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Expression Differentiation of *BPI* Gene in Post-Weaning Piglets of Yorkshire, Sutai and Meishan Breeds

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Abstract: The Bactericidal/Permeability-Increasing protein (BPI) plays a very important role in the animal body's natural defense. It has a series of biological functions such as killing Gram-negative bacteria, the neutralization of endotoxin or lipopolysaccharides, etc. To investigate the effect and mechanism of porcine *BPI* gene on enterotoxigenic *Escherichia coli* F18, particularly whether the expression of *BPI* gene is related to the different breeds. In this study, real-time PCR was used to analyze the expression of *BPI* gene expression in post-weaning piglets of Yorkshire, Sutai (Resource Population of resistance to *E. coli* F18) and Meishan. The results showed that there was almost no expression or very low expression of *BPI* gene in heart, liver, spleen, lung, kidney, stomach, muscle, thymus and lymph nodes but there was a high expression in the duodenum and jejunum and the expression levels were significantly different from those of the other 9 organs. It also showed that the jejunum and duodenum expression of *BPI* gene in Sutai was significant higher than Yorkshire and Meishan ($p<0.05$). The researchers can speculate that the expression of *BPI* gene was significant difference in different breeds. The researchers can further indicates that the expression of the *BPI* gene in Sutai piglets could be beneficial to the infection of *E. coli*. *BPI* gene might have a direct role against diarrhea and edema disease caused by ECF18 in weaned piglets, the resistance was related to the upregulation of *BPI* gene expression in the intestine. *BPI* gene can be identified as a genetic marker for future breeding against *E. coli* and Salmonella related diseases.

Key words: *BPI* gene, gene expression, pig, real-time PCR, bacteria, human

INTRODUCTION

Bactericidal/Permeability-Increasing protein (BPI) is a 55 kDa cationic antimicrobial polypeptide of neutrophil primary (Azurophilic) granules of humans, rabbits and cows (Levy *et al*., 2000). In humans this protein is also expressed, to a lesser extent in human monocytes, eosinophils, fibroblasts and epithelial cells (Calafat *et al*., 1998; Reichel *et al*., 2003; Canny *et al*., 2002). Among AMPs (Levy, 2004), BPI is notable for its high affinity (nM) for the lipid A region common to all LPS (lipopolysaccharide) of gram-negative bacteria. Besides having the ability of killing gram-negative bacteria neutralization of endotoxin or Lipopolysaccharide (LPS), BPI has a series of biological functions such as an opsonic function, promoting complement activation, opsonization for increased phagocytosis inhibiting angiogenesis inhibiting the release of inflammatory mediators, anti-fungal and protozoan and it plays a very important role in the natural defense in animal body (Elsbach, 1998; Weiss *et al*., 1978; Iovine *et al*., 1997). BPI's crystal structure shows a boomerang shaped bi-partite molecule (Beamer *et al*., 1997) that includes a cationic, lysine-rich N terminus containing the antibacterial and LPS (endotoxin)-neutralizing activities of the molecule (Ooi *et al*., 1991) and a C terminus that contributes to the opsonic activity of BPI (Iovine *et al*., 1997). BPI also facilitates delivery of gram-negative bacterial outer membrane blebs to human DCs (Schultz *et al*., 2007). Natural BPI_{55} and recombinant BPI_{55} showed bactericidal and Lipopoly-Saccharide (LPS) binding activity similar to a 23 kD fragment of

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recombinant BPI N-terminal 199 amino acid ($rBPI_{23}$ or BPI_{1-199}). Natural BPI_{55}, recombinant BPI_{55} and $rBPI_{23}$ can kill many kinds of GNB and neutralize bacterial endotoxin but have no adverse effects on eukaryotic cells (Kong *et al*., 2006). It has been demonstrated that the N-terminus of BPI is identical to natural BPI in the effect on LPS and GNB (Li *et al*., 2006). Christopher *et al*. (2004) detected that there were AvaII and HpaII restriction polymorphisms in *BPI* gene exons 4 and 10, respectively in York, Hampshire, Duroc, Landrace, Large White, wild boar and Meishan pig. Their drug testing showed that the genotype was related to the susceptibility of swine Salmonella. They also identified *BPI* gene as the candidate gene for disease resistance breeding.

Diarrhea and edema disease are two major infectious diseases which cause the death of post-weaning piglets and are the diseases that lead to huge economic loss in the swine business. The major pathogen of these two diseases is enterotoxigenic *E. coli* F18 (ECF18). ECF18 relies on its fimbriae to adhere to the piglet small intestine epithelial cell surface and binds to porcine small intestinal epithelial cell brush F18 receptor. It then settles, reproduces and produces enterotoxin causing piglet diseases (Da Silva *et al*., 2001). Enterotoxigenic *Escherichia coli* and Salmonella are the main swine intestinal gram-negative bacteria. Therefore, real-time PCR was used to detect tissue expression differentiation of *BPI* gene in Post-weaning Piglets of Yorkshire, Sutai and Meishan breeds which were foreign pig breeds hybrid lines bred with foreign lineages and Chinese domestic pig breeds. The role and mechanism of the *BPI* gene in porcine ECF18 was discussed to find out whether the expression of *BPI* gene was associated with the different breeds or not. Moreover, it will provide the foundation for further research in the breeding of ETEC F18-resistant pigs in the future.

MATERIALS AND METHODS

Experimental materials: The Sutai pig is a new hybrid between the Duroc and Taihu breeds that produces high quality lean meat. In previous studies, the researchers identified a few FUT1 AG animals (9.2%) in a Sutai pig population and selectively bred them to generate the prized Sutai FUT1 AA individuals (ETEC F18 resistant) (Bao *et al*., 2008). After 5 years of continuous selection and breeding, two pig resource populations were established with one carrying the ETEC F18-resistant AA genotype and the other harboring ETEC F18-sensitive AG or GG genotypes; each has a population of over 200 animals. Simultaneously, the researchers also constructed a type V secretion system to express ETEC F18 adhesin. The display of functional adhesin through the type V secretion system was combined with receptor binding experiments to further analyze and verify the resistance/sensitivity to the ETEC F18 strain among these pig resource populations (Wu *et al*., 2007). Yorkshire piglets were collected from Engineering Research Centre for Molecular Breeding of Pig in Changzhou city of Jiangsu province. Meishan were collected from Meishan pigs Conservation Breeding company. About 19 Sutai pigs , 4 Meishang pigs and 4 Yorkshire after sacrifice, the following organs, jejunum and duodenum were collected in 1.5 mL Eppendorf nuclease-free tubes and stored immediately in liquid nitrogen and then placed in a low temperature freezer (-80°C) until further study.

Design and synthesis of real-time PCR primer: Real-time PCR primers were used according to the published porcine *BPI* and *GAPDH* gene sequences. Using the software of primer express 2.0, BPI primers were designed based on the sequence of NM_001159307 in GenBank and synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. A 136 bp BPI fragment was amplified with forward primer 5'-ATATCGAATCTGCGCTCCGA-3' and reverse primer 5'-TTGATGCCAACCATTCTGTCC-3'. Primers were designed across the exon to avoid genomic DNA contamination. *GAPDH* gene as internal reference, forward primer: 5'-ACATCATCCCTGCTTCTACTGG-3'; reverse primer: 5'-CTCGGACGCCTGCTTCAC-3', its amplified fragment length was 187 bp.

Total RNA extraction and real-time PCR reaction: RNA was extracted from all the tissues of the piglet using Trizol RNA extraction kit (Invitrogen, Gaithersburg, MD, USA). The extraction step was operated strictly according to the kit instruction. After electrophoresis with 1% agarose gel, the RNA purity and concentration were detected by ultraviolet spectrophotometry and then stored at -70°C. For cDNA synthesis, 10 μL reaction mixtures contained 2 μL 5×PrimerScript Buffer reaction mixture, 0.5 μL PrimerScript RT Enzyme Mix I, 0.5 μL Oligo dT, 0.5 μL Random 6 mers, 500 ng total RNA and RNase free H_2O were added to10 μL. Reaction condition was set at 37°C for 15 min, 85°C for 5 sec and the product was preserved at 4°C.

Fluorescent quantitative PCR reaction system and reaction conditions: Reaction system was 20 μL: 1 μL cDNA, upstream and downstream of each primer (10 μmol L^{-1}), 0.4 μL ROX Reference Dye II (50×), 10 μL SYBR Green Real-time PCR Master Mix (2×), 7.8 μL ddH_2O. PCR reaction condition was 95°C for 15 sec; 40 cycles of 95°C for 15 sec and 62°C for 34 sec and then,

persevered at 4°C, melting curve was analyzed after amplification. The unity of PCR amplification was determined with the use of melting curve (85±0.8)°C Tm peak. Each sample was tested in real-time PCR for 3 times and the average was taken.

Statistical analysis: The $2^{-\Delta\Delta Ct}$ method was suitable for processing the relative quantification results. The following formula was used: ΔΔCT = (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. Data were analyzed by SPSSV13.0. Differences among breeds were analyzed by independent-samples t-test. The $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The analysis of RNA purity and integrity: Total RNA was detected by ultraviolet spectrophometer, A260/A280 of samples was 1.8-1.9 indicating high quality RNA extraction which could be used for subsequent tests. RNA formaldehyde degeneration gel electrophoresis shows that 28S and 18S bands were bright clear and sharp and the brightness of 28S band was 2 times of that of 18S band.

Relative expression of *BPI* gene in different tissues: SYBR Green real-time fluorescence quantitative PCR method was used in this experiment to detect change of *BPI* gene expression in all 11 individual tissues including heart, liver, spleen, etc. With the internal reference gene *GAPDH* to normalize the expression level and define the *BPI* gene expression level of heart tissue as 1 and calculate the relative quantitative levels of gene expression in other tissues. It showed that there was almost no expression or very low expression of *BPI* gene in heart, liver, spleen, lung, kidney, stomach, muscle, thymus and lymph nodes but there was a high expression in the duodenum and jejunum and the expression levels were significantly different from those of the other 9 organs.

The amplification curve and melting curve of fluorescence quantitative PCR: After the reaction according to the changes of fluorescence, system automatically generated the curves for cycle number and the detection of changes in the amplification reaction of the fluorescence kinetics. The *BPI* gene PCR amplification curve was shown in Fig. 1. The analysis of the melting curve of *BPI* gene mRNA real-time PCR products was shown in Fig. 2. The figure showed that there was one specific peak in *BPI* gene real-time PCR product, without primer dimer and the formation of non-specific product.

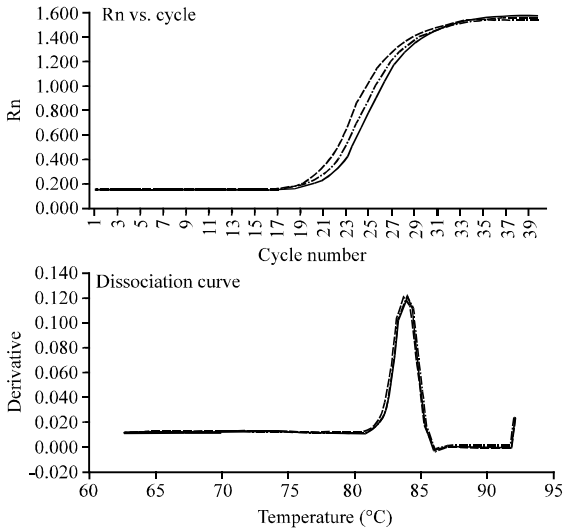

Fig. 1: Real-time PCR amplification curve and dissociation curve for the *BPI* gene in jejunum and duodenum

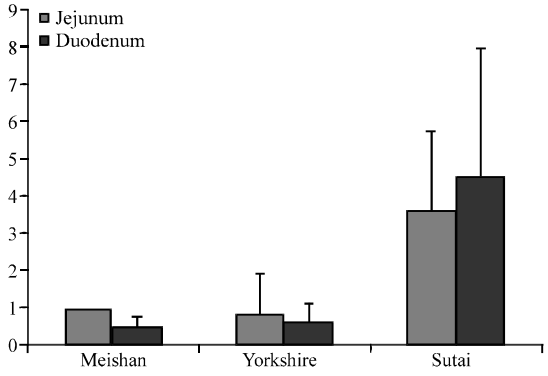

Fig. 2: Expression levels of *BPI* gene among different tissues of three breeds

Expression analysis of *BPI* gene in post-weaning Piglets of Yorkshire, Sutai and Meishan breeds: *BPI* gene in post-weaning Piglets of Yorkshire, Sutai and Meishan breeds, the researchers detected the expression of *BPI* gene in jejunum and duodenum. The study comprised 27 individuals. The data indicated that the average expression levels of *BPI* gene in jejunum and duodenum existed in Yorkshire, Sutai and Meishan breeds.

Table 1: Comparison of *BPI* gene expression between jejunum and duodenum of three breeds

Part of small intestine	Meishan	Yorkshire	Sutai
Jejunum	1.000±0.000[b]	0.851±1.080[b]	3.623±2.107[a]
Duodenum	0.531±0.290[b]	0.638±0.513[b]	4.516±3.430[a]

The results showed that the jejunum and duodenum expression of *BPI* gene in Sutai was significant higher than Yorkshire and Meishan breeds ($p<0.05$) (Table 1 and Fig. 2).

Neutrophil is an important component in the body's innate immune system. It is the first line of defense against foreign microorganisms, especially pathogens. In recent years, proteins or peptides with anti-microbial activity have been found in a variety of neutrophils. Bactericidal/Permeability-Increasing protein (BPI) is a pluripotent protein located in neutrophils and tissue that likely plays a pivotal role in host defense against GNB and their endotoxin by means of its antibiotic and endotoxin-neutralizing and disposing functions (Schultz *et al*., 2007). Lipopolysaccharide (LPS) is the main component in gram-negative bacterial outer membrane. When the bacteria dies and is dissolved or the bacterial cell is destroyed by artificial means, free LPS also known as endotoxin will be released. LPS molecule consists of three parts: the O side-chain core oligosaccharide and lipid A. LPS is toxic to the host and the main toxic component is lipid A. Since, the N terminal fragment of BPI molecule is highly cationic, when BPI protein binds to lipid A of LPS on the bacterial cell wall, the affinity and binding become very strong. At the beginning, the binding of the BPI protein and the bacterial outer membrane activates a series of enzymatic systems which promotes the degradation of membrane phospholipids and peptidoglycan and leads to the change in the bacterial outer membrane permeability and growth inhibition. But the bacteria can also repair and grow. In the later stage as the length of time of activity increases, the concentration of BPI protein increases and damages the bacterial cell membrane; as a result, the structural and functional changes appear which leads to irreversibly growth inhibition and ultimately lysis and death (Weiss *et al*., 1980; Mannion *et al*., 1990).

E. coli is the main Gram-negative bacteria in the gut, the main pathogen that leads to the diarrhea and edema disease in weaned piglets. When intestine is infected with *E. coli*, a large number of bacteria grow and reproduce, accompanied by death and the release of endotoxin. On the other hand, ECF18 can also rely on its fimbral adhesion to the surface of the epithelial cells in the small intestine and binds to the epithelial cell brush F18 receptors in porcine small intestine and settle in it thus reproduce itself in large amount and produces endotoxin to cause tissue damage and pig disease. Combining the effect of the biological activity of BPI protein, *BPI* gene is directly and closely related to the resistance to intestinal ECF18 and other gram-negative bacteria. When the piglets are invaded by ECF18 strains, the bactericidal effect of *BPI* gene and a series of cascading immune effects are activated. Since it has a cytotoxic effect on gram-negative bacteria and neutralizes endotoxin, it kills pathogens before the attachment of ECF18 strains to the small intestine and the release of intestinal toxins which reduces the probability of diarrhea and edema disease in the infected piglets.

Christopher *et al*. (2004) reported that there were *AvaII* and *HpaII* restriction polymorphisms in BPI gene exons 4 and 10, respectively toxicity test showed that the genotype was related to the susceptibility to swine Salmonella and classify the *BPI* gene as the disease-resistance candidate gene for breeding. The research studied the expression of *BPI* gene in all 11 individual tissues including heart, liver, spleen, etc. The results showed that there was almost no expression or very low expression of the *BPI* gene in the weaned piglet heart, liver, spleen, lung, kidney, stomach, muscle, thymus and lymph nodes but there was a high expression in the duodenum and jejunum. The results indicated that BPI not played an important role in both immune response and control function in preventing weaning piglets from being infected by *E. coli* F18. But there might be a direct role of *BPI* gene on intestinal disease in weaned piglets caused by ECF18. Therefore, real-time PCR was used to detect jejunum and duodenum expression of *BPI* gene in post-weaning Piglets of Yorkshire, Sutai and Meishan breeds. The results showed that the jejunum and duodenum expression of *BPI* gene in Sutai was significant higher than Yorkshire and Meishan ($p<0.05$). The researchers can speculate that the expression of *BPI* gene was significant difference in different breeds. Sutai pig, a new, high-quality, lean-meat pig breed, following 15 years of breeding. The piglets tested were from the ECF18-resistant resource populations stated. Stronger adaptation and immune function may cause the difference between Sutai and the other two breeds in jejunum and duodenum. *Escherichia coli* and Salmonella are the main Gram-negative bacteria in the intestine.

CONCLUSION

The result indicates the relationship between the porcine *BPI* gene and the infectiousness of Gram-negative bacteria such as ECF18 and Salmonella. This study showed that porcine *BPI* gene might have a direct role against diarrhea and edema disease caused by ECF18 in weaned piglets. The resistance was related to the

upregulation of *BPI* gene expression in the intestine. The *BPI* gene can be identified as the candidate gene for breeding against Gram-negative bacteria.

RECOMMENDATIONS

More research needs to be done to investigate the potential role of *BPI* gene in the intestine and the study provides further support for the notion that BPI plays an important anti-disease role in weaned piglets caused by ECF18 and the expression of *BPI* gene is associated with the breeds.

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