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Developmental expression of *LTβR* and differential expression in *Escherichia coli* F18 resistant/sensitive piglets

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ABSTRACT. We analyzed *LTβR* mRNA expression in piglets from birth to weaning and compared the differential expression between *Escherichia coli* F18-resistant and sensitive populations to determine whether this gene could be used as a genetic marker for *E. coli* F18 resistance. Sutai piglets of different age groups (8, 18, 30, and 35 days; N = 4 each) and piglets demonstrating resistance/sensitivity to *E. coli* F18 were used. *LTβR* expression levels were determined by real-time PCR. The *LTβR* expression levels in the lymph node, duodenum, and jejunum were significantly higher in 8-day-old piglets than in the other age groups ($P < 0.01$), and the expression levels were significantly

higher in the lungs of 8-day-old piglets than in 35-day-old piglets ($P < 0.01$) and 30 day-old piglets ($P < 0.05$). In liver tissue, the expression level was significantly higher in the 35-day-old piglets than in other age groups ($P < 0.01$). In the stomach tissue, the expression level was significantly higher in 35-day-old piglets than in 18-day-old piglets ($P < 0.05$). *LTβR* expression in the lymph nodes was significantly higher in the resistant group than in the sensitive group ($P < 0.01$), but there was no significant difference in the other tissues ($P > 0.05$). These results indicate that 8 days after birth is a crucial stage in the formation of mesentery lymph nodes and immune barriers in pigs, and increased expression of *LTβR* may be beneficial for developing resistance to *E. coli* F18.

Key words: Pigs; *LTβR* gene; *E. coli* F18; mRNA

INTRODUCTION

Escherichia coli is known to cause post-weaning diarrhea (PWD) in pigs, and the most predominant pathogenic strain associated with this condition is *E. coli* F18 (Boldin, 2008). Studies have shown that the pathogenicity of *E. coli* F18 depends on the presence of specific receptors expressed on the small intestinal surface of piglets (Bertin and Duchet-Suchaux, 1991). Furthermore, the genes encoding *E. coli* F18 receptors are functional, and contribute to the specific binding and absorption of bacterial ligands on the histiocytes of the host. Thus, mutations in these genes can result in changes or total loss of protein structure and function of the pilus receptor, disrupting the binding of *E. coli*, consequently leading to resistance to PWD. The α1-fucosytransferase (*FUT1*) gene was identified as an important candidate gene for the *E. coli* F18 receptor protein using the candidate gene approach and genetic linkage analysis (Vögeli et al., 1997). Meijerink et al. (1997; 2000) reported that a mutation in the M307 locus of *FUT1* confers resistance to *E. coli* infections. However, the distributions of genotypes at this locus are highly skewed among Chinese porcine breeds; therefore, this gene is not suitable for breeding disease resistance (Bao et al., 2008). So screening and identifying the gene conferring resistance to *E. coli* F18 and search for novel genetic markers are very important.

Lymphotoxin (LT) is a heteromeric complex of LTα, and is also called TNFβ and LTβ. LTβ mainly exists in a dimeric form and combines with LTβ to form $LT\alpha_1\beta_2$. $LT\alpha_1\beta_2$ does not bind to the known TNF receptors, referred to here as TNF-R55 and TNF-R75; rather, it interacts with another receptor in the TNF family called the LT-β receptor (LTβR) (Crowe et al., 1994; Sudhamsu et al., 2013). Browning et al. (1996) found that the signaling event mediated by *LTβR* plays an important role in the immune response, and indicated that activation of *LTβR* might have an application in tumor therapy. Wege et al. (2014) found that *LTβR* expressed on hematopoietic cells seemed to be involved in the downregulation of acute inflammatory reactions paralleled by the appearance of immature myeloid cells. Vondenhoff et al. (2009) found that LTβR on mesenchymal cells controlled the formation of lymph nodes, through the combination of $LT\alpha_1\beta_2$ with LTβR leading to the induction of chemokines, which attract hematopoietic cells. At the same time, Hehlgans and Pfeffer (2005) indicated that the *LTβR* gene belongs to the TNF family of receptors, which play an important role in regulating

the immune response, the formation of the lymphonodus, and the maintenance of the intestinal barrier. The intestines represent a first line of defense for preventing infection by pathogenic bacteria. Studies have indicated that gut-associated lymphoid tissue, including the mesenteric lymph nodes (MLNs), is the primary intestinal barrier protecting the intestinal tissue from infection (Nagler-Anderson, 2001). Therefore, as *LTβR* is an immunological gene required for the formation of lymph nodes, further research is needed to determine its ability to confer resistance to *E. coli* F18.

Therefore, in this study, we studied the *LTβR* expression profile and its association with PWD in response to *E. coli* F18 in piglets of different ages, and compared the differential *LTβR* expression between *E. coli* F18-resistant groups and *E. coli* F18-sensitive groups. Through this study, we aimed to provide an experimental and theoretical basis to verify the function of this gene and to determine its role as a novel genetic marker for resistance to *E. coli* F18.

MATERIAL AND METHODS

Ethics statement

The animal study proposal was approved by the Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments Ethics Committee under permit No. SYXK(Su) IACUC 2012-0029. All piglet experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

Animals and sample collection

Sutai pigs produce high quality lean meat and are bred from Duroc and Meishan pigs. For this experiment, we used 24 Sutai piglets obtained from the Center of Sutai Pig Breeding, Suzhou, China. Of these, 16 pigs of different ages (8, 18, 30, and 35 days; N = 4 in each group; weaning time, 30 days) were selected for tissue sampling. The animals were allowed access to food and water *ad libitum* under normal conditions, and were humanely sacrificed by an intravenous injection of sodium pentobarbital as necessary to ameliorate suffering. Piglets within the same age group had similar feeding conditions, weight, shape, and health status. In addition, we selected eight pigs that showed resistance and sensitivity to *E. coli* F18 and were similar in terms of birth weight, weaning weight, shape, and coat color (N = 4 each) (Wu et al., 2007; Liu et al., 2013). Tissue samples from 11 organs, including the heart, liver, spleen, lung, kidney, stomach, muscle, thymus, MLNs, duodenum, and jejunum, were collected, immediately stored in liquid nitrogen, and transferred into a -70°C freezer in our laboratory until analysis.

Design and synthesis of real-time PCR primers

Real-time PCR primers were designed using the Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the porcine *LTβR* gene sequences published in the GenBank database (Table 1). *GAPDH* was used as the reference gene. The primers were synthesized by Shanghai Invitrogen Biotechnology Co.

Table 1. Real-time PCR primers and their sequences.

Gene	Primers sequences (5'→ 3')	Length (bp)	Annealing temperature (°C)
LTβR	F: GGTGCTACAACAGCAAAGCC	219	62
	R: TCAGGGATGGGGTATGGAGG		
GAPDH	F: ACATCATCCCTGCTTCTACTGG	187	62
	R: CTCGGACGCCTGCTTCAC		

Total RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted from the 11 tissue samples using TRIzol (TaKaRa). The integrity, purity, and concentration of total RNA were determined by denaturation gel electrophoresis with 2.2% formaldehyde and UV spectrophotometry. The extracted total RNA was stored at -70°C prior to reverse transcription and generation of cDNA.

The reverse transcription reaction mixture (10 µL) consisted of 2 µL 5X PrimerScript buffer reaction solution, 0.5 µL PrimerScript RT enzyme mix I, 0.5 µL oligo dT primers, 0.5 µL random hexamers, 500 ng total RNA, and RNase-free H_2O. The conditions for reverse transcription were 37°C for 15 min and 85°C for 5 s. The resulting cDNA was stored at 4°C.

The cDNA was amplified by real-time PCR. The real-time PCR reaction mixture (20 µL) consisted of 1 µL template, 0.4 µL 10 µM of each forward and reverse primers, 0.4 µL 50X ROX Reference Dye II, 10 µL 2X SYBR green Real-time PCR Master Mix, and 7.8 µL double-distilled water. The real-time PCR protocol was as follows: one cycle at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s, and a final step at 4°C. The dissociation curve was analyzed following amplification. A dissociation curve T_m of 85° ± 0.8°C was used to determine the specificity of the amplification. Each sample was amplified three times, and the average values were calculated.

Statistical analysis

The real-time PCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt = (average Ct value of target genes in the test group - geometric mean value of Ct values of reference genes in the test group) - (average Ct value of target genes in the control group - geometric mean value of Ct values of reference genes in the control group). Statistical analyzes were performed with SPSS 16.0. Differences in the levels of *LTβR* expression among piglets of different age groups were analyzed by one-way analysis of variance, and differences between *E. coli* F18-resistant and *E. coli* F18-sensitive groups were analyzed by *t*-test.

RESULTS

Analysis of RNA purity and integrity

Total RNA samples were assayed using 1% agarose gel electrophoresis. The results revealed the presence of 28, 18, and 5S bands with no significant bands of DNA contamination or degradation, indicating that the extracted total RNA was of high purity. RNA purity was evaluated with ultraviolet spectrophotometry. The A_{260}/A_{280} ratio of the samples was 1.8-1.9, indicating that the extracted RNA was of high purity and could be used for subsequent tests.

Expression profiles of the *LTβR* gene in various tissues among piglets of different age groups

The results of the real-time PCR study revealed that the *LTβR* gene was expressed in all tissue samples studied in Sutai pigs of different ages. The expression profiles were slightly changed across the different age groups and were lower in the heart, muscle, and thymus than in the other tissues studied (Figure 1).

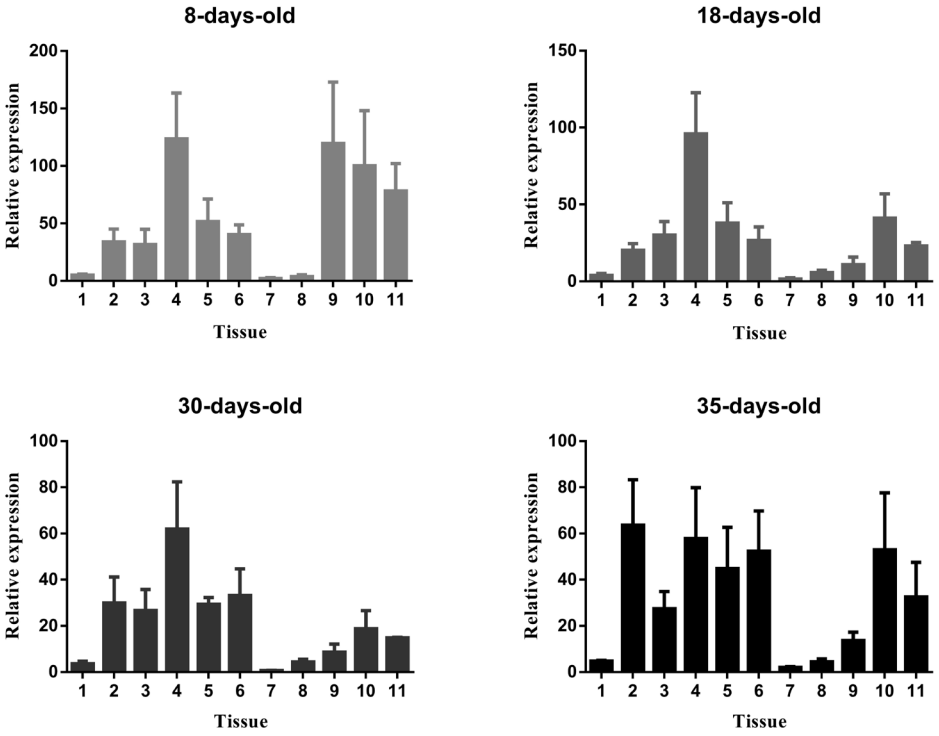

Figure 1. Expression profiles of the Lymphotoxin β receptor (*LTβR*) gene in 11 tissues sampled from Sutai piglets of different age groups; Numbers 1-11 denote the heart, liver, spleen, lung, kidney, stomach, muscle, thymus, lymph node, duodenum, and jejunum, in that order.

LTβR gene expression in tissues of piglets in different age groups

Expression levels of the *LTβR* gene in the lymph node, duodenum, and jejunum were somewhat similar and was significantly higher in 8-day-old piglets than in the other age groups ($P < 0.01$); however, no differences were observed among the other ages ($P > 0.05$). Furthermore, *LTβR* expression was significantly higher in the lungs of 8-day-old piglets than in the lungs of 35-day-old piglets ($P < 0.01$) and 30-day-old piglets ($P < 0.05$). *LTβR* expression was significantly higher in the livers of 35-day-old piglets than in the livers of the other age groups ($P < 0.01$). *LTβR* expression was significantly higher in the stomach tissues of 35-day-old piglets than in the stomach tissues of 8-day-old piglets ($P < 0.05$; Figure 2).

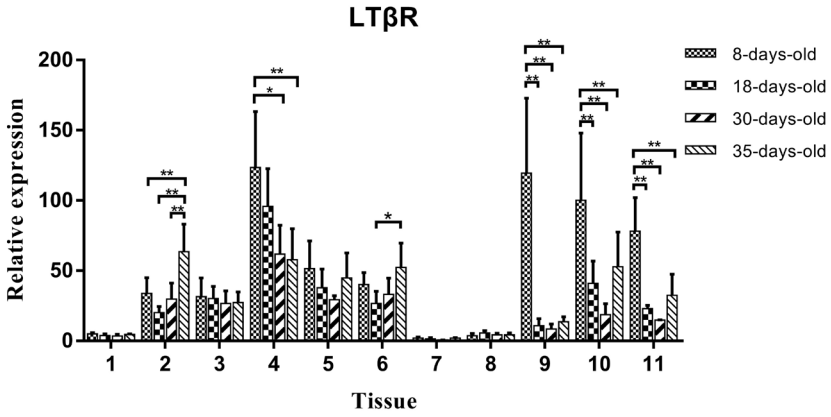

Figure 2. Expression levels of the *LTβR* gene in 11 different tissues on days 8, 18, 30, and 35. The average ΔCt value in muscle from each age was used as a reference; Numbers 1-11 denote the heart, liver, spleen, lung, kidney, stomach, muscle, thymus, lymph node, duodenum, and jejunum, in that order; *significant difference ($P < 0.05$); **very significant difference ($P < 0.01$).

LTβR gene expression in tissues of piglets resistant and sensitive to *E. coli* F18

Expression levels of the *LTβR* gene were examined in various tissues. Generally, mRNA expression of the *LTβR* gene was higher in the individuals resistant to *E. coli* F18 than in the sensitive ones, except for the mRNA expression in liver tissue. In lymph nodes, mRNA expression of the *LTβR* gene was significantly higher in the resistant group than in the sensitive group ($P < 0.01$), but there was no difference in mRNA expression among the other tissues ($P > 0.05$; Figure 3).

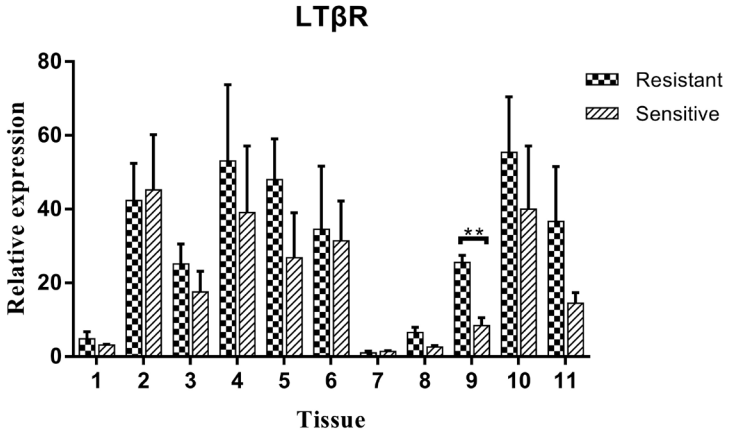

Figure 3. Expression of the *LTβR* gene between *Escherichia coli* F18-resistant and sensitive piglets. The average ΔCt value of muscle from each age was used as a reference. Numbers 1-11 denote the heart, liver, spleen, lung, kidney, stomach, muscle, thymus, lymph node, duodenum, and jejunum, in that order; **very significant difference ($P < 0.01$).

DISCUSSION

The biological function of LT is similar to that of TNFα, and is involved in killing various tumor cells, regulating the immuno-inflammatory response, and inducing antigen expression in various differentiated cells. LT plays an efficient role as a polymer of $LT\alpha_1\beta_2$, whose specific receptor is LTβR. Lymph node formation is a complex process, which is controlled by the induction of the *LTβR* gene in mesenchymal cells by $LT\alpha_1\beta_2$-expressing lymphoid tissue inducer (LTi) cells. Extravasation of the first hematopoietic cells at future lymph node locations occurs independently of LTα, and these cells expressing TNF-related activation-induced cytokine (TRANCE) are the earliest LTi cells. $LT\alpha_1\beta_2$ expression is first induced by paracrine signaling. Subsequent LTβR activation in mesenchymal cells leads to their differentiation to stromal organizers, which then begin to express TRANCE, interleukin 7 (IL-7), and vascular endothelial growth factor-C (VEGF-C) in addition to induced adhesion molecules and chemokines. Both TRANCE and IL-7 further induce the expression of $LT\alpha_1\beta_2$ in newly arrived immature LTi cells, resulting in further activation of LTβR and the generation of a positive feedback loop. Thus, the activation of LTβR by LTi cells during lymph node development creates a local environment that attracts hematopoietic precursors, in which they locally differentiate into fully mature $LT\alpha_1\beta_2$-expressing LTi cells. Furthermore, the same signals may regulate lymphangiogenesis to the lymph node through induction of VEGF-C (Vondenhoff et al., 2009). Therefore, as a receptor-encoding gene, the role of *LTβR* in regulating the body's immune response is inseparable from its role in lymphonodus formation. It is important to investigate the association between the function of the *LTβR* gene and the resistance of piglets to *E. coli* F18, as MLNs form part of the main intestinal barrier. The results indicate that the *LTβR* gene is expressed in all tissues studied, and that its expression levels were lower in the heart, muscle, and thymus than in the other tissues. The thymus is an important lymphoid organ whose function is closely related to the immune system. In addition, T cells differentiate, develop, and mature in the thymus. Therefore, low *LTβR* expression in the thymus could indicate that the *LTβR* gene does not exert some of its effects during early thymic development in piglets at birth. Tumanov et al. (2009) found that LT produced by T cells migrating to the liver contributed to tissue regeneration of the liver. Behnke et al. (2014) found that a lack of *LTβR* signaling contributes to changes in liver morphometry, cytokine levels, and blood parameters. These data indicate that the *LTβR* gene plays an important role in the regeneration of liver tissue. Expression levels of *LTβR* were significantly higher in the livers of 35-day-old piglets than in piglets of other age groups ($P < 0.01$). This suggests that the liver forms rapidly during the first 30-35 days of life, and is stabilized before the piglets are 30 days old. Mice lacking the *LTα* gene have abnormal phenotypes, and lack lymphonodus and T and B cells in the spleen (De Togni et al., 1994; Banks et al., 1995). In the present experiment, the higher level of *LTβR* expression in the spleen could indicate that this gene plays a key role in its development. Immune signaling pathways consisting of LTβR, LIGHT, and TNF are conducive to resistance to pathogen invasion (Ware, 2005). In lung tissue, the expression level of this gene in 8-day-old piglets was significantly higher than the corresponding levels in 35- ($P < 0.01$) and 30-day-old piglets ($P < 0.05$), and expression in the tissues tended to drop gradually with age. Within the uterus, embryos obtain oxygen from the placenta, and after birth, there is a sudden shift following which the organism is required to obtain oxygen from the air. Lung tissue can be invaded by many bacteria that contribute to the organism's immune response. Therefore, the significantly higher level of *LTβR* expression in the lung tissues of 8-day-old piglets may be due to an immunological reaction at birth. Unweaned piglets

are not susceptible to *E. coli* because the antibodies in breastmilk protect the piglets from enteric pathogen infection. At 35-days-old, the piglets will have been weaned for approximately 1 week; at this age, newly weaned piglets would be most susceptible to *E. coli* F18 infection regardless of the phenotype or of the status of their immune system. *LTβR* expression in the stomach tissue was significantly higher in 35-day-old piglets than in 8-day-old piglets ($P < 0.05$), which may be necessary to generate immune responses in the stomachs of weaned piglets.

The intestines (duodenum and jejunum) are an important site where *E. coli* F18 cells adhere to the intestinal epithelium. Therefore, we compared the expression of the *LTβR* gene in the lymph node, duodenum, and jejunum. The results indicated that the expression levels were similar in all tissues; the expression level was significantly higher in 8-day-old piglets than in piglets of the other age groups ($P < 0.01$), but there were no differences among the other age groups ($P > 0.05$). This higher expression could be due to the development of the intestinal immune system at this stage, and MLNs and the intestinal barrier may be rapidly formed at 8 days. Moreover, diarrhea occurs at a lower incidence in breastfed piglets regardless of the presence of *E. coli* F18 receptors; this is because breastmilk offers protection against *E. coli* F4 and F18 infections (Deprez et al., 1986). Therefore, we speculated that the intestinal immune system formed rapidly around 8 days of age due to protection gained from breastmilk antibodies in piglets, which may contribute to *E. coli* F18 resistance after weaning. The *LTβR* expression profile in resistant/sensitive groups was somewhat similar among piglets in different age groups. mRNA expression of the *LTβR* gene was examined in various tissues and was found to be higher in resistant individuals than in sensitive ones in all tissue samples except for the liver. mRNA expression of the *LTβR* gene in the lymph nodes was significantly higher in resistant individuals than in sensitive individuals ($P < 0.01$), but there was no difference in the expression of the other tissues ($P > 0.05$). We previously reported serum biochemical values in resistant/sensitive groups and found that immune responses and disease resistance were better in resistant pigs than in sensitive pigs (Bao et al., 2010). The higher expression of *LTβR* may be indicative of MLN formation, and improved immune signaling pathways contribute to better immunity in an organism. High *LTβR* gene expression contributes to the formation of the intestinal immune system and may be indicative of piglets developing resistance to *E. coli* F18. In conclusion, 8 days of age is a crucial stage during piglet development during which MLNs and immune barriers are formed. Further study should aim to investigate the pattern of *LTβR* expression in piglets aged 8 to 18 days in order to explore the molecular mechanisms involved in establishing the intestinal barrier. The expression profiles of the *LTβR* gene in *E. coli* F18-resistant and *E. coli* F18-sensitive groups indicate that increased expression of *LTβR* in lymph nodes may be beneficial for piglets and aid in developing resistance to *E. coli* F18.

Conflicts of interest

The authors declare no conflict of interest.

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