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Comparative Study on BPI Gene Expression in Various Tissues between Rongchang Pig and Landrace

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FYZ conceived and designed the research. RW analyzed the data. YSH, JXM and GZW performed the experiments. JXM wrote the manuscript, FYZ revised it while BZ lingually revised it.

Key words
Rongchang pig, Landrace, Tissue expression, BPI gene, Real-time fluorescence quantitative

ABSTRACT

BPI is a pluripotent protein located in neutrophils and tissue that likely plays a vital role in host defense against GNB (gram-negative bacteria) and their endotoxin by means of its antibiotic and endotoxin neutralizing and disposing functions. BPI has several biological functions in mammals such as human, bovine, pig and rabbit, it also has major influence in the natural defense of the animal body. In this paper, by comparative study on BPI gene expression in various tissues between Rongchang pig and Landrace, we confirmed the expression of pig BPI gene in various tissues to determine the difference expression of antibacterial related gene among indigenous breed (Rongchang pig) and artificially cultivated breed (Landrace), The Rongchang pig (0 day old, 28 day old, 120 day old) and Landrace BPI (0 day old, 28 day old, 120 day old) gene were expressed in normal liver, heart, lung, kidney, spleen and small intestine. The results showed that: The expression level of BPI in lung of Rongchang pig was relatively higher level compared to that in heart, liver, kidney, spleen and small intestine. The expression level of BPI in liver of Landrace was relatively higher level compared to that in heart, lung, kidney, spleen and small intestine. Real-time fluorescence quantitative PCR analysis revealed that the native breed (Rongchang pig) has a better resistance to disease than Landrace. When Rongchang pig and Landrace were 0 day old, the expression level of BPI of Rongchang pig was higher than Landrace in heart, liver, spleen, kidney, small intestine, lower than Landrace in lung. When Rongchang pig and Landrace were 28 day old, the expression level of BPI of Rongchang pig was higher than Landrace in spleen ($p<0.05$), kidney ($p<0.05$), small intestine ($p<0.05$); lower than Landrace in lung, heart, liver. When Rongchang pig and Landrace were 120 day old, the expression level of BPI of Rongchang pig was higher than Landrace in spleen, lung ($p<0.05$), kidney ($p<0.05$), small intestine ($p<0.05$); lower than Landrace in heart, liver. The research trials showed that the expression of BPI gene increased with its increasing age in different growth stages. Ages and tissues are the main factors that influence the gene expression significantly.

INTRODUCTION

Disease has brought a great deal of breeding risk to swine production and increased the cost of epidemic prevention treatment, and the prevention and treatment of a large number of drugs, resulting in the potential threat of pork food safety (Zhong *et al*, 2016). Utilize the genetic method, it can be a good solution to reduce the disease susceptibility and improve disease resistance (Henryon *et al*., 2014). BPI gene has been studied in many other species, and it is expected to be a candidate gene for disease resistance breeding. As a local pig variety, the Rongchang pig is one of the Chinese indigenous and famous breeds which produces high quality meat, it also has been characterized with high disease resistance (Liu *et al*., 2009). The landrace is artificially cultivated breed with steady genetic feature and higher production level. We considered that it is necessary to compare those two typical indigenous and traditional swines in different genetic expressions research in order to enabling further investigation into the expression profiles and functional classifications of genes in Rongchang pig and landrace (Zou *et al*., 2014).

Bactericidal/permeability-increasing protein (BPI) is a highly cationic protein which is located mainly in the primary granules of polymorphonuclear leucocyte (PMN). BPI has a high affinity for LPS of gram-negative bacteria

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(Elsbach and Weiss, 1993; Weiss, 2003). BPI plays an important role in the natural defense of the animal body (Schultz and Weiss, 2007; Balakrishnan *et al*., 2013). It was reported that high expression of BPI may contribute to host immune defense against gram-negative bacterial infections in ark shell *Scapharca broughtonii* (Mao *et al*., 2013). There are few domestic and international reports regarding porcine BPI gene polymorphisms and their associated impacts on disease resistance. In recent years, BPI was started to be identified as a candidate gene for disease-resistance breeding in pig (Christopher *et al*., 2004). A researcher found that the porcine BPI gene may be the direct factor that caused resistance to ETEC F18 in weaning piglets (Keuzer *et al*., 2013). Currently, although various kind of swine tissues transcriptomic data have been obtained, more studies focused on muscle to gain insights into improving meat quality and enhancing the ability of disease resistance from the perspective of agricultural economy in pig remains limited (Zhu *et al*., 2013) .

In this study, we used real-time fluorescence quantitative PCR technology (Tobias *et al*., 2012) and took β-actin as an internal reference gene (Tesson *et al*., 2002) to compare the differences of expression of BPI gene in tissues between Rongchang pig and Landrace. This study formed a foundation for the correlation of disease resistance and selective breeding on Rongchang pig and Landrace. Using molecular biology technology to provide an important theoretical significance and applicable value for livestock production and breeding.

Table I.- Ingredient composition of the diet.

Ingredient	% of diet	Ingredient	% of diet
Ground corn	47.60	Calcium carbonate	1.50
Soybean meal	37.00	Phosphoric acid	0.80
Whey	10.00	Vitamin premix	0.05
Poultry fat	3.00	Mineral mix	0.05
Antibiotic	1.00		

MATERIALS AND METHODS

Animal care and dietary treatments

Animal care, handling, sampling and administration procedures were approved by the Southwest University (Chongqing, China) Animal Care and Use Committee prior to initiation of the experiment. The basal diet was formulated based on NRC (1998) recommendations, the ingredient composition of the diet has shown in Table I (Zou *et al*., 2014). All the pigs were housed and maintained according to the standard protocol and in an environmentally controlled nursery and provided ad libitum access to both feed and water.

Tissue sample preparation

All pigs (Rongchang pig and Landrace, 0 day old, 28 day old, 120 day old, 9 per age group) included in the study were healthy and raised in the same conditions with similar birth weights, weaning weights, and body sizes. Heart, liver, spleen, lung, kidney, small intestine were extracted immediately, and quick-frozen in liquid nitrogen tanks after butchering, then kept at -80 °C in the refrigerator for the extraction of total tissue RNA.

Primer design

The real-time fluorescence quantitative PCR primers used in the study were based on the published sequences of the porcine BPI and GAPDH genes. Referenced to mRNA sequence of pig's BPI gene and EST sequences, and BPI mRNA sequences of other animals provided by NCBI database (http://www.ncbi.nlm.nih.gov/), the specific primers were designed in highly conserved position. The primers were synthesized by Invitrogen Corporation (Carlsbad, CA, USA) and the sequence of the primers was presented in Table II.

Table II.- Sequence of the primers.

Primer	Sequence
Oligodt-Aaptor	TTTTTTTTTTTTTTTTT
PCR BPI	F: AGTCTGCGGCTGGGTTAT
	R: GGAGGACACGGTATTGGTC
PCR β-actin	F: TCTGGCACCACACCTTCT
	R: TGATCTGGGTCATCTTCTCAC

Total RNA extraction and detection

Total RNA was extracted from the tissues of the Rongchang pig and Landrace using a Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. In order to detect the integrity of RNA extracted, the RNA samples were electrophoresis at 260 V in 1% agarose gel for 7-8 min. Then 1 μL of the total RNA was mixed with 99 μL of DEPC water, the concentrations and OD values at 260 nm and 280nm were determined in the protein nucleic acid analyzer. An OD260/OD280 value of 1.8-2.0 indicated that the total RNA achieved the quality for the reverse transcription experiment (Cury and Koo, 2008).

Reverse transcription

Single-stranded cDNA was generated using the primescript RT-PCR Kit (TaKaRa) following the manufacturer's instructions. The 10 μL of reagents containing 1 μL of RT buffer (10×), 3.75 μL of RNase free dH_2O, 0.5μL of AMV reverse transcriptase, 0.25

μL of RNase inhibitor, 0.5 μL of oligo dT, 1μL of dNTP mixture (10 mM) and 1μL of positive control RNA were added successively into 0.2 mL of PCR tube without RNA enzyme for reverse transcription reaction. The PCR reverse transcription process was conducted as following: 30 °C for 10 min, 42 °C for 30 min, 99 °C for 5 min, 5 °C for 5 min, then preserve at -20 °C. Then, the PCR amplification program was conducted according to the reference (Zhu *et al*., 2013).

Real-time fluorescence quantitative PCR

The 25 μl reaction system consisted of 2 μL of cDNA (10 μM/L), 1μL of BPI-F, 1 μL of BPI-R, 12.5 μL of SYBR Green Real-time PCR Master Mix (2×), and 8.5 μL of dH_2O. The PCR reaction conditions included 40 cycles at 95 °C for 330 s, 56 °C for 30 s and 72 °C for 30 s. Each sample was tested using real-time PCR three times, and the average of three measurements was used. Real-time fluorescence quantitative PCR was performed using a Bio-Rad IQ5 sequence-detection system (Bio-Rad, CA, USA) with SYBR Green PCR Master Mix (TaKaRa), according to the manufacturer's instruction. The BPI fragment was amplified using the primers listed in Table II.

Statistical analysis

Triplicate PCR amplifications were performed for each sample. Samples without cDNA template were used as negative controls and pig's β-actin gene download from NCBI database (http://www.ncbi.nlm.nih.gov/) was used as an internal reference. The real-time fluorescence quantitative PCR results were processed using the $2^{-\Delta\Delta Ct}$ method (ΔCt=the mean expression level of BPI-the mean expression level of β-actin). The expression levels of this gene in other groups could be quantified since the group with the least ΔCt was defined as 1 (ΔΔCt=ΔCt of the other group-group with the least ΔCt in each experiments). Based on the mathematical derivation of the Livak sehmittgen, when the amplification efficiency of target gene and reference gene are consistent, the expression of the target gene in the experimental group was compared with that of the control group. The standard curve for BPI was Y= -3.353X-1.839 with a correlation coefficient of 0.998, and the amplification efficiency was 92.3%. The standard curve for the β-actin gene was Y= -3.341X-1.827 with a correlation coefficient of 0.997, and the amplification efficiency was 91.2% (Livak and Sehmittgen, 2001). The consistent amplification efficiencies indicated that the $2^{-\Delta\Delta Ct}$ method can be used for quantitative calculations. Analysis of data were performed by ANOVA for a completely randomized design using procedure of SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The LSD was used to analyze the significance of differences in BPI expression.

RESULTS

Real-time fluorescence quantitative PCR amplification results

The standard curve chart (Fig. 1) shows that the dilution gradient can be in a straight line, the correlation coefficient of the standard curve is between 0.99 and 1, indicate that less error and higher reliability. Melt curve analysis of BPI and β-actin PCR product was shown in Figure 2. It can be seen that only single peak curve was presented, indicating that the amplified products was unitary and no primer-dimers and other non-specific amplification was produced.

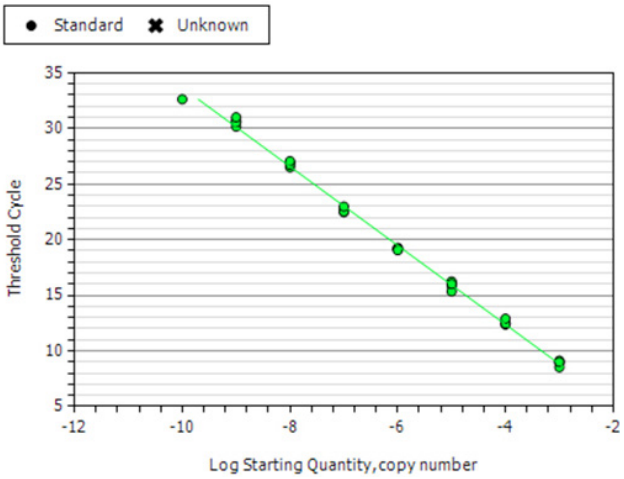

Fig. 1. Real-time fluorescence quantitative PCR standard curve chart of BPI gene and β-actin.

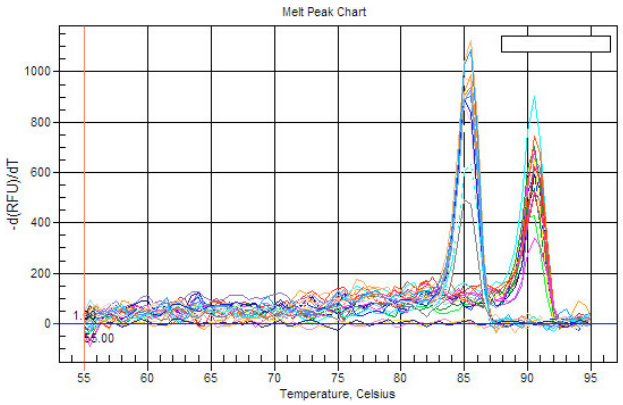

Fig. 2. Melt curve analysis of BPI and β-actin PCR product.

Expression of BPI gene in different tissues of Rongchang pig and Landrace

Figure 3 was the relationship between expression and day old in tissues of Rongchang pig. From the Figure

3, it revealed that the BPI gene increased with the age of the pig and the increase trend of BPI gene in lung was more significant than that in other tissues. In addition, the expression of BPI gene in spleen was higher than that in other tissues in the embryonic (0 day old), the initial growth (28 day old) and growth (120 day old) stages. Figure 4 is the relationship between expression and days in tissues of Landrace. It revealed that the expression of BPI gene increased with the age of the pig and the increase trend of BPI gene in liver was more significant than that in other tissues. Figure 5 is the BPI expression of varies tissues between Rongchang pig and Landrace at different ages. It revealed that the expression of BPI gene increased with its increasing age in different growth stages. Figure 5A shows that the expression levels of BPI gene when Rongchang pig and Landrace were 0 day old; Figure 5B shows that the

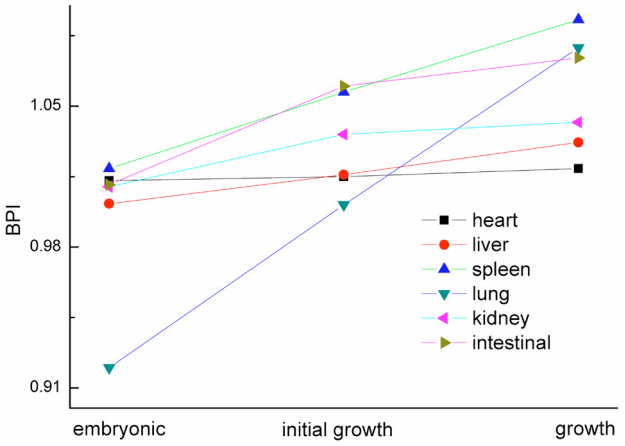

Fig. 3. The relationship between BPI expression and days in tissues of Rongchang pig.

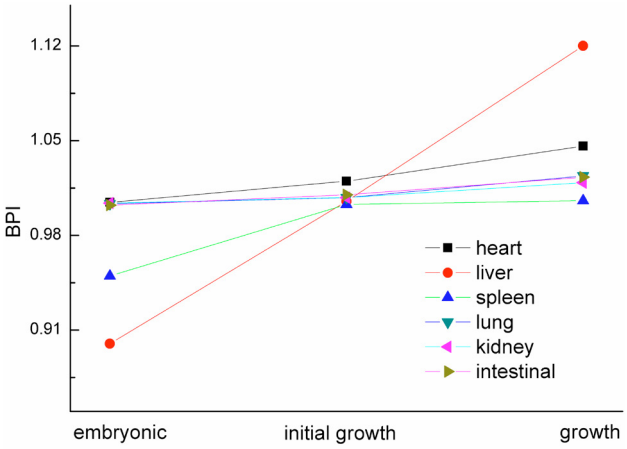

Fig. 4. The relationship between BPI expression and days in tissues of Landrace.

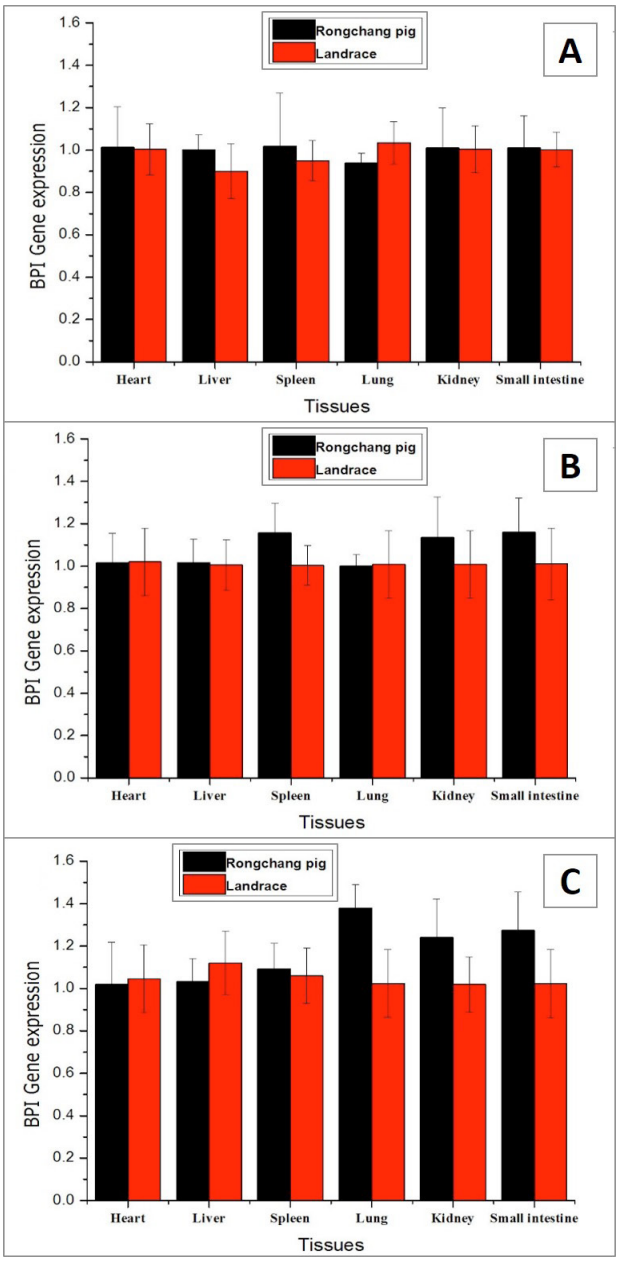

Fig. 5. The BPI expression of varies tissues between Rongchang pig and Landrace at different ages. A, 0 day old Rongchang pig and Landrace of varies tissues; B, 28 day old Rongchang pig and Landrace of varies tissues; C, 120 day old Rongchang pig and Landrace of varies tissues.

expression levels of BPI gene when Rongchang pig and Landrace were 28 day old; Figure 5C shows that the expression levels of BPI gene when Rongchang pig and Landrace were 120 day old.

DISCUSSION

In our study, cross-intron primer design method is used to eliminate the influence of DNA. The intron length of BPI and β-actin crossed are more than 500 bp and the genomic DNA cannot amplify at such a length of intron (Liang and Pardee, 1992). The specificity of RT-PCR depends on the specificity of the banding of primers and template DNA. Sequence alignment and structure function prediction indicate that the structure and function BPI gene of pig is similar as that of human and cow (Eckert *et al*., 2006). The design of the primers refers the sequence of the cDNA of pig and BPI exon of human.

Given its ability to neutralize endotoxin and protect against gram-negative bacteria (Zhou *et al*., 1999), the BPI has application prospects widely, it may be considered as a "super antibiotic". Porcine BPI also has these functions (Tuggle *et al*., 2006). In our study, we have confirmed the findings from studies done in Rongchang and Landrace piglets, that BPI gene expression levels are higher in spleen than in other tissues. We also have reconfirmed the findings from studies done in Rongchang and Landrace adult swines, that BPI gene expression levels are higher in lung and liver than in other tissues (Gan *et al*., 2015). The increase of BPI gene in lung is more significant than that in other tissues with the increase of the age of Rongchang pig. The reason could be that the experiments are conducted in July and August, which is the hottest period in Chongqing, China. Thus the high incidence of respiratory disease leads to such a trend. On the other side, the BPI has the function of protection of lung. The researchers found that recombinant BPI administration protects pigs against endotoxemia-induced acute lung injury (Vandermeer *et al*., 1994). While the reason for the increase trend of BPI gene in liver of Landrace is more significant could be attributed to the weak constitution and poor resistance of the Landrace. This is similar to the study of reseacher (Gan *et al*., 2015) in Rongchang pig. Since the Landrace is a species from Denmark and usually lives in relatively cold place, they are easy to have an attack of illness and then many drugs are fed. Therefore, the BPI gene increases more since liver is a detoxification and excretion organ (Gaur *et al*., 2014). BPI is vital in the innate immune response, our findings may indicate that the high expression of BPI gene may be related to age in the lung and liver. In our trials, we found that the expression level of BPI gene in spleen of Rongchang pig was higher than other tissues (heart, liver, lung, kidney, small intestine). The spleen is one of the vital immune organs in body and is mainly responsible for making antibodies, regulating immune responses, filtering aging erythrocytes, storing blood. The normal structure and function of immune organs are connected with swine immunity (Wu *et al*., 2016). It has a high BPI expression level in spleen indicate that the Rongchang pig has a high resistance to disease.

We tested differential performances of BPI genes in 6 tissues, further molecular level exploration is required. In the embryonic stage of pigs, the BPI gene in lung of Rongchang pig is lower than Landrace, while in other tissues are higher than those of Landrace. In the initial growth stage, the BPI gene in spleen, kidney and small intestine of Rongchang pig is higher than that of Landrace, while others are lower. In the growth stage, the BPI gene in spleen, lung, kidney and small intestine of Rongchang pig is higher than that of Landrace, while others are lower. The studies show that the BPI gene also shows similar tissue-specific expression in a different breed of weaned piglets and adult swines. BPI can directly kill gram-negative bacteria such as E.coli F18, and that up-regulated expression is closely related to resistance to intestinal E.coli F18 (Keuzer *et al*., 2013), also the BPI gene tissue specificity has vital implications in biological and engineering measures to utilize the activity of porcine endogenous BPI protein. Many researches indicated that BPI has the effect of neutralize porcine endogenous endotoxin and gram-negative bacteria (Bingle and Jeremy, 2004). We can see a difference in expression of antibody specific genes in two different pig breeds clearly, which indicate towards the underlying differences in genetic make-up of resistant and susceptible animals. The findings of this study provided a new insight for present research, the use of BPI as a disease resistance candidate gene for breeding still needs further study in mammals.

CONCLUSION

Real-time fluorescence quantitative PCR analysis revealed that Rongchang pig has a better resistance to disease and immunity than Landrace. The research trials showed that the expression of BPI gene increased with its increasing age in different growth stages, ages and tissues are the main factors that influence the gene expression significantly. BPI as a disease resistance candidate gene may play an important role for breeding, the resistance was related to the upregulation of BPI gene expression in the spleen, lung and liver.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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