Analysis of polymorphism in the porcine *TLR4* gene and its expression related to *Escherichia coli* F18 infection

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ABSTRACT: The genetic variation in exon 1 of the *TLR4* gene was detected among a total of 893 animals, including Asian wild boars, 3 imported commercial and 10 Chinese indigenous pig breeds. The expression of *TLR4* was assayed by RT-PCR and different expression between resistant and sensitive resource populations to ETEC F18 was analysed to discuss the role that the *TLR4* gene plays in resistance. In this study, new alleles were detected in exon 1 of the *TLR4* gene. These polymorphisms are significantly different between Chinese indigenous breeds and imported breeds. Based on the published *TLR4* gene sequence (AB232527) in GenBank, a 93G > C mutation was found in 5'UTR and only a 194G > A synonymous mutation was found in the coding sequence of exon 1. In addition, *TLR4* gene was broadly expressed in 11 tissues with the highest level in lung. The expression was relatively high in the lymph nodes, kidney and spleen. Generally, the expression of *TLR4* gene in sensitive individuals was higher than that in resistant individuals. The results indicated that the downregulation of the mRNA expression of *TLR4* gene had reduced the transmembrane signal transduction of LPS and then led to the responsive ability of the host to ETEC F18 in piglets.

Keywords: pigs; *TLR4* gene; polymorphism; expression profile; ETEC F18

Toll-like receptors (*TLRs*) exist in the cells of lymphoid tissues and non-lymphoid tissues. The expression levels of different types of *TLRs* in respective tissues and cells are different. The genetic polymorphism in *TLRs* gene among species and individuals has a great influence on immune responses to infections caused by various pathogens (Medzhitov, 2001). To date, 13 members of the *TLR* family have been identified in mammals which make up a large family of type I transmembrane receptor proteins (Beutler, 2005). *TLRs* have been shown to play an important role in the activation of innate immunity and in the induction of adaptive immunity, especially in the innate immune system. They are critical for the recognition of PAMPs (pathogen associated molecular patterns) in pathogens of humans and various animals (Hirschfield et al., 2000; Ozinsky et al., 2000). In the large *TLR* family, most researches focused on *TLR4*. As a major receptor for the recognition of bacterial lipopolysaccharides (LPS), *TLR4* can recognize many pathogenic microorganisms, such as Gram-negative bacteria,

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Chlamydia, spirochetes, and viruses. The genetic difference in these receptor genes can cause differences in the resistance to various pathogens (Werling and Jungi, 2003; Machida et al., 2006).

It is notable that the genetic variations at the *TLR4* locus of humans and mice affect the susceptibility and resistance to diseases to a certain extent (Takeuchi et al., 1999; Poltorak et al., 2000). SNPs (single nucleotide polymorphisms) of *TLR4* also commonly exist in other animals, such as chicken, pig, and cow. *TLR4* genotype is usually related to the susceptibility and resistance to diseases in animals. For example, Leveque et al. (2003) proposed that the polymorphism of chicken *TLR4* was related to the susceptibility to salmonella; Goldammer et al. (2004) reported that the copy number of *TLR4* in the mammary gland of dairy cows increased with mastitis; Wang et al. (2007) analysed the genetic polymorphism of *TLR4* gene and its relationship to mastitis; and Shinkai et al. (2006) and Zhou et al. (2008) discussed polymorphism in the coding region of porcine *TLR4* gene that consists of 3 exons, and SNPs were found only in exon 3. To date, no mutation has been detected in exon 1.

The pathogenesis of F18 antigen is that after *E. coli* strains enter the pig's intestinal tract, they strongly adhere to intestinal epithelial cells via their pili to bind to the F18 receptors in the brush border membrane of intestinal epithelial cells. They then settle, propagate, produce enterotoxins, and cause diseases in piglets (Benin and Ducher-Suchaux, 1991; Nagy and Fekete, 1999). Using a candidate gene approach and linkage analysis, Vogeli et al. (1997) demonstrated that alpha(1,2)-fucosyltransferase gene (*FUT1*) in chromosome 6q11 is the candidate gene controlling the adhesion to F18 receptor. The research of Meijerink et al. (1997) showed that there exists a G/A mutation at position M307 of *FUT1* gene, with *G* predominant over *A*. In other words, pigs with *AA* genotype are resistant to ETEC F18 and pigs with *GG* genotype or the *AG* heterozygote are sensitive to ETEC F18. Breeding for disease resistance could be implemented by marker-assisted selection using the *FUT1* gene as ETEC F18R candidate gene. Using a small amount of *AG* type (9.2%) individuals in the *FUT1* gene detected from Sutai pigs (Bao et al., 2008), our group conducted proper selection and assortative mating. Small intestinal epithelium cells of resistant (*AA* genotype) and sensitive (*AG* and *GG* genotypes) pigs were selected to test the adhesion capability of the wild type *E. coli* expressing F18ab fimbriae, recombinant *E. coli* expressing F18ac fimbriae or recombinant *E. coli* secreting and surface-displaying the FedF subunit of F18ab fimbriae (Wu et al., 2007). After 5 years of breeding, we have now established resistant (*AA* genotype) and sensitive (*AG* and *GG* genotypes) resource populations to ETEC F18 in our Sutai pig population.

Currently, *TLR4* is known to play an extremely important role as a key link between innate immunity and adaptive immunity. This study compared and analysed the polymorphism of porcine *TLR4* exon 1 in wild boars and 14 domestic and international pig breeds using the PCR-SSCP method. Sequencing of different DNA fragments was conducted, and SNPs in *TLR4* were screened. In addition, the expression of this gene in different tissues of pigs was examined by fluorescence quantitative PCR aiming to analyse the differential expression between resistant and sensitive resource population to ETEC F18 of Sutai pigs. Our results provide an experimental basis for further research on the functions of the *TLR4* gene.

MATERIAL AND METHODS

Experimental material

Among the experimental animals, Duroc, Yorkshire, and Landrace pigs were from the Xingtai (Yangzhou) Agriculture and Animal Husbandry Technology Development Co., Ltd. (originating from the Wanjiang Pig Breeding Farm in Taiwan). Huai pigs were collected from the pig-breeding farm of the Academy of Agricultural Sciences. Meishan, Erhualian, Fengjing, and Sutai pigs were from the Sutai Pig Breeding Centre in Suzhou, Jiangsu province. Wuzhishan pigs were obtained from the Wuzhishan Pig Breeding Centre at the Hainan Academy of Agricultural Sciences. Ronchang pigs were from the Ronchang Pig Breeding Centre at the Chongqing Academy of Animal Sciences. Xiushuihang pigs were from the livestock breeding field in Xiushui county, Jiangxi province. Wanan spotted pigs were from the livestock breeding field in Wanan county, Jiangxi province. Lepin spotted pigs were from the livestock breeding field in Leping city, Jiangxi province. Tibetan pigs were from Nyingchi county, Tibet Autonomous Region. Wild boars were from Jinzhai county, Anhui province. Approximately 1.0 g of ear tissue was collected from each pig and placed into a 1.5-ml Eppendorf

tube. Samples were kept on ice before assay in the laboratory.

Sutai pig is a new variety of high-quality lean-meat type pig bred by Sutai pig breeding centre in the city of Suzhou. It is a product of 15-year hybridization of Duroc and Taihu pigs. The resistant and sensitive resource population to ETEC F18 of Sutai pigs used for RT-PCR analysis was from the Centre of Sutai Pig Breeding. The tissue samples of 11 organs, including the heart, liver, spleen, lung, kidney, stomach, muscle, thymus gland, lymph nodes, duodenum and jejunum, were collected from sixteen 35-day-old piglets (8 resistant individuals and 8 sensitive individuals) that were raised under the same conditions. Samples were stored in liquid nitrogen immediately after collection and then transferred into a –70°C freezer in our laboratory.

PCR-SSCP analysis

Based on the published porcine *TLR4* gene sequence (AB232527) in GenBank, primers were designed as P1: 5'-TTCTCACTTCCTCTTACC-3' and P2: 5'-AGACTCCTACCACATACC-3'. The predicted amplified fragment is 277 bp. The specific primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). *Taq* DNA polymerase and dNTP were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The PCR reaction mixture contained the following components: 1.0 µl of genomic DNA (100 ng/µl), 2.0 µl of 10× buffer (Mg^{2+}, 25 mmol/l), 1.5 µl of dNTP mix (10 mmol/l), 1.0 µl of each primer (10 µmol/l), 0.2 µl of *Taq* polymerase (5 IU/µl), and sterilized distilled water to make up the final volume of 20 µl. The PCR amplification program was as follows: initial denaturation at 95°C for 10 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s before the final extension at 72°C for 8 min. The products were stored at 4°C until use. The PCR amplification products were examined by electrophoresis on a 1% agarose gel. As the gel loading sample, 7 µl of loading buffer was mixed with 15 µl of PCR product, denatured at 98°C for 15 min, and then incubated on ice for 10 min. All the denatured ice-cold mixtures were loaded onto 12% non-denaturing polyacrylamide gel (Acr:Bis = 29:1) for electrophoresis. The electrophoresis was carried out at 130 V overnight, and the gel was subsequently silver stained.

Table 1. Sequences of primers used in RT-PCR reactions

Primer name	Sequence 5' to 3'
TLR4-F	CAGATAAGCGAGGCCGTCATT
TLR4-R	TTGCAGCCCACAAAAAGCA
GAPDH-F	ACATCATCCCTGCTTCTACTGG
GAPDH-R	CTCGGACGCCTGCTTCAC

According to the results of PCR-SSCP, homozygotes of different genotypes were selected for sequencing. After electrophoresis detection, the targeted fragments in the PCR products were recovered by a gel extraction kit (Bio Basic Inc., Toronto, Canada). After purification, these fragments were sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing on an ABI PRISM 377 automatic DNA sequencer.

RT-PCR primers

Using the Primer Express 2.0 software, *TLR4* primers were designed based on the sequence of AB232527 in GenBank and synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China). *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* are listed in Table 1.

RNA extraction

Total RNA was extracted from various pig tissues (50–100 mg) using Trizol reagent (TakaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. 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

An amount of 10 µl of cDNA synthesis reaction mixture contained the following components:

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 hexamers, 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 s.

Real-time PCR amplification was performed in 20 µl of reaction mixture containing 1 µl of cDNA, 0.4 µl each of the upstream and downstream primers (10 µmol/l), 0.4 µl of ROX Reference Dye II (50×), 10 µl of SYBR Green Real-time PCR Master Mix (2×), and 7.8 µl of ddH_2O. PCR reaction conditions were 95°C for 15 s followed by 40 cycles of 95°C for 5 s and 62°C for 34 s. The dissociation curve was analysed 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.

Standard curve

A certain amount of cDNA template was sequentially diluted 10-fold. Then the real-time PCR was run using these diluted products in gradient as a template. The values of the concentration gradient were put into the fluorescence quantitative PCR cycler. Monitoring the real-time reaction data, the system software generated the standard calibration curves for *TLR4* and *GAPDH* mRNA.

Data processing and analysis

The $2^{-\Delta\Delta Ct}$ method was suitable for processing the relative quantification results. The following formula was used:

$\Delta\Delta C_T$ = (average *Ct* value of the target gene in the tested group – 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 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 the SPSS 11.0 software.

RESULTS AND ANALYSIS

Polymorphism analysis in exon 1 of *TLR4* gene

PCR amplification was carried out using the designed primers. According to the 1% agarose gel electrophoresis, the length of the amplified fragment was in agreement with the predicted fragment length, without nonspecific bands. Based on the PCR products, SSCP analysis was performed. In this study, 6 genotypes were detected in exon 1 of the *TLR4* gene. They were defined as *AA*, *BB*, *CC*, *AB*, *AC* and *BC* with 3 alleles *A*, *B*, and *C* (Figure 1).

DNA sequencing was conducted for the PCR products from *AA*, *BB*, and *CC* homozygotes. With an identical sequence from GenBank (AB232527), *CC* genotype was defined as the wild type. Compared to *CC* genotype, *BB* genotype had a G/C substitution mutation at nucleotide 93, and *AA* genotype had a G/A substitution at nucleotide 194. 93G > C mutation was located in 5'UTR and only 194G > A mutation was located in the coding sequence of exon 1. SNP 194G > A is a synonymous mutation.

The frequencies were calculated for the alleles of each breed. As shown in Table 2, three alleles were detected with six genotypes. In Duroc pigs, *AA*, *BB*, *CC*, *AB*, *AC* and *BC* genotypes were detected. In Sutai pigs with the Duroc lineage, *BB*, *CC* and *BC* genotypes were detected. In Landrace and Yorkshire pigs, *CC* and *BC* genotypes were detected. *TLR4* exon 1 is highly conserved in wild boars and all 10 Chinese indigenous pig breeds, with only the *CC* genotype detected. According to the χ^2 test, the distribution of genotypes in the Sutai population deviated from the Hardy-Weinberg equilibrium ($P < 0.01$).

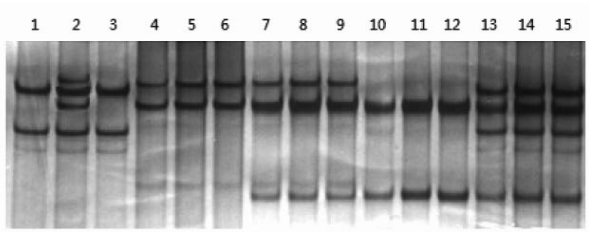

Figure 1. SSCP analysis on PCR amplified products
Lanes 4–6 = genotype AA; Lanes 1, 3 = genotype BB; Lanes 10–12 = genotype CC; Lane 2 = genotype AB; Lanes 7–9 = genotype AC; Lanes 13–15 = genotype BC.

Table 2. Allele frequencies in the *TLR4* gene in different swine breeds

Breed	Number of pigs	Allele frequency			χ^2
		A	*B*	*C*	
Duroc	77	0.15	0.19	0.66	1.46
Yorkshire	68	0	0.13	0.87	1.27
Landrace	95	0	0.02	0.98	0.18
Sutai	219	0	0.05	0.95	25.64*
Meishan	43	0	0	1.00	–
Erhualian	46	0	0	1.00	–
Fengjing	38	0	0	1.00	–
Huai	39	0	0	1.00	–
Ronchang	48	0	0	1.00	–
Wanan spotted	31	0	0	1.00	–
Xiushuihang	34	0	0	1.00	–
Lepin spotted	35	0	0	1.00	–
Wuzhishan	44	0	0	1.00	–
Tibetan	43	0	0	1.00	–
Wild boar	33	0	0	1.00	–

*Hardy-Weinberg equilibrium, $P < 0.01$

Expressions of *TLR4* gene in different tissues

Total RNA samples extracted from 11 tissues were assayed by 1% agarose gel electrophoresis. Three bands, representing 28S, 18S, 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 the high quality of the extracted RNA that was sufficient for subsequent experiments.

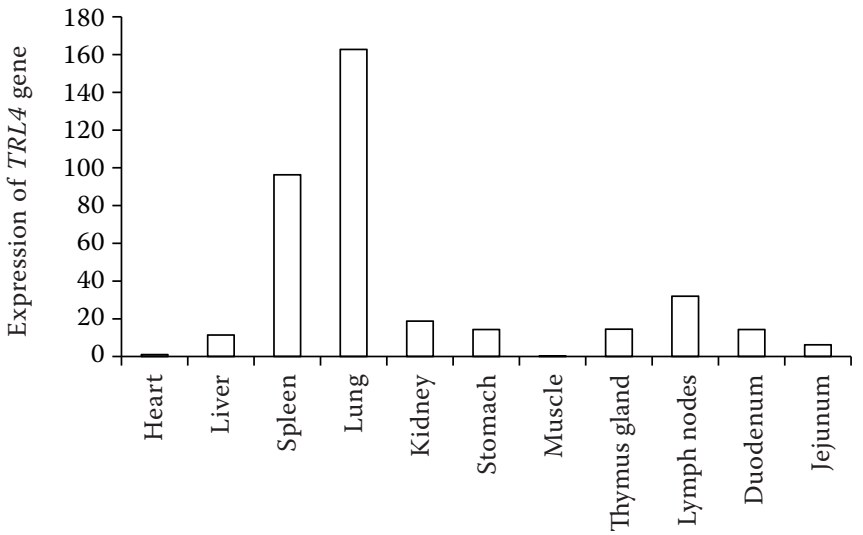

Figure 2. The expression levels of *TLR4* in various tissues

Table 3. Differences in *TLR4* mRNA expression ($2^{-\Delta\Delta Ct}$) between resistant and sensitive resource populations to ETEC F18

Populations	Heart	Liver	Spleen	Lung	Kidney	Stomach	Muscle	Thymus	Lymph	Duodenum	Jejunum
Resistant pigs	1.000 ± 0.000	17.35 ± 3.290	119.512 ± 29.640[a]	207.270 ± 46.562[a]	26.844 ± 9.364[a]	28.051 ± 9.722	0.252 ± 0.189	19.581 ± 3.180[a]	28.278 ± 7.188[a]	15.683 ± 6.155	8.440 ± 1.310
Sensitive pigs	1.623 ± 0.198	18.79 ± 3.984	161.210 ± 32.891[b]	275.369 ± 52.650[b]	42.765 ± 9.426[b]	23.356 ± 9.297	0.662 ± 0.347	42.248 ± 5.101[b]	84.280 ± 8.660[b]	31.432 ± 10.203	10.011 ± 1.513
Ratio (sensitive/ resistant)	1.623	1.083	1.349	1.329	1.593	0.833	2.626	2.158	2.980	2.004	1.424

Means with the different superscripts within the same column differ significantly ($P < 0.05$)

The PCR amplification curve and the dissociation curve for the *TLR4* gene showed the good reproducibility and a single specific peak was observed with the RT-PCR products for the *TLR4* gene with no primer dimers or nonspecific reaction products. 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.

Using the established SYBR green real-time quantitative PCR method described above, 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. The results of the analysis are summarized in Figure 2. *TLR4* was expressed in all tested tissues, with the highest expression in lung and a relatively high expression level in spleen, lymph nodes, and kidney. The order was as follows: lung > spleen > lymph nodes > kidney > thymus gland > stomach > duodenum > liver > jejunum > heart > muscle. As shown in Table 3, the average expression level of *TLR4* in heart of resistant individuals was defined as 1.0 and we analysed the differentiation of *TLR4* mRNA expression between resistant and sensitive resource population to ETEC F18. Generally, the mRNA expression of *TLR4* gene in sensitive individuals was higher than that in resistant individuals, and the ratio of sensitive individuals to resistant individuals in all tissues apart from stomach ranged from 1.083 to 2.980. In tissues of lung, lymph node, kidney and spleen, the mRNA expression of *TLR4* gene in sensitive individuals was significantly higher than that in resistant individuals ($P < 0.05$).

DISCUSSION

Previous research has demonstrated that the polymorphism or difference in the Toll-like receptor (TLRs) family as an immune factor is highly related to the susceptibility and resistance of animals to pathogens (Giorgio and Alan, 2007). As a member of the TLRs, TLR4 is the major receptor in the innate immune recognition of the lipopolysaccharide (LPS) endotoxins expressed by Gram-negative bacteria (Lazarus et al., 2002). A mutation at certain conservative nucleotides in the *TLR4* gene could lead to a serious loss of the responsive ability of the host to Gram-negative bacteria (Poltorak et al., 2000). Therefore, the polymorphism study on the porcine *TLR4* gene can provide a genetic basis for the resistance mechanism to Gram-negative bacteria in pigs. Analysis of the porcine *TLR4* cDNA sequences published in the GenBank database of the NCBI revealed that SNPs were detected only in *TLR4* exon 3. Shinkai et al. (2006) reported 7 amino acid substitutions caused by mutations in exon 3 of the *TLR4* gene in 11 pig breeds. Zhou et al. (2008) investigated the SNPs in the fragments of exon 3 of the *TLR4* gene among Meishan, Xinhuai, Yorkshire, and Duroc breeds. As a result, five SNPs were detected. In this study, SNPs were found in exon 1 of the *TLR4* gene for the first time in 3 imported commercial breeds (Duroc, Yorkshire, and Landrace) and in Sutai pigs with the 50% Duroc lineage. Because in the population of Sutai pigs used in this study proper selection and assortative mating had been conducted for more than 5 years, the results of χ^2 test showed that all detected loci deviated from the Hardy-Weinberg equilibrium only in Sutai pigs among all populations ($P < 0.01$). The 194G > A mutation in exon 1 was detected only in Duroc, and it is note-

worthy that three *AA* homozygous Duroc animals were found. Further investigations will be needed to clarify whether this mutation has an effect on the expression level and resistance/susceptibility to pulmonary inflammatory diseases and whether it can be used as a marker for disease resistance.

The polymorphisms in exon 1 of the porcine *TLR4* gene were significantly different between Chinese indigenous pig breeds and imported breeds in this study. In the production practice of pig farming, the growth performance of Chinese indigenous pig breeds is not as good as that of the imported breeds in general. However, they show very strong stress resistance and disease resistance. This common phenomenon confirmed the genetic difference in disease resistance between Chinese indigenous pig breeds and imported breeds. Furthermore, it is worth thorough discussion and analysis to find out whether this difference in resistance is related to the remarkable difference in the polymorphism in exon 1 of the *TLR4* gene. In this study, exon 1 of the *TLR4* gene was also highly conserved for the Asian wild boar. According to the study of Palermo et al. (2009), the phylogenetic analysis of pig *TLR4* identified three clusters of variation (ancestral, Asian, European), also supporting the difference between *TLR4* of Chinese indigenous pig breeds and imported breeds. Therefore, it is believed that the genetic differences in the *TLR4* gene between Chinese indigenous breeds and imported breeds may have different origins (Palermo et al., 2009).

Regarding the expression of the porcine *TLR4* gene, Qiu et al. (2007), as the only report published in China, located the pig *TLR4* gene at SSC1q2.9–q2.13 and found the highest expression in the lung. Our study agrees with the previous finding of Qiu et al. (2007) *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 that is responsible for infections in various organs, such as inflammation in the lung. The infectious Gram-negative bacteria, including *Pasteurella pneumotropica*, *Haemophilus influenzae*, and *Klebsiella pneumoniae*, can all cause 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). Therefore, it can be deduced that the function of the *TLR4* gene may be related to innate immune recognition, especially in lung diseases of pigs. In tissues of lung, lymph node, kidney and spleen, the mRNA expression of *TLR4* gene in sensitive individuals was significantly higher than that in resistant individuals in this study ($P < 0.05$), which showed that the *TLR4* gene has a certain impact on porcine Gram-negative bacteria such as ETEC F18. The pathogenicity 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, while the levels of the mRNA expression of *TLR4* gene were rather low in piglet's small intestine. This result further demonstrated that the mRNA expression of *TLR4* gene has no significant influence on the existence of *E. coli* F18 receptor in piglet's small intestine. The impact of *TLR4* gene on resistance to ETEC F18 may be related to its regulation and control of innate immune recognition. That is to say, as a major receptor for the recognition of bacterial LPS, the downregulation of the mRNA expression of *TLR4* gene had reduced the transmembrane signal transduction of ETEC F18 bacterial LPS and then led to the responsive ability of the host to ETEC F18 in piglets.

Acknowledgements

W.B. Bao and L. Ye contributed equally to this study.

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