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Differentiation of Porcine *TLR4* Gene Expression in Piglets of Different Ages

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Abstract: Toll Like Receptors (TLRs) play an important role in innate and adaptive immunity, however the expression of TLR4 in piglets of different ages is still unknown. In this study, the tissue samples of 11 organs including heart, liver, spleen and so on were collected from 32 piglets of 4 different ages (8, 18, 30 and 35 days old). Real-time PCR was used to compare and analyze the expression of TLR4 both in different tissues and growing periods of piglets which aimed at discussing the function of TLR4 in immune responses in the piglets of development periods as well as showing the relationship between the expression level and piglets sensitivity to different subtypes *E. coli*. The results showed that *TLR4* gene was expressed in all the tissues and high levels of expression were detected in immune organs such as lung, lymph node, thymus gland and spleen. In addition in 8 days old piglets, the expression level of TLR4 in immune organs such as lung, spleen, kidney and thymus gland was relatively high. Then the whole expression quantity continually increased reaching the highest level in 35 days old of weaning period, especially in thymus gland and lung. The expression of TLR4 in thymus gland in 35 days old piglets was significantly higher than that in piglets of other ages ($p<0.05$) and the expression in lung was significantly higher than that in 8 days old piglets ($p<0.05$). The results indicated that TLR4 played the extremely important role in connecting the natural immunity and specific immunity. Besides, TLR4 not only played an important role in both immune response and general resistance to lung diseases but also could have a significant control function in preventing weaning piglets from being infected by *E. coli* F18.

Key words: Piglet, *TLR4* gene, real-time PCR, *E. coli* F18, thymus gland, piglets, China

INTRODUCTION

Toll Like Receptors (TLRs) is type I transmembrane receptor protein. To date, 13 members of the TLR family have been identified in mammals. Researches showed that TLRs is the pathogen pattern recognition receptors of initiate innate and adaptive immune. It can identify the PAMPs, send a signal causing the release of the inflammatory mediator and it also plays an important role in innate immune defense, finally activating the acquired immune system (Vaidya and Cheng, 2003). The bacterial endotoxin Lipopolysaccharide (LPS) is the major component of gram-negative bacteria. It is a main target for parasitifer to recognize and attack the pathogenic bacteria. Besides, TLR4 is the major receptor for the recognition of bacterial Lipopolysaccharide (LPS) (Chow *et al*., 1999; Lien *et al*., 2000). The activated TLR4 could stimulate the expression of proinflammatory cytokine including IL-1b, IL-6, IL-8 and TNF-a (Senthilselvan *et al*., 1997), therefore taking part in the innate immune response. Thats maybe the reason why *TLR4* gene received great attention. Now-a-days, many reports showed that TLR4 is generally related with various inflammations. Hammad *et al*. (2009) found that airway epithelial cells would express TLR4 to activate the dendritic cells after inhaled the extract of house dust mites and then lead to allergic inflammation. Penders *et al*. (2010) considered that the polymorphism of TLR4 influenced atopic dermatitis diseases. Wang *et al*. (2011) reported that the up-regulation expression of TLR4 had a closely relationship with the occurrence and development of Acute Lung Injury (ALI).

There are many researches about the porcine *TLR4* gene all over the world. Alvarez *et al*. (2006) have cloned swine TLR4 cDNA from the alveolar macrophage. Shinkai *et al*. (2006) and Zhou *et al*. (2008) have analyzed

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the polymorphism of TLR4 coding region. Qiu *et al.* (2007) located the pig *TLR4* gene at SSC1 q2.9-q2.13 and found that the highest expression was in the lung. Miguel *et al.* (2010) found that the TLR4 and most of the proinflammatory genes were also up-regulated in discrete brain areas of PRRSV-infected pigs.

To date, there are no reporters about the expression of *TLR4* gene in pigs of different ages. Demonstrated in current researches, piglets in different ages have different sensitivity to the different subtypes *E. coli*. Piglets within 1 week are high-risk to get acute diarrheal diseases yellow dysentery which is caused by pathogenic *E. coli* K88 (Jones and Rutter, 1972); 10-30 days old piglets are easy to get a white scour; 35 days old piglets on weaning time are easily infected by *E. coli* F18 suffering from the diarrhea (Verdonck *et al*., 2002).

This study compared and analyzed the expression of *TLR4* gene both in different tissues and pigs in different periods by the real-time PCR method, aiming at discussing the role of TLR4 plays in immune responses in the piglets of development periods as well as the relationship between TLR4 expression and piglets' sensitivity to different sub-types *E. coli*. The results provided some experimental basis for further research on the functions of the *TLR4* gene.

MATERIALS AND METHODS

Experimental materials: Experimental Sutai pigs were from the Sutai Pig Breeding Center in Suzhou, Jiangsu province. Sutai pig is the hybridization product of Duroc and Taihu pigs after 15 years of practices. In 1999, it was approved by National Committee of Livestock and Poultry species as a new breed. The tissues samples of 11 organs including the heart, liver, spleen, lung, kidney, stomach, muscle, thymus gland, lymph nodes, duodenum and jejunum were collected from 32 healthy Sutai pigs aged 8, 18, 30 or 35 days old which were raised under the same conditions. Samples were stored in liquid nitrogen immediately after collection and then transferred into a -70°C freezer in the laboratory.

Real-time PCR primer design: Using the software of Primer Express 2.0, TLR4 primers were designed based on the sequence of AB232527 in GenBank and synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. GAPDH was used as an internal control to normalize all of the threshold Cycle (Ct) values of other tissue products. Primer sequences for amplification of TLR4 and GAPDH were shown in Table 1.

RNA extraction: Total RNA was extracted from various swine tissues (50-100 mg) using Trizol reagent (TaKaRa Biotechnology Dalian Co., Ltd.) according to the manufacturer's instruction. Precipitated RNA was resuspended in 20 μL of RNase-free H_2O and then stored at 80°C. RNA quality and quantity were assessed by agarose gel electrophoresis and UV spectrophotometer, respectively.

Reaction system and conditions for fluorescence quantitative PCR: The 10 μL of cDNA synthesis reaction mixture contained the following parts: 2 μL of 5× PrimerScript Buffer, 0.5 μL of PrimerScript RT Enzyme Mix I, 0.5 μL of Oligo dT, 0.5 μL of random 6 mers, 500 ng of total RNA and RNase-free H_2O to make up the final volume of 10 μL. The reaction was carried out at 37°C for 15 min and then at 85°C for 5 sec.

Real-time PCR amplification was performed in 20 μL of reaction mixture containing 1 μL cDNA (100-500 ng), 0.4 μL 10 μmol L^{-1} each of the forward and reverse primers, 0.4 μL 50× ROX Reference Dye II, 10 μL 2× SYBR Green Real-time PCR Master Mix and 7.8 μL ddH_2O. PCR reaction condition was 95°C for 15 sec followed by 40 cycles of 95°C for 5 sec and 62°C for 34 sec. The dissociation curve was analyzed after amplification. A peak of Tm at 85±0.8°C on the dissociation curve was used to determine the specificity of PCR amplification. The Tm value for each sample was the average of the real-time PCR data for triplicate samples.

Data processing and analysis: The $2^{-\Delta\Delta Ct}$ method was suitable for processing the relative quantification results. The following formula was used:1 ΔΔCT = (average Ct value of the target gene in the tested group Δ-average Ct value of the housekeeping gene in the tested group), (average Ct value of the control gene in the control group, average Ct value of the housekeeping gene in the control group). Ct (initial cycles) is the abscissa value of the intersection between the amplification curve and the

Table 1: Primers used for real-time PCR

Genes	Sequence	Expected length (bp)
TLR4	Forward primerΔ 5'-CAGATAAGCGAGGCCGTCATT-3'	113
	Reverse primerΔ 5'-TTGCAGCCCACAAAAAGCA-3'	
GAPDH	Forward primer: 5'-ACATCATCCCTGCTTCTACTGG-3'	187
	Revers primer: 5'-CTCGGACGCCTGCTTCAC-3 '	

threshold line and it refers to the number of cycles at which the fluorescence signal strength reaches the required threshold during PCR amplification. The statistical analyses were carried out using SPSS 11.0 software.

RESULTS AND DISCUSSION

The purity and integrity of total RNA: Total RNA samples extracted from 11 tissues were assayed by 1% agarose gel electrophoresis. Three bands, representing 28, 18 and 5S were observed with no bands from DNA contamination or significant degradation. This indicates the high purity of the extracted total RNA. RNA purity was also examined on a UV spectrophotometer. The A260/A280 ratios of the samples were 1.8-1.9 indicating a high quality of the extracted RNA that was sufficient for subsequent experiments.

Fluorescence quantitative PCR amplification curve and melted curve: The PCR amplification curve and the dissociation curve for the *TLR4* gene showed the good repetition and a single specific peak was observed with the real-time PCR products for the *TLR4* gene with no primer dimers or nonspecific reaction products (Fig. 1). The standard curves for the *TLR4* and *GAPDH* genes indicated that the amplification efficiencies of the target gene and the reference gene were almost the same so that the $2^{-\Delta\Delta Ct}$ method could be applied for quantitative calculation. Data was analyzed by SPSS 11.5 showed as x±SD. T-test was used to inspect the significant of expression in different developmental periods.

Results of expression *TLR4* gene in different tissues and different day-old piglets: Using the established SYBR green real-time quantitative PCR method described, the expression levels of TLR4 were examined in various tissues in this study. The expression level of TLR4 in heart was defined as 1.0 so that the expression levels of this gene in other tissues could be quantified. As was shown in Table 2, TLR4 was expressed in all tested tissues. As a whole, the gene expressed higher in immune organs like lung, thymus gland, lymph node and spleen with the highest expression in lung in all the tested periods (Fig. 2). During the time of 35 days old weaning period, the expression of TLR4 in lung and thymus gland had a tremendous increase. The expression of TLR4 in thymus gland of 35 days old piglets was significantly higher than that in piglets of other ages ($p<0.05$) and the expression in lung was significant higher than that in

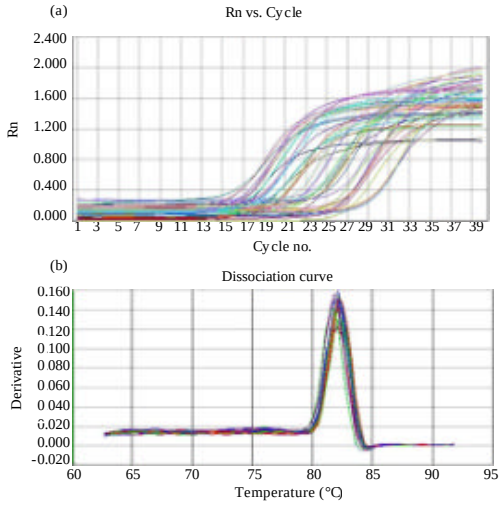

Fig. 1: Real-time PCR amplification curve and dissociation curve for the *TLR4* gene in various tissues

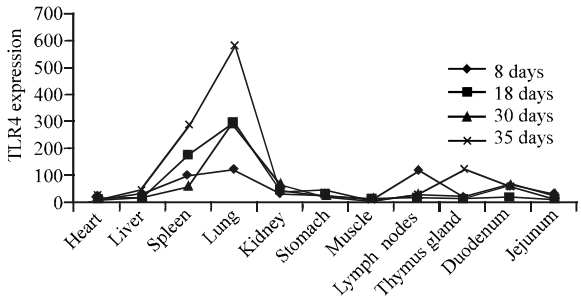

Fig. 2: Differentiation of TLR4 mRNA expression among different old-day piglets

8 days old piglets ($p<0.05$). Recent researches prompted that the activated TLR4 not only starts and regulates the congenital immune but also activates the acquired immune so that it played an important role in both innate and adaptive immune (Medzhitov, 2001; Beutler, 2005). The TLR family which is relying on MyD88 has a synergistic effect with the antigen recognition receptor then it mediates the autoreactive B cells to express IgG, thus involves in regulating the acquired immune response (Leadbetter *et al*., 2002).

The recent research demonstrated that the piglets absorbed antibodies from colostrum in 24 h after birth and acquired congenital immune by storing maternal antibody in the blood. So, there was a relatively higher blood passive antibody level in 1 day old piglets. In this study, the expression of TLR4 in immune organs of 8 days old piglets was relatively high. However with increasing age, the level of maternal antibody will gradually decline. Meanwhile with the

Table 2: Differentiation of TLR4 mRNA expression among different old-day piglets

Tissues	Heart	Liver	Spleen	Lung	Kidney	Stomach	Muscle	Lymph nodes	Thymus gland	Duodenum	Jejunum
8 days expression ($2^{-\Delta\Delta Ct}$)	2.811	28.737	103.5359	121.510	24.264	21.684	1.000	57.931	18.760	54.098	31.259
	±0.504	±10.295	±30.7850	±11.902^{a}	±17.927	±4.490	±0.000	±12.535	±1.036^{a}	±13.575	±5.696
18 days expression ($2^{-\Delta\Delta Ct}$)	3.770	13.739	172.8120	291.540	38.393	25.762	1.000	15.414	12.605	16.622	10.140
	±0.105	±9.715	±68.9030	±42.085^{ab}	±8.149	±1.544	±0.000	±13.495	±10.687^{a}	±4.647	±2.123
30 days expression ($2^{-\Delta\Delta Ct}$)	7.531	20.538	63.2790	287.540	60.818	20.543	1.000	27.271	19.929	57.154	21.090
	±0.199	±13.295	±16.1740	±58.140^{ab}	±15.564	±1.113	±0.000	±8.960	±5.044^{a}	±1.750	±7.287
35 days expression ($2^{-\Delta\Delta Ct}$)	2.948	41.859	285.0350	573.043	47.094	39.464	1.000	26.400	121.930	53.076	18.157
	±0.629	±14.304	±28.7640	±72.970^{b}	±4.692	±9.653	±0.000	±14.781	±4.822^{b}	±27.461	±8.215

Means with the different superscripts within the same column differs significantly ($p<0.05$)

stimulation of external pathogens, the piglets will begin to establish its own immune response system, particularly during the time of weaning. The acquired immunity will be fully opened due to the stimulation of external environment factors, especially the stress reaction of weaning. At the same time, the expression of TLR reached its highest level in immune organs such as lung, spleen and thymus. The test results further reminded that TLR4 played the extremely important role in connection with the natural immunity and specific immune. The infection of *E. coli* F18 bacteria depends on the existence of *E. coli* F18 receptor in the brush border membranes of piglet's small intestinal mucosa (Shi *et al*., 2002) while the levels of mRNA expression of *TLR4* gene were rather low in piglet's small intestine. This result further demonstrated that the expression of *TLR4* gene probably had no significant influence on the existence of *E. coli* F18 receptor in piglet's small intestine. Impact of *TLR4* gene on resistance to ETEC F18 may be related to its regulation and control on innate immune recognition.

Regarding the expression of the *TLR4* gene, Medzhitov *et al*. (1997) first identified and cloned human *TLR4* genes and then showed that TLR4 expressing a higher degree in spleen, heart, endothelial cells, macrophages, neutrophils and Dendritic Cells (DC). Qiu *et al*. (2007) as the only report published in China, located the pig *TLR4* gene at SSC1q 2.9-q2.13 and found the highest expression in the lung. TLR4 is the major receptor for the recognition of bacterial Lipopolysaccharide (LPS).

Lipopolysaccharide also called endotoxin is the major component in the cell wall of gram-negative bacteria. It is also the key pathogenic factor of bacteria which is responsible for infections in various organs such as inflammation in the lung.

The infectious caused by gram-negative bacteria including *Pasteurella pneumotropica*, *Haemophilus influenzae* and *Klebsiella pneumoniae* can all result in inflammation in the lung. The LPS from these bacteria leads to chronic airway inflammation such as Chronic Obstructive Pulmonary Disease (COPD). In addition, LPS is the main factor causing other types of inflammatory lung diseases such as Acute Lung Injury (ALI). In this study as was reported, the expression of TLR4 was expressed highest in lung. And it had higher expression in immune organs include lymph node, kidney and spleen. Current reports showed that newborn piglets were not susceptible to *E. coli* F18 but piglets in 35 days around weaning were the most susceptible to *E. coli* F18. It reminded that the resistance to *E. coli* F18 is stronger in 8 days old piglets but weaker in 35 days old piglets. During the time from birth to weaning, different aged piglets obviously have different sensitivity to different sub-types *E. coli* (Dean, 1990; Willemsen and de Graaf, 1992). This study showed that during the time of 35 days old around weaning period, the expression of TLR4 in lung and thymus gland were significantly higher than that in 8 days old piglets.

The results indicated that the expression of TLR4 in weaning piglets was closely related with the resistance to *E. coli* F18. Furthermore, this study revealed that the TLR4 was a main receptor which could induced immune response to gram-negative bacteria endotoxin-lipopolysaccharide in recognitive microbial mechanism of natural immune. It not only played an important role in both immune response and general resistance to lung diseases but also had a significant control function in preventing weaning piglets from being infected by *E. coli* F18. It was needed to do the further research on the function of TLR4 to provide guidance and basis for the resistance breeding of swine.

CONCLUSION

In this study, the expression of TLR4 was detected in all the tissues showing higher levels in immune organs like lung, lymph node, thymus gland and spleen. In addition, the expression of TLR4 in thymus gland of 35 days old pigs was significantly higher than that in the piglets of other periods and in the lung it was significantly higher than that in 8 days old piglets. The results indicated that TLR4 played the extremely important role in connection with the natural immunity and specific immune. Besides, TLR4 not only played an important role in both immune response and general resistance to lung diseases but also had a significant control function in preventing weaning piglets from being infected by *E. coli* F18.

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REFERENCES

Alvarez, B., C. Revilla, S. Chamorro, M. Lopez-Fraga, F. Alonso, J. Dominguez and A. Ezquerra, 2006. Molecular cloning, characterization and tissue expression of porcine Toll-like receptor 4. J. Dev. Comp. Immunol., 30: 345-355.

Beutler, B., 2005. The Toll-like receptors: Analysis by forward genetic methods. Immunogenetics, 57: 385-392.

Chow, J.C., D.W. Young, D.T. Golenbock, W.J. Christ and F. Gusovsky, 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J. Biol. Chem., 274: 10689-10692.

Dean, E.A., 1990. Comparison of receptors for 987P pili of enterotoxigenic *Escherichia coli* in the small intestines of neonatal and older pig. Infect. Immun., 58: 4030-4035.

Hammad, H., M. Chieppa, F. Perros, M.A. Willart, R.N. Germain and B.N. Lambrecht, 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat. Med., 15: 410-416.

Jones, G.W. and J.M. Rutter, 1972. Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by *Escherichia coli* in piglets. Infect. Imm., 6: 918-927.

Leadbetter, E.A., I.R. Rifkin, A.M. Hohlbaum, B.C. Beaudette, M.J. Shlomchik and A. Marshak-Rothstein, 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. J. Nature, 416: 603-607.

Lien, E., T.K. Means, H. Heine, A. Yoshimura and S. Kusumoto *et al*., 2000. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J. Clin. Invest., 105: 497-504.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. J. Nat. Rev. Immunol., 1: 135-145.

Medzhitov, R.P., C.A. Preston-Hurlburt and C.A. Janeway, 1997. A human homologue of the drosophila toll protein signals activation of adaptive immunity. Nature, 388: 394-397.

Miguel, J.C., J. Chen, W.G. van Alstine and R.W. Johnson, 2010. Expression of inflammatory cytokines and Toll-like receptors in the brain and respiratory tract of pigs infected with porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol., 135: 314-319.

Penders, J., C. Thijs, M. Mommers, E.E. Stobberingh and E. Dompeling *et al*., 2010. Host-microbial interactions in childhood atopy: Toll-like receptor 4 (TLR4), CD14 and fecal *Escherichia coli*. J. Allergy Clin. Immunol., 125: 231-236.

Qiu, X.T., Y.H. Li, H.J. Li, Y. Yu and Q. Zhang, 2007. Localization and tissue expression of Swine Toll-like receptor 4 (TLR4). Chinese J. Agric. Biotechnol., 15: 37-40.

Senthilselvan, A., Y. Zhang, J.A. Dosman, E.M. Barber and L.E. Holfeld *et al*., 1997. Positive human health effects of dust suppression with canola oil in swine barns. Am. J. Respir. Crit. Care Med., 156: 410-417.

Shi, Q.S., X.M. Xie, X.C. Liu , S.Q. Huang and C.Q. He, 2002. Experimental results on enterotoxigenic *E. coli* F18 receptor genotypes. Yi Chuan., 24: 656-658.

Shinkai, H., M. Tanaka, T. Morozumi, T. Eguchi-Ogawa and N. Okumura *et al*., 2006. Biased distribution of single nucleotide polymorphisms (SNPs) in porcine Toll-like receptor 1 (TLR1), TLR2, TLR4, TLR5 and TLR6 genes. Immunogenetics, 58: 324-330.

Vaidya, S.A. and G. Cheng, 2003. Toll-like receptors and innate antiviral responses. J. Curr. Opin. Immunol., 15: 402-407.

Verdonck, F., E. Cox, K. van Gog, Y. van der Stede, L. Duchateau, P. Deprez and B.M. Goddeeris, 2002. Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic *Escherichia coli* strain or an F18 verotoxigenic *Escherichia coli* strain. Vaccine, 20: 2995-3004.

Wang, Q., H. Zhu, J. Zhu and L.N. Zhou, 2011. Dynamic expression of Toll-like receptor 4 in septic acute lung injury in rats. Chinese J. Chin Prac Diagand Ther., 25: 157-161.

Willemsen, P.T.J. and F.K. de Graaf, 1992. Age and serotype dependent binding of K88 fimbriae to porcine intestinal receptors. Microb. Pathogenesis, 12: 367-375.

Zhou, B., C.W. Liu, D.B. Yu, R.H. Huang, H.L. Liu and L.Y. Wang, 2008. Detection of SNPs in exon 3 of the Swine TLR4 gene using PCR-SSCP method. Chinese J. Anim. Husbandry Vet. Med., 40: 26-30.