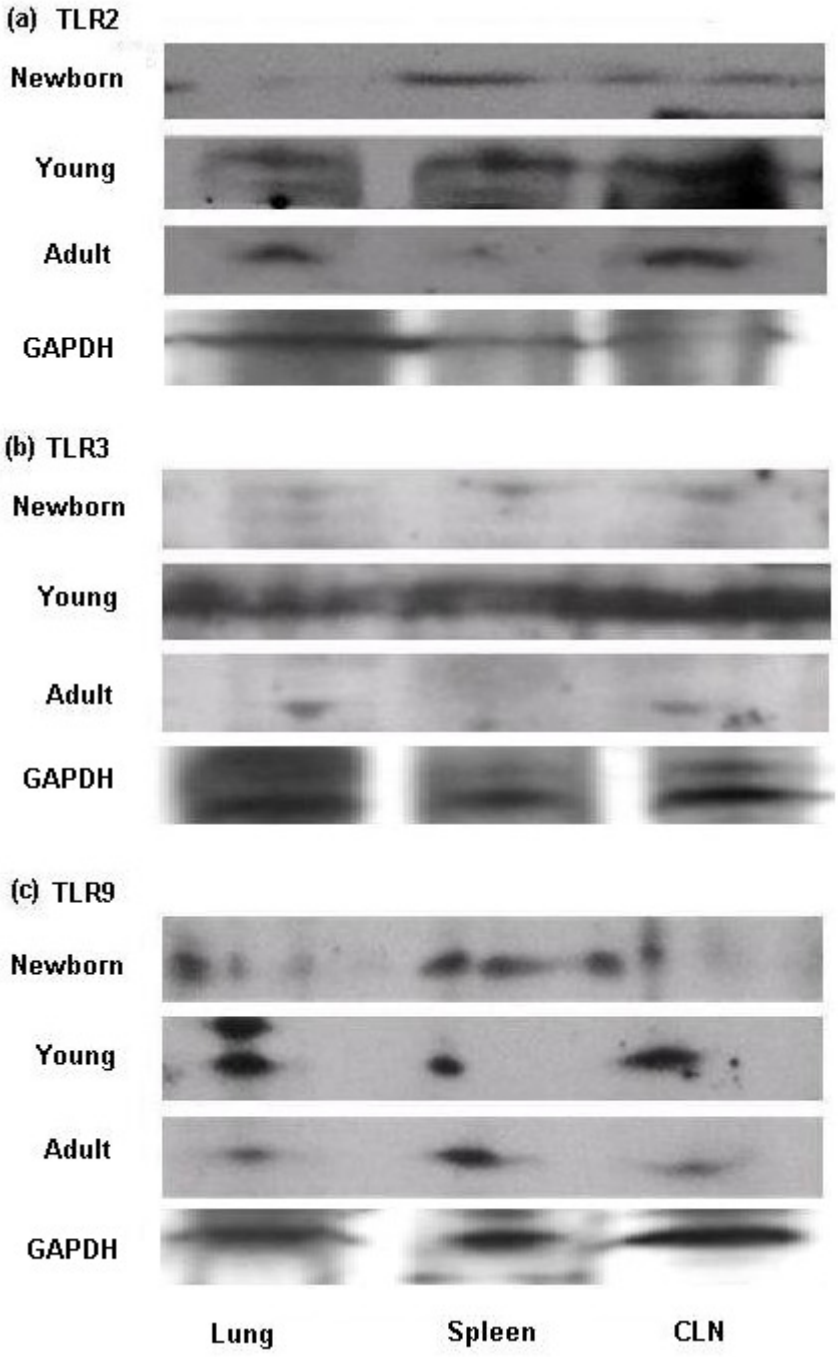

Figure 3: Expression of TLR2, TLR3 and TLR9 protein in gut-associated lymphoid tissues and lymph node. The expression of (a) TLR2 (b) TLR3 and (c) TLR9 protein in lungs, spleen and cervical lymph node tissues collected from 1 day old newborn piglets, 2 month old young pigs and 5 month old adult pigs.

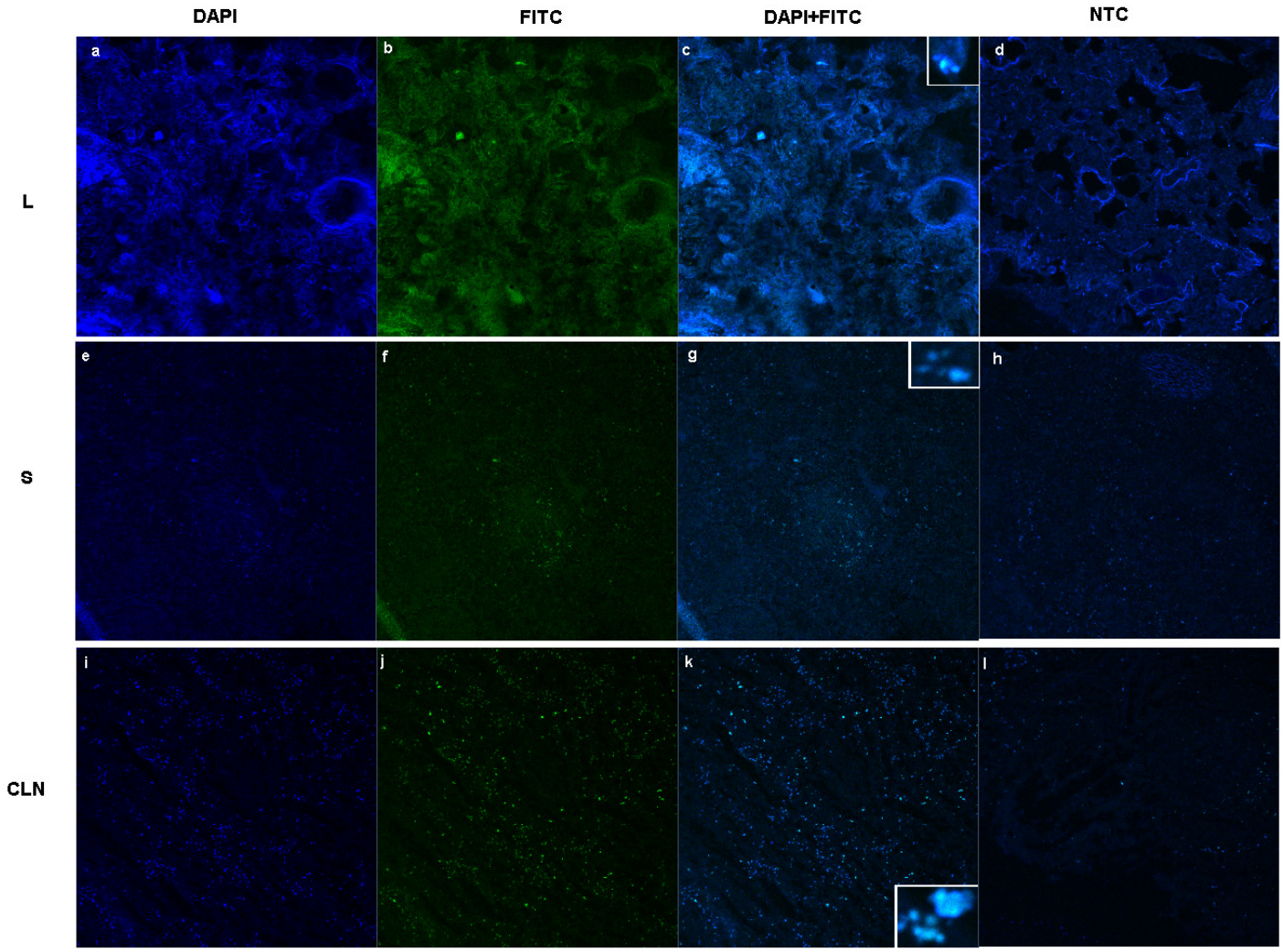

Figure 4: Localization of TLR2 protein in porcine lymphoid tissues. (4b) Immunofluorescence detection of TLR2 proteins in the alveolus, lining cells of bronchioles and in the smooth muscle layer surrounding the bronchioles. (4f) TLR2 protein localized in the lymphoid cells in the white pulp and in the cells within the follicles in spleen. (4j) TLR2 proteins were stained in the lymphocytes and macrophages in the lymph node especially in the lymphoid follicle in the cortex in spleen. (4a, e, and i) The cell nuclei were counterstained with DAPI. (4c, g and k) Merged images. (4d, h, and l) Negative control. Magnification 10X (L: Lung, S: Spleen, CLN: Cervical lymph node).

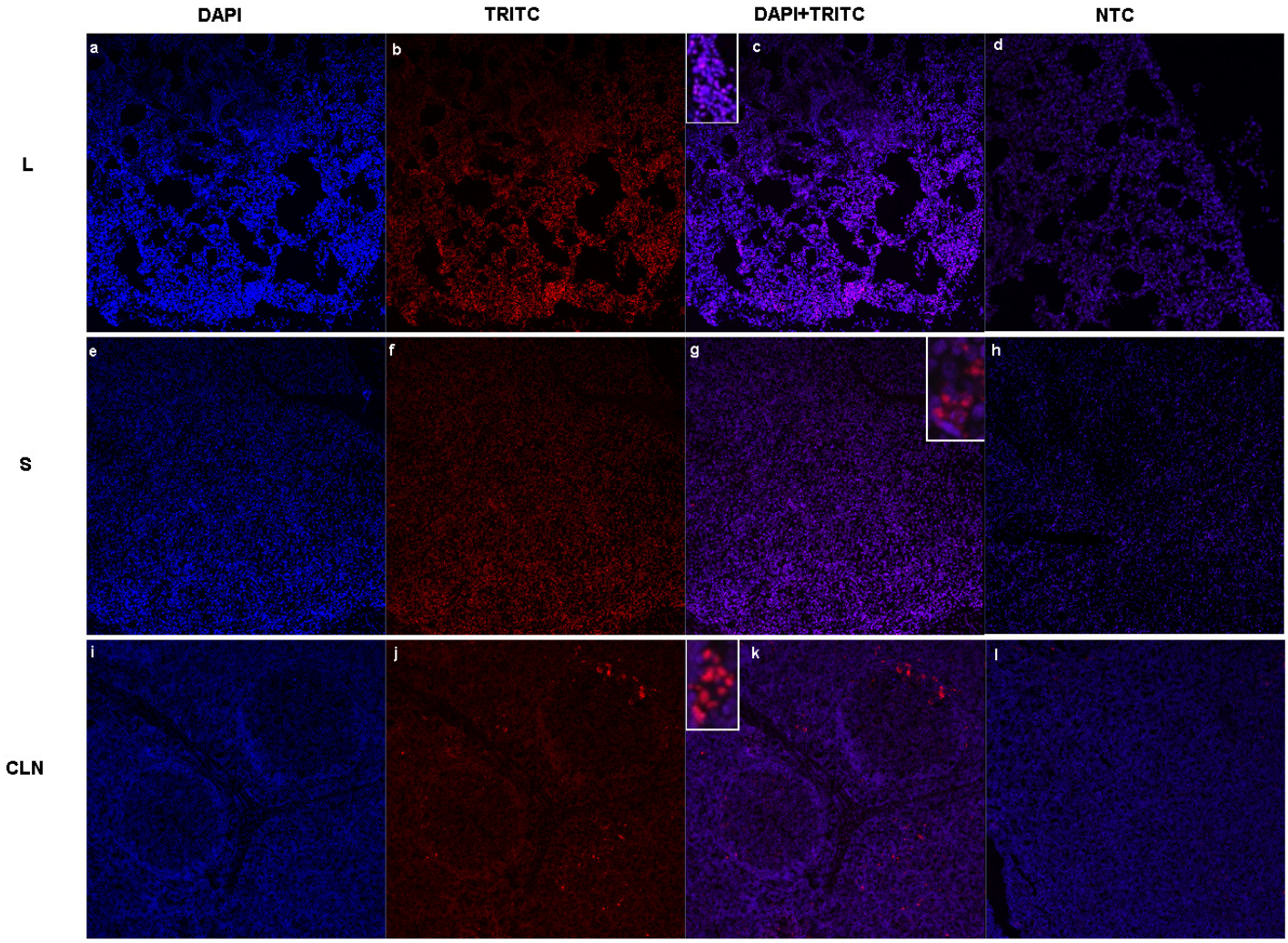

Figure 5: Localization of TLR3 protein in porcine lymphoid tissues. (5b) Immunofluorescence detection of TLR3 in the squamous cells of alveolus in lungs. (5f) TLR3 proteins were localized homogeneously in the lymphoid cells within both the red pulp and white pulp in spleen. (5j) TLR3 proteins were stained in the lymphocytes and macrophages in the lymph node especially in the lymphoid follicle in the cortex, and remarkably localized in the germinal centre as well as in the lymphoid follicle in the lymph node. (5a, e, and i) The cell nuclei were counterstained with DAPI. (4c, g and k) Merged images. (5d, h, and l) Negative control. Magnification 10X (L: Lung, S: Spleen, CLN: Cervical lymph node).

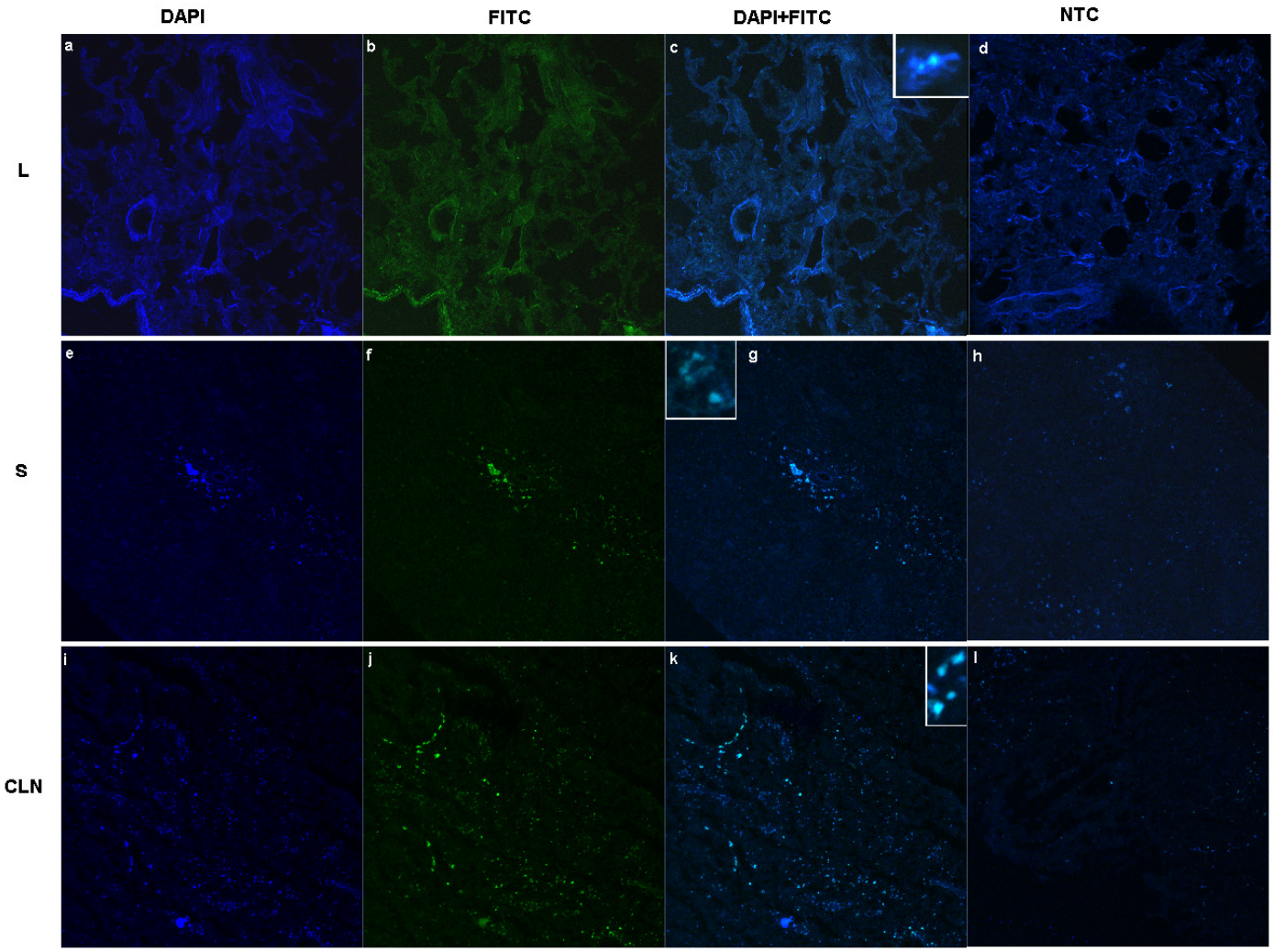

Figure 6: Localization of TLR9 protein in porcine lymphoid tissues. (6b) Immunofluorescence detection of TLR9 in the alveolus, lining cells of bronchioles and in the smooth muscle layer surrounding the bronchioles. (6f) TLR9 protein localization in cells across the spleen and remarkable staining was detected around the artery and in the trabecules of the spleen. (6j) TLR9 proteins were stained in the lymphocytes and macrophages in the lymph node especially in the lymphoid follicle in the cortex. (6a, e, and i) The cell nuclei were counterstained with DAPI. (4c, g and k) Merged images. (6d, h, and l) Negative control. Magnification 10X (L: Lung, S: Spleen, CLN: Cervical lymph node).