Differential expression of Toll-like receptor 4 signaling pathway genes in *Escherichia coli* F18-resistant and – sensitive Meishan piglets

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Abstract

The Toll-like receptor 4 (*TLR4*) signaling pathway is an important inflammatory pathways associated with the progression of numerous diseases. The aim of the present study was to investigate the relationship between *TLR4* signaling and resistance to *Escherichia coli* F18 in locally weaned Meishan piglets. Using a real-time PCR approach, expression profiles were determined for key *TLR4* signaling pathway genes *TLR4*, *MyD88*, *CD14*, *IFN-α*, *IL-1β* and *TNF-α* in the spleen, thymus, lymph nodes, duodenum and jejunum of *E. coli* F18-resistant and -sensitive animals. *TLR4* signaling pathway genes were expressed in all the immune organs and intestinal tissues, and the expression was generally higher in the spleen and lymph nodes. *TLR4* transcription was higher in the spleen of sensitive piglets (*p* < 0.05), but there was no significant difference in *TLR4* mRNA levels in other tissues. Similarly, *CD14* transcription was higher in lymph nodes of sensitive animals (*p* < 0.05) but not in other tissues. *IL-1β* expression was higher in the spleen and in the duodenum of resistant piglets (*p* < 0.05, *p* < 0.01, respectively), and there were no significant differences in other tissues. There were also no significant differences in the expression of *MyD88*, *TNF-α* and *IFN-α* between sensitive and resistant piglets (*p* > 0.05). These results further confirm the involvement of the *TLR4* signaling pathway in resistance to *E. coli* F18 in Meishan weaned piglets. The resistance appeared to be mediated via downregulation of *TLR4* and *CD14*, and upregulation of *MyD88* that may promote the release of cytokines *TNF-α*, *IL-1β*, *IFN-α* and other inflammatory mediators which help to fight against *E. coli* F18 infection.

Key words: pig, TLR4, cytokine, immune response, *E. coli* F18

Introduction

Post-weaning diarrhea (PWD) in piglets is the most frequent acute and fatal disease affecting the swine industry at present, and the enterotoxigenic *Escherichia coli* (ETEC) F18 strain is the main pathogen causing the disease (Imberechts et al. 1992, Rippinger et al. 1995, Imberechts et al. 1997). The F18 fimbriae expressed by porcine toxigenic *E. coli* strains are comprised of 1-2 um long filaments that mediate adhesion to enterocytes and once adhered, bacteria enter the cell where they multiply and mature, releasing...
enterotoxins in the process (Hahn et al. 2000). While the use of antibiotics and vaccines can reduce diarrhea resulting from *E. coli* infections, long-term treatment results in increased antibiotic-resistant ETEC, and large numbers of pathogenic bacteria continue to reside both in piglets and the surrounding environment. It is therefore preferable to improve resistance to PWD through genetic approaches in order to solve the problem at source. Some research on ETEC F18 resistance has been reported; Vogeli et al. (1997) have argued that the FUT1 G(M307)A point mutation is a useful marker for identifying *E. coli* F18 adhesion-resistant in pigs. However, multiple studies have indicated that this functional site is not usually suitable for Chinese indigenous pig breeds (Yan et al. 2003, Bao et al. 2008). Thus, screening for candidate genes that may be responsible for resistance to *E. coli* F18 in Chinese native pig breeds and identifying effective genetic markers remains a priority.

Mammalian Toll-like receptors (TLRs) derive their name from the Drosophila Toll protein, and TLRs are responsible for the activation of innate immunity, induction of the adaptive immune defense mechanism, and are associated with congenital innate immunity (Medzhitov et al. 2001, Beutler et al. 2005). Mammalian TLRs constitute a large family of transmembrane receptors, of which TLR4 has received the most attention and has been found to induce the production of inflammatory factors and cytokines during anti-infection and immunity processes (Takeda and Akira 2005, Zacharowski et al. 2006, Pan et al. 2012). We previously used Agilent two-color microarray-based gene expression profiling to investigate differential gene expression, and found that the TLR4 signaling pathway may regulate resistance to *E. coli* F18 in weaned piglets (Bao et al. 2012). Meishan pigs, a popular Chinese local breed, were infected with *E. coli* F18 and examination of intestinal tissue, bacterial counts and binding assays revealed two distinct resistant and sensitive phenotypes (Wu et al. 2014). The present study investigated the expression profiles of resistant and sensitive pigs, and assessed the differential expression of TLR4 signaling pathway genes TLR4, MyD88, CD14, IFN-α, IL-1β and TNF-α in particular. We chose three distinct types of immune organs (spleen, thymus, lymph node) and two distinct types of intestinal tissues (duodenum and jejunum). These results provide a theoretical basis for screening potential resistance-associated genes and may help to identify genetic markers of *E. coli* F18 infection in pigs.

**Materials and Methods**

**Experimental materials and sample collection**

Meishan pigs (35 days old) were obtained from the Meishan Pig Conservation Breeding Company. Prior to the main study, we selected weaning piglets at 35 days of age with almost the same birth weight and weaning weight as model animals, those piglets were from four families and tested their susceptibility to *E. coli* F18 by challenging with the pathogens through feeding F18ab and F18ac strains (Wu et al. 2014), and then we obtained 27 piglets with diarrhea and 15 piglets without diarrhea. The intestinal tract of these pigs were used to carry out series of verification tests, such as *E. coli* F18 bacteria counting, histopathological detection and adhesion test of the pathogens to the epithelial cells of the small intestine in vitro (Liu et al. 2013). Finally we attained confirmed individuals with the resistance and susceptibility to *E. coli* F18, from which we selected two resistant and two sensitive full-sib piglets from four families respectively (in total eight resistant and eight sensitive piglets, eight full-sib pairs). The spleen (the central part of spleen), thymus (the whole thymus), mesenteric lymph nodes, duodenum (the forepart of duodenum) and jejunum (the forepart of jejunum) tissues were sampled from all the individuals. About 100 mg of the sample was placed in 1.5 mL nuclease-free Eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C until further experimentation.

**Real-time PCR primer design**

Primers for real-time PCR amplification of TLR4, MyD88, CD14, IFN-α, IL-1β and TNF-α were designed based on gene sequences obtained from the GenBank database. GAPDH, TBP1 and ACTB were used as an internal control to normalize threshold cycle (Ct) values. Primers (Table 1) were synthesized by Shanghai Invitrogen Biotechnology (Shanghai, China).

**Total RNA extraction and fluorescence quantitative PCR**

Total RNA was extracted from the tissue samples (50-100 mg) using Trizol reagent (TaKaRa Biotechnology Dalian Co., Ltd, China) according to manufacturer’s instructions. Qualitative and quantitative assessment of RNA was carried out by agarose gel
Table 1. Primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequence (5’→3’)</th>
<th>Length (bp)</th>
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| TLR4      | AB232527      | F: CAGATAAGCGAGGCCGTCATT  
                       | R: TTGCAGCCACAAAAAGCA                                    | 113         |
| CD14      | AB267810.1    | F: CCTCAAGTCCTGAATGTG  
                       | R: CCGGGATTGTCAGATAGG                                     | 180         |
| MyD88     | EF198416.1    | F: GCTGGAACAGACAACTAT  
                       | R: TCCCTTGCTTTGCAGGTAAT                                   | 153         |
| TNFα      | KS4001        | F: CGACTCAGTCGCGAGATCA  
                       | R: CCGGCCCCAGATCATCAGCAAG                                  | 58          |
| IL-1β     | NM_001005149  | F: TGAATGTGCGCAAAGAGGA  
                       | R: TTGGGTACATCATCAGACG                                     | 63          |
| IFN-α     | X57191        | F: CCTGGACCACAGAGGGGA  
                       | R: TCTCATGCACCAGAGCCA                                     | 92          |
| GAPDH     | AF017079.1    | F: ACATCATCCCCTGCTCTACTGG  
                       | R: CTCGGACGCTTCTCCAC                                      | 187         |
| TBP1      | DQ845178.1    | F: AACAGTTCAGTGTTATGAGC  
                       | R: AGATGTTCTCAAACAGCCTCG                                   | 153         |
| ACTB      | XM_00312428.3 | F: TGGCGCCCAGCAAGTGAG  
                       | R: GATGGAGGGGCGAGACTCG                                    | 149         |

Fig. 1. Expression of TLR4, MyD88, CD14, IFN-α, IL-1β and TNF-α in different tissues of E. coli F18-sensitive and -resistant Meishan piglets. Samples were collected from the resistant group (n=8) and sensitive group (n=8) of piglets; three technical repetitions in qPCR analysis of every gene were performed.
electrophoresis and NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, U.S.), respectively. Precipitated RNA was dissolved in 20 μL of RNase-free H2O and stored at -70°C.

cDNA was synthesized using a Reverse Transcription System (TaKaRa Biotechnology Dalian Co., Ltd., China) according to manufacturer’s instructions. Briefly, reactions contained 2 μL of 5 × PrimeScript buffer, 0.5 μL of PrimeScript RT Enzyme Mix I, 0.5 μL of Oligo dT, 0.5 μL of random hexamers, 500 ng of total RNA, and RNase-free Water to a final volume of 10 μL. Reactions were carried out at 37 for 15 min and at 85 for 5 s.

Real-time PCR amplification was performed in 20 μL reactions containing 1 μL of cDNA (100-500 ng), 0.4 μL of forward and reverse primer (10 μL of each), 0.4 μL of 50 × ROX Reference Dye II, 10 μL of 2 × SYBR Green Real-time PCR Master Mix, and 6.8 μL of double-distilled H2O. Thermal cycling was performed as follows: 95 for 15 s, followed by 40 cycles of 95 for 5 s and 62 for 34 s. The dissociation curve was analyzed, and a Tm peak at 85 ± 0.8 was used to determine the specificity of amplification. Tm values are averages of triplicate experiments.

Data processing and analysis

The 2−ΔΔCt method, where ΔΔCt = (average Ct value of the target gene – average Ct value of the housekeeping gene) – (average Ct value of the control gene – average Ct value of the housekeeping gene), was used to process real-time PCR data (Livak et al. 2001). Statistical analysis was carried out using SPSS 16.0 software (SPSS Inc, USA), and the Student’s t-test was applied to determine significant differences in mRNA expression between different animal groups.

Results

Using the established SYBR green real-time quantitative PCR method described above, the expression levels of TLR4 signaling pathway genes (TLR4, MyD88, CD14, IFN-α, IL-1β and TNF-α) were examined in five tissues (spleen, thymus, lymph node, duodenum and jejunum) in E. coli F18-resistant and -susceptible individuals. The expression level of each gene in the thymus tissue of the resistant group was defined as 1. TLR4 signaling pathway genes were expressed in all tissues tested, and expression was highest in the spleen and lymph nodes (Fig. 1). TLR4 expression was higher in the spleen of sensitive piglets (p<0.05), but there was no significant difference in TLR4 mRNA levels in other tissues. CD14 transcription was also higher in lymph nodes in sensitive animals (p<0.05) but not in other tissues. IL-1β expression was higher in the spleen and in the duodenum of resistant pigs (p<0.05, p<0.01 respectively). No significant differences were found in the expression of IL-1β in other tissues, and no significant differences were detected in the expression of MyD88, TNF-α and IFN-α between sensitive and resistant piglets in any tissues (p>0.05).

Discussion

TLRs are cell membrane pattern recognition receptors expressed predominantly in cells that participate in the host defense against bacteria (Pasare and Medzhitov 2004). TLRs recognize and bind to highly conserved molecular structures present on or associated with particular pathogens, and in doing so trigger signal transduction cascades that lead to the release of inflammatory mediators. TLRs play an important role in the innate and acquired immune response systems. Innate immune responses caused by TLR4 stimulation are mediated through two signaling pathways, called the MyD88-dependent and MyD88-independent pathways, which utilize adaptor proteins MyD88/TIRAP and TRIF/TRAM, respectively. TLR4 is the most widely-studied member of the TLR receptor family, and the TLR4/CD14 signaling pathway is one of the most important pathways involved in mediating the inflammatory response to bacterial endotoxins. This receptor also influences the development of other diseases. The TLR4 signaling pathway functions primarily through classical MyD88-dependent pathway activation. The subsequent downstream releases inflammatory cytokines, which in turn trigger the host immune response. The signal can also be conducted through MyD88-independent pathway. The MyD88-independent pathway can generate surprisingly robust IgG antibodies to play an role in antiviral effects (Yamamoto et al. 2003a,b, Rogers et al. 2015). Identification of drugs that can block or inhibit nodes in the TLR4 signaling pathway are therefore of great clinical interest. A number of studies on TLR4 signaling pathway genes in humans and mice have demonstrated an important role in immunity, disease and infection (Takeda et al. 2005, Zacharowski et al. 2006, Pan et al. 2012), and they may therefore be associated with resistance in other animals.

In the present study we investigated the expression key TLR4 signaling pathway genes in the spleen, thymus, lymph nodes, duodenum and jejunum
of E. coli F18-resistant and -sensitive Meishan piglets as a representative Chinese breed. The expression was found to be higher in the spleen and lymph nodes, and since these both are important immune organs, the TLR4 signaling pathway does appear to contribute to the host immune response in pigs. Differential expression profiling showed that TLR4 mRNA levels were higher in the spleen of the sensitive individuals (p < 0.05), while there was no significant difference in other tissues, although the expression of TLR4 in the sensitive pigs was generally higher than that in resistant animals. This result may reflect the fact that TLR4 is the main receptor that identifies lipopolysaccharide (LPS) and so mediates the inflammatory response against a range of pathogenic bacterial species. Indeed, we previously demonstrated that TLR4 elicits the host innate immune recognition mechanism to Gram-negative bacteria including E. coli F18, and downregulation of TLR4 expression may be related to resistance (Bao et al. 2011).

CD14 mRNA levels were significantly higher in the lymph nodes of the sensitive pigs (p < 0.05), but there was no significant difference in other tissues, although as observed with TLR4, the expression of CD14 was generally higher in sensitive animals CD14 may have a particularly high affinity for LPS, since they are known to be specific recognition sites for LPS (Peri et al. 2012). CD14 combines with LPS binding protein (LBP) and passes LPS on to TLR4 receptor complexes (Wright et al. 1990). Previous studies have shown that CD14-/− in mice are resistant to peritoneal infection with some clinical isolates of E. coli (Haziot et al. 2001). Thus the increased expression of CD14 may increase the risk of infection (Metkar et al. 2012), and downregulation of CD14 may therefore be associated with resistance to E. coli F18.

The expression of MyD88, TNF-α, IL-1β and IFN-β was generally higher in all the tissues in the resistant pigs, and levels of IL-1β mRNA in resistant spleen and duodenum tissue were particularly elevated (p < 0.05; p < 0.01). Myeloid differentiation factor 88 (MyD88) is an essential adaptor in the TLR-dependent pathways (Kawai et al. 1999, Hoebe et al. 2003). This signaling pathway therefore controls the expression of cytokines that activate the host immune system (Dauphinee and Karsan 2006, Liu et al. 2006). Together, these results and previous findings indicate that up-regulation of MyD88 in the resistant pigs leads to a series of inflammatory responses including increased release of cytokines such as IL-1β and other inflammatory mediators, which accelerate chemotaxis and aggregation of neutrophils and macrophages, increase capillary permeability, and assists infiltration of lymphocytes. All of these inflammatory responses may contribute to resistance to E. coli F18 infection.

The present results indicate that resistance to E. coli F18 in pigs is associated with downregulation of TLR4 and CD14, and possible upregulation of MyD88. We intend to further investigate the regulatory functions of TLR4, CD14, IL-1β and other genes at the cellular level using RNAi and overexpression approaches. Resistance-associated TLR4 signaling pathway SNPs will also be probed to identify genetic markers for the diagnosis and treatment of E. coli F18 infections in pigs and other animals.

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References


