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Genetic variation in exon 10 of the BPI gene is associated with *Escherichia coli* F18 susceptibility in Sutai piglets

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ABSTRACT

Our aim was to investigate the effect of the porcine bactericidal/permeability-increasing protein (BPI) on the susceptibility to enterotoxigenic *Escherichia coli* F18 (ETEC F18). Specifically, we wanted to determine whether the *Hpa*II restriction polymorphism in exon 10 of BPI mediates susceptibility to ETEC F18. Thirty verified ETEC F18-resistant and thirty susceptible Sutai (Duroc × Taihu) piglets were identified using the receptor binding assay. Exon 10 of the BPI gene produced the AA, BB, and AB genotypes after *Hpa*II digestion. The genotype distribution among ETEC F18-resistant piglets was significantly different from that among susceptible piglets. Among piglets with the AA genotype, 90% were ETEC F18-resistant; this percentage of resistant piglets was significantly higher than the percentage of resistant piglets with the AB (57.1%) and BB genotypes (17.4%). There was high expression only in the tissues of the duodenum and jejunum, wherein the expression levels in the ETEC F18-resistant group were significantly higher than those in the susceptible group ($P < 0.05$). The average expression levels in individuals with the AA genotype were significantly higher than those in individuals with the AB or BB genotype ($P < 0.05$), while the results of Western blot show the same evidences as real time PCR. These results indicate that the upregulation of porcine BPI gene expression in the small intestines plays a direct role in resistance to ETEC F18 infection. The AA genotype for the *Hpa*II site in exon 10 of the porcine BPI gene was demonstrated to be an anti-ETEC F18 marker and could be used for selective breeding to enhance ETEC F18 resistance.

1. Introduction

Bactericidal/permeability-increasing protein (BPI) is an endogenous cationic protein found in humans and other mammals and is primarily present in the aniline blue particles of polymorphonuclear leukocytes (PMNs). In addition to killing gram-negative bacteria and neutralizing endotoxin and lipopolysaccharide (LPS) (Akin et al., 2011), BPI has several biological functions, such as promoting complement activation and opsonization for increased phagocytosis, inhibiting angiogenesis, inhibiting the release of inflammatory mediators, and inhibiting infection by fungi and protozoan. BPI plays an important role in the natural defense of the animal body (Elsbach, 1998; Nicole et al., 1997; Weiss et al., 1978). There are few domestic and international reports regarding porcine BPI gene polymorphisms and their associated impacts on disease resistance/susceptibility. In the United States patent literature, there is only one report on the sequence of porcine BPI gene number 10, which is 1452 bp in length; however, there are no clear remarks, and this sequence is now referred to as the patented BPI gene sequence. The information in this patent demonstrates that the *Ava*II and *Hpa*II restriction fragment length polymorphism (RFLP) sites in exons 4 and 10 of BPI are related to the susceptibility of swine to *Salmonella*. BPI was identified to be a candidate gene for disease-resistance breeding (Christopher et al., 2004).

Diarrhea and edema disease are two major causes of mortality in postweaning piglets, and these conditions lead to huge economic losses in the swine industry. *Escherichia coli* F18 (*E. coli* F18) is the primary causative agent of these two diseases. *E. coli* F18 relies on its fimbriae to adhere to the surface of epithelial cells in the piglet's small intestine and to bind to the F18 receptor on porcine small intestinal epithelial brush cells. Then, the bacterium settles, reproduces, and produces enterotoxin, which causes disease in piglets (Da Silva et al., 2001).

The present study used groups of Sutai pigs that were previously identified as resistant and susceptible to *E. coli* F18 using the V-secretion system and the receptor binding assay. Then, the single nucleotide polymorphism (SNP) in the *Hpa*II restriction site in exon 10 of porcine BPI was analyzed, and the distribution of different

Abbreviations: BPI, bactericidal/permeability-increasing protein; *E. coli*, *Escherichia coli*; PMNs, polymorphonuclear leukocytes; LPS, lipopolysaccharide; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

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genotypes for the *Hpa*II restriction site was determined for the resistant and susceptible groups. In addition, the expression levels of BPI were detected in different tissues of weaning piglets in both the *E. coli* F18-resistant and *E. coli* F18-susceptible groups. Then, the expression levels of the different genotypes were compared. In addition, expression of BPI protein was analyzed by Western blot. The data obtained in this study highlight the close relationship between the *Hpa*II restriction site and *E. coli* F18 susceptibility and the key role of BPI in inhibiting ETEC F18 infection.

2. Materials and methods

2.1. Samples and bacterial strain

Existing studies have shown that the α-(1, 2) fucosyltransferase gene (*FUT1*) is a candidate gene for the control of *E. coli* F18 strain adhesion. G/A point mutations exist in M307 of *FUT1*, and G is dominant over A. The AA genotype is related to resistance to *E. coli* F18, whereas the GG and AG genotypes are related to susceptibility (Meijerink et al., 1997; Vogeli et al., 1997). The Sutai pig, which was generated by crossing Duroc pigs with Taihu pigs, is a high-quality and lean-meat breed. In a previous study (Bao et al., 2008), we detected a small number (9.2%) of *FUT1* AG genotype individuals in Sutai pig populations that were adequate for selective breeding, and by breeding these homozygotes, we obtained the valuable *FUT1* AA genotype (*E. coli* F18-resistant type) in the Sutai pig. Through five years of continuous breeding, *E. coli*-resistant and *E. coli*-susceptible resource groups have been created in a population of more than 200 Sutai pigs. At the same time, we also constructed a V-secretion system based on the expression of *E. coli* F18 adhesin; using the V-secretion system and the receptor binding assay, further analysis and verification of *E. coli* F18 resistance and susceptibility were performed for these resource groups (Wu et al., 2007).

The F18ab fimbriae standard strain 107/86 (O139:K12:H1) was provided as a gift by the veterinary laboratory at the Institute of Microbiology, University of Pennsylvania. The recombinant *E. coli* expression F18 fimbriae which carried the F18 operon in the pET22b vector was induced by the optional condition of IPTG so that the expression of F18ac fimbriae that contained the fed operon of recombinant *E. coli* r*E.coli*1534 was induced, and the surface expression of the F18ab fimbriae FedF subunit of *E. coli* pnirBMisL-fedF was constructed and stored (Zhang and Zhu, 2007). Anaerobic bacteria were washed with phosphate buffer saline (PBS) for three times, and the bacterial concentration was adjusted to approximately 1×10^9 CFU/ml. After adding 1% mannose (w/v) to 0.5 ml of wild type or recombinant bacteria, the suspensions were incubated at 37 °C for 30 min, mixed with 0.5 ml of small intestinal cells, incubated at 37 °C for 30 min, and finally centrifuged at 900 ×*g* for 5 min. At this time, 50 μl of the preparation was extracted after being suspended in PBR (0.24 g KH_2PO_4, 1.44 g Na_2HPO_4, 0.42 g KCl, 9 g NaCl, 0.25 g $CaCl_2$ and distilled water was added to 1 l), and the mixture (50 μl) was deposited on a glass slide, air dried, heat-fixed and stained with methylene blue for 3–5 min (Wu et al., 2007). Then, villi were taken with an oil immersion lens at 1000× magnification and the adhesion of bacteria was evaluated quantitatively by counting the number of bacteria adhering along a 50 m villous brush border at 20 randomly selected places for each piglet, after which the mean bacterial adhesion was calculated. Adhesion of less than 5, and more than 30 bacteria per 250 mm brush border length was noted as resistant or susceptible, respectively (Coddens et al., 2007).

The samples in this study included thirty *E. coli* F18-resistant piglets and thirty *E. coli* F18-susceptible piglets that were obtained from the aforementioned verified *E. coli* F18-resistant and susceptible groups. Piglets selected from the *E. coli*-resistant and susceptible resource groups and raised in the same environment were sacrificed postweaning (35 days). Fifteen centimeters of the duodenum and jejunum was obtained according to the approach that Alwan et al. (1998) used for the isolation and preparation of intestinal epithelial cells. Tissue samples were also taken from 11 organs, including the heart, liver, spleen, lung, kidney, stomach, muscle, thymus, lymph nodes, duodenum, and jejunum, and were stored in liquid nitrogen on-site and then transferred to a −70 °C freezer. Approximately 1.0 g of ear tissue was collected from each individual and stored in a 1.5-ml Eppendorf tube in an ice box. DNA was extracted using the conventional phenol–chloroform method and stored at −20 °C for later use.

2.2. PCR–RFLP analysis of the HpaII polymorphism in exon 10 of BPI

Using the published porcine BPI sequence in the patent (Christopher et al., 2004), BPI exon 10 primers were designed (Table 1). The PCR product size was 445 bp in length. The primers were synthesized by the engineering service of Shanghai Biological Engineering Technology Co., Ltd.

The PCR reaction system consisted of 2.5 μl of 10× PCR buffer, 1.5 μl of 2.5×10^3 μM/l dNTPs, 1 μl of 10 μM/l primers (upstream), 1 μl of 10 μM/l primer (downstream), 0.2 μl of *Taq* enzyme (5 U/μl) (TaKaRa Biotechnology Dalian Co., Ltd), 1 μl of 100 ng/μl DNA template, and 17.8 μl of ddH_2O; the total volume was 25 μl. The PCR amplification program consisted of denaturing at 94 °C for 4 min; 32 cycles of denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s; a final extension at 72 °C for 7 min, and preservation at 4 °C.

After amplification, the mixture was digested with *Hpa*II for 3 h. The digestion reactions contained 3 μl of the PCR product, 0.2 μl of *Hpa*II (5 U/μl) (TaKaRa Biotechnology Dalian Co., Ltd), 1 μl of 10× buffer, and 5.8 μl of ddH_2O. Homozygotes with different genotypes were selected for direct sequencing based on the PCR–RFLP results.

2.3. Design and synthesis of real-time PCR primers

The real-time PCR primers used in the study were based on the published sequences of the porcine BPI and *GAPDH* genes. The NM001159307 sequence of BPI in the NCBI database (http://www.ncbi.nlm.nih.gov/) was used as the reference. A 136 bp BPI fragment was amplified using the primers listed in Table 1, and the *GAPDH* gene was used as an internal reference.

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted from homogenized tissues (50–100 mg) using a Trizol reagent (TaKaRa Biotechnology Dalian Co., Ltd) according to the manufacturer's instructions. The electrophoresis of the total RNA that was extracted from 11 tissues on 1% agarose gels revealed that the extracted total RNA was highly pure. The result of UV spectrophotometry indicated that the extracted RNA was of high quality and could be used for subsequent tests. RNA was reverse transcribed in a final reaction volume of 10 μl using the PrimeScript RT Reagent Kit (TaKaRa Biotechnology Dalian Co., Ltd) containing 2 μl

Table 1
Primers used in this study.

Primer no.	Oligonucleotide sequence 5′ — 3′
BPI-F[a]	CCCAACATGGAGATGCAGTTC
BPI-R[a]	CAATGAATCAATGAGCACACC
BPI-RT-F[b]	ATATCGAATCTGCGCTCCGA
BPI-RT-R[b]	TTGATGCCAACCATTCTGTCC
GAPDH-F[c]	ACATCATCCCTGCTTCTACTGG
GAPDG-R[c]	CTCGGACGCCTGCTTCAC

[a] Primers used for PCR–RFLP. The products were digested with the *Hpa*II restriction enzyme.
[b] Primers used for real-time PCR to amplify BPI cDNA.
[c] *GAPDH* was the reference gene in the real-time PCR.