doi: 10.17221/84/2015-CJAS

Gene Expression and Tissue Distribution of β-Defensins in Chinese Min Pigs and Landrace Pigs

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ABSTRACT

Jiao W., Ma Q., Lv X., Shan A., Li Z. (2017): **Gene expression and tissue distribution of β-defensins in Chinese Min pigs and Landrace pigs.** Czech J. Anim. Sci., 62, 178–183.

β-Defensins are a major group of mammalian antimicrobial peptides and play an important role in innate and adaptive immunity due to their antimicrobial, chemotactic, and regulatory activities. In this study, the tissue distribution of porcine β-defensin (*pBD-1*, *pBD-2,* and *pBD-3*) gene expression was determined using real-time polymerase chain reaction in 14-day-old Chinese Min pigs and Landrace pigs. The results showed that in the two breeds, *pBD-1* and *pBD-2* were primarily expressed in the tongue and kidney, respectively. The *pBD-3* genes in Min pigs and Landrace pigs were abundant in the heart and tongue respectively. The mRNA expression levels of the three peptide genes in most tissues were much higher in Min pigs than in Landrace pigs. Collectively, higher defensin gene expressions were observed in some organs and tissues of Min pigs, presumably related to their higher resistance to disease.

Keywords: antimicrobial peptides; swine; mRNA abundance

Antimicrobial peptides are promising antibacterial agents with a broad spectrum of antimicrobial activity that participate in multiple aspects of immunity (Hancock and Sahl 2006). In mammals, there are two major classes of antimicrobial peptides: defensins and cathelicidins (Cederlund et al. 2011). Defensins show high antimicrobial activity against pathogenic bacteria, fungi, mycobacteria, enveloped viruses (Auvynet and Rosenstein 2009), and are considered chemotactic for T-lymphocytes and immature dendritic cells (Soman et al. 2009). Differing in their size and alignment of disulfide bonds, defensins in mammals can be grouped into α-, β-, and θ-defensins (Wang et al. 2003). β-Defensin, characterized by 3 pairs of disulfide bonds (C1–C5, C2–C4, and C3–C6), has been isolated from epithelial cells, blood plasma, urine, leukocytes, and tissues of vertebrates, including humans and domestic animals (Bagnicka et al. 2010). In pigs, neither α- nor θ-defensin have been found and 13 porcine β-defensins have been identified and partially characterized (Sang and Blecha 2009). These β-defensins are either constitutively expressed or induced by infection by *Bordetella pertussis* or proinflammatory cytokines (Elahi et al. 2006).

Supported by grants from the National Key Technology R&D Program (2013BAD10B03), "Young Talents" Project of Northeast Agricultural University (14QC16), the China Postdoctoral Science Foundation (2012M510082, 2013T60342), the Research Fund for the Doctoral Program of Higher Education of China (20132325120003), the National Natural Science Foundation of China (31601949, 31472104, 31272453), and the Program for Universities in Heilongjiang Province（1254CGZH22, 2012TD003).

Wenjing Jiao and Qingquan Ma contributed equally to this paper.

doi: 10.17221/84/2015-CJAS

Min pigs are a Chinese indigenous breed that is distributed in the northeast of China. They are adapted to harsh climates and extensive feeding. Native to China, Min pigs have higher immunity, stronger disease resistance and adaptability (Xu 1989). We have previously reported that expression of four cathelicidin genes (*PMAP-23*, *PMAP-37*, *PR-39*, and *protegrin-1*) in most tissues was higher in Min pigs than in Landrace pigs (Ma et al. 2014), suggesting that the high expression of antimicrobial peptides might be a contributing factor in which the Min pigs display strong disease resistance. We hypothesized that these characteristics may be related to the greater expression of β-defensins genes. Accordingly, we chose the well-characterized β-defensins *pBD-1*, *pBD-2*, and *pBD-3*, determined the mRNA gene expression in various tissues, and compared the expression patterns between Min pigs and Landrace pigs.

MATERIAL AND METHODS

Animals. The animal experiment was conducted according to the animal experiment guidelines approved by the National Institute of Animal Health. Randomly selected healthy Landrace pigs (n = 3) and Min pigs (n = 3) were fed in the same environment. At the age of 14 days they were slaughtered by exsanguination for sampling.

Tissue sample collection. The cerebral cortex, epithelium of tongue, trachea, lymph node, thymus, spleen, heart, liver, lung, kidney, duodenum, jejunum, and ileum were removed and snap frozen in liquid nitrogen. All tissues were stored at –80°C before total RNA extraction.

Total RNA extraction and reverse transcription reaction. Total RNA was extracted using the E.Z.N.A.™ Total RNA Kit (Omega Bio-tek, USA) according to the manufacturer's instructions. The quality of RNA was checked by gel electrophoresis. The purity of RNA was measured by a spectrophotometer at 260 nm and 280 nm, with a value of 1.8 to 2.0 for the ratio of OD_{260} : OD_{280}. RNA was reverse-transcribed in a final volume of 10 µl using the PrimeScript™ RT reagent Kit (TaKaRa, China) according to the manufacturer's manual. The RT products (cDNA) were stored at –20°C before relative quantitative real-time PCR.

Relative quantitative real-time PCR (RT-qPCR). The primers used for RT-qPCR were designed based on known sequences deposited in GenBank – *DEFB1* (known as *pBD-1*, Acc. No. NM_213838), *DEFB1* (known as *pBD-2*, Acc. No. NM_214442), *LOC404703* (known as *pBD-3*, Acc. No. NM_214444) (Table 1). The specificity of the primer set for each gene was tested by electrophoresis of amplified products on 2% high-grade agarose gel. Furthermore, direct sequencing of amplicons was performed to confirm validity of primers for RT-qPCR. Sequencing of both directions was done to confirm entire sequence of amplicons. The resultant sequences were analyzed for homologous counterparts in the GenBank database using the BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST). RT-qPCR was conducted on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with the SYBR *Premix Ex Taq*™ Kit (TaKaRa). The cDNA from the reverse transcription reaction of total RNA was used as a template for PCR amplification in a total volume of 25 µl. An abundantly expressed gene, *β-actin*, was used as the reference gene. The parameters for RT-qPCR were as follows: a pre-run incubation at 95°C for 30 s, 40 cycles with a 5 s denaturation step at 95°C, and a 60°C annealing step for 34 s. All reactions were performed in triplicate, and the samples were distributed into 96-well plates. The specificity of each product was confirmed by analyzing the melt curve. Standard curves for serial dilutions of target genes and *β-actin* RNA were generated. The mean quantity values were calculated from the cycle threshold (C_t) values using SDS 1.3.1 software by the relative standard curve method (Applied Biosystems). All target mRNA levels were normalized to the *β-actin* RNA levels and expressed as a relative level (target mRNA/ *β-actin* RNA). The ratios of expression levels of genes in different tissues between the two breeds were compared.

Table 1. Primer sequences used for real-time PCR

Target gene	Primer sequences	Product size (bp)
β-actin	F: 5'-ATGCTTCTAGGCGGACTGT-3' R: 5'-CCATCCAACCGACTGCT-3'	211
pBD-1	F: 5'-CCGCCTCCTCCTTGTATT-3' R: 5'-GGTGCCGATCTGTTTCAT-3'	145
pBD-2	F: 5'-ACCTGCTTACGGGTCTTG-3' R: 5'-CTCTGCTGTGGCTTCTGG-3'	168
pBD-3	F: 5'-GAAGTCTACAGAAGCCAAAT-3' R: 5'-GGTAACAAATAGCACCATAA-3'	102

F = forward, R = reverse

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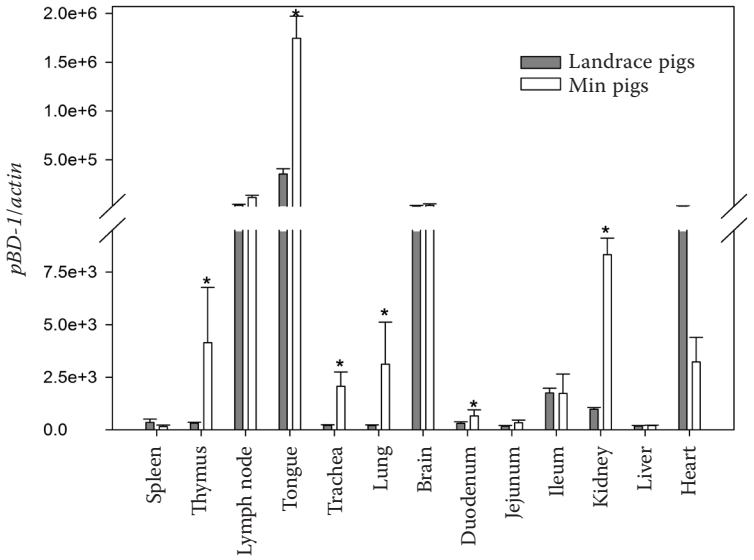

Figure 1. Tissue distribution of *pBD-1* mRNA in Landrace pigs and Min pigs
values are depicted as relative *pBD-1*/β-*actin* levels plus SEM
*significant differences of gene expressions ($P < 0.05$) between breeds

Statistical analysis. Evaluation of relative gene expression in different tissues was performed using the SPSS software (Version 13.0) and one-way ANOVA analysis, followed by the *t*-test to assess the differences between the two breeds of pigs. Data were presented as the mean ± SEM. Significance was defined as $P < 0.05$.

RESULTS

mRNA expression of β-defensins in porcine tissues. Electrophoresis results showed a single band was observed and no non-specific products or primer-dimers were generated during the PCR amplification cycles (data not shown). The PCR products were confirmed by sequencing the PCR amplicons and by performing a BLAST database search against the GenBank sequences. All sequences were found to be 100% identical with the *pBD* genes in the GenBank database.

The mRNA expressions of the three β-defensins were detected in all tested tissues in both breeds of pigs (Figures 1–3). In both breeds, a relatively higher mRNA expression of *pBD-1* was observed in the tongue, lymph node, brain, and heart. The relatively lower level of *pBD-1* expression was found in the spleen, liver, duodenum, and jejunum. mRNA of *pBD-2* was highly expressed in the liver, brain, kidney, and tongue, but less expressed in the lung, heart, and spleen. *pBD-3* was highly expressed in the heart, tongue, brain, kidney, but less expressed in the spleen, liver, ileum, and lymph node.

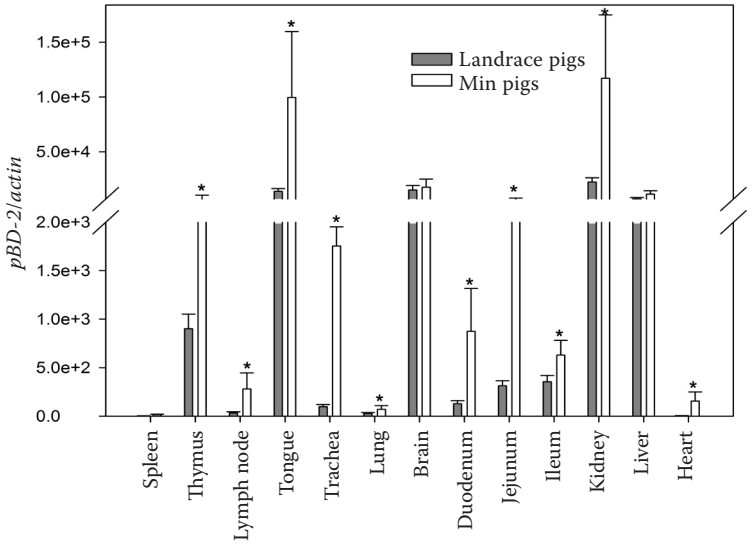

Figure 2. Tissue distribution of *pBD-2* mRNA in Landrace pigs and Min pigs
values are depicted as relative *pBD-2*/β-*actin* levels plus SEM
*significant differences of gene expressions ($P < 0.05$) between breeds

doi: 10.17221/84/2015-CJAS

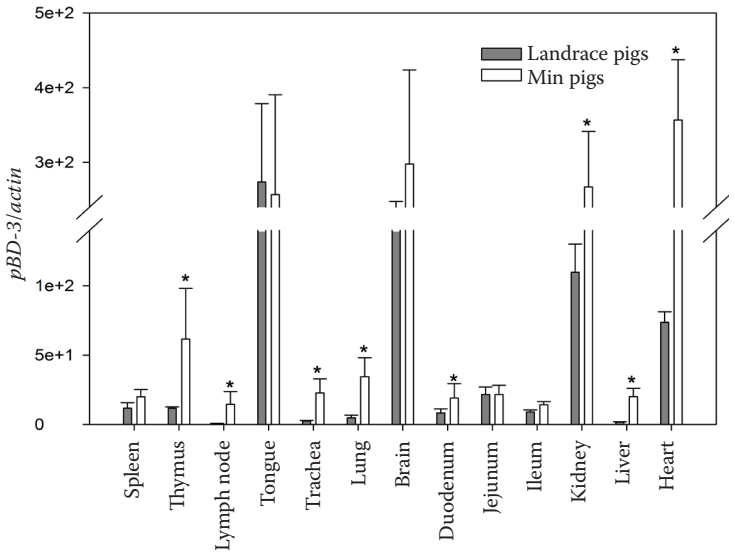

Figure 3. Tissue distribution of *pBD-3* mRNA in Landrace pigs and Min pigs
values are depicted as relative *pBD-3*/β-*actin* levels plus SEM
*significant differences of gene expressions ($P < 0.05$) between breeds

Comparison of tissue distribution of β-defensins between the two breeds. As shown in Figures 1–3, the mRNA expression of the three β-defensins in most tissues in Min pigs was higher than their expression in Landrace pigs. For *pBD-1*, the expression values in the brain, tongue, trachea, lymph node, thymus, liver, lung, kidney, duodenum, and jejunum in Min pigs were higher than in Landrace pigs. For *pBD-2*, the expression values of all tissues in Min pigs were higher than in Landrace pigs. For *pBD-3*, the expression values of all tissues except the tongue in Min pigs were higher than in Landrace pigs.

Correlation of expression between genes. As shown in Table 2, *pBD-1* and *pBD-2* were significantly correlated in Min pigs, and the Pearson's correlation coefficient was 0.597 ($P = 0.031$). *pBD-1* and *pBD-3* were significantly correlated in Landrace pigs, and the Pearson's correlation coefficient was 0.785 ($P = 0.001$). For *pBD-2* and *pBD-3*, gene expression levels were significantly correlated in both Landrace and Min pigs, and the coefficients were 0.738 ($P = 0.004$) and 0.553 ($P = 0.050$), respectively.

Table 2. Pearson's correlation coefficients of *pBDs* gene expression

Genes	Pig breed	*pBD-2*	*pBD-3*
pBD-1	Landrace	0.367 ($P = 0.218$)	0.785 ($P = 0.001$)
	Min	0.597 ($P = 0.031$)	0.337 ($P = 0.26$)
pBD-2	Landrace	–	0.738 ($P = 0.004$)
	Min	–	0.553 ($P = 0.05$)

DISCUSSION

β-Defensins are thought to have the most conserved tertiary structure (Sang et al. 2006), and may be implicated in adaptive immunity. Cloning and sequence analysis revealed that the mutations of the three peptide cDNAs in Chinese Min pigs were not observed.

β-Defensins serve as host defense substances not only by direct antimicrobial activity, but also as mediators in linking innate and adaptive immunity (McCormick and Weinberg 2010; Dommisch and Jepsen 2015). In the present study, mRNA of three β-defensins was diversely expressed in the tissues that require strong mucosal defenses, particularly the tongue, which was consistent with the expression pattern described previously in pigs (Zhang et al. 1998; Veldhuizen et al. 2007), sheep (Huttner et al. 1998), and cattle (Stolzenberg et al. 1997). Previously we found that the cathelicidin molecules, the other important antimicrobial peptides family, were generally expressed at low levels in the tongue in both breeds (Ma et al. 2014). That means that cathelicidins and β-defensins evolved in different directions.

In this study, β-defensins were shown to be highly expressed in organs that are rarely exposed to bacteria, such as the brain (Qi et al. 2009; Chen et al. 2010), which was in agreement with previous reports in mice (Huttner et al. 1997), rats (Hiratsuka et al. 2001) and cattle (Stolzenberg et al. 1997). A previous study showed the inducible expression of cathelin-related antimicrobial peptide in endothelial cells of the blood-brain barrier and

doi: 10.17221/84/2015-CJAS

in cells of the meninges in *Neisseria meningitides*-infected mice (Bergman et al. 2006), suggesting the possible important roles of pBD-2 and pBD-3 in maintaining the defense functions of the brain.

The expression of *pBD-1*, *pBD-2,* and *pBD-3* was prevalent in the tissues that control the immune system, such as the spleen, thymus, and lymph node. Ma et al. (2014) reported that the cathelicidins were generally expressed at high levels in the thymus and the spleen in both pig breeds, which was consistent with the present study. This agreement showed that cathelicidins and β-defensins play important role in these immune organs, which are essential to the maturation of the immune system. Defensins produced by cells in the course of innate host defense serve as signals that initiate, mobilize, and amplify adaptive immune host defenses (Oppenheim et al. 2003) as well as play extensive immunoregulatory roles in modulating early innate immune responses and induce adaptive immune processes (Scott and Hancock 2000; Selsted and Ouellette 2005). Human β-defensin has been reported to link innate and adaptive immunity through selective chemotaxis for cells stably transfected to express human chemokine receptor 6 (CCR6) (Yang et al. 1999). Similarly, pBDs might participate in both the innate and adaptive immune response due to their direct antimicrobial activities against invading pathogens and chemotactic activities on immune cells (Hazlett and Wu 2011).

In this study, we must take into account the fact that the expression of defensins was tested only at the level of transcripts. The level of mRNA does not necessarily correlate with the contents of the corresponding peptides in the tissues and organs of pigs.

We found that the mRNA expression levels of the three β-defensins in most tissues were much higher in Min pigs than in Landrace pigs, which was in agreement with our previous results about cathelicidins (Ma et al. 2014). Similarly, another two typical indigenous breeds in China, Meishan pigs and Tibetan pigs, exhibited relatively high expression of *pBD-1*, *pBD-2,* and *pBD-3* compared with crossbred pigs (Qi et al. 2009; Chen et al. 2010). Tibetan pigs are distributed in high altitude areas, and Meishan pigs are distributed in southern China where there is a warmer climate. Because Min pigs are adapted to a harsh climate, the habitat environment may be correlated to the diversity of β-defensins expression. However, only 3 animals of each breed were used in this study and inter-individual differences in the level of appropriate transcripts may be quite large. The correlation analysis of mRNA expression levels showed that the β-defensin expressions, that were significantly correlated in one breed, had no connection in the other breed. This suggested that, similarly to cathelicidins, β-defensin may be constitutively expressed in some tissues and inducibly expressed in other tissues (Ma et al. 2014).

In summary, mRNA expression of the three β-defensins in Min pigs and Landrace pigs was determined. In addition, the tissue distribution of *pBD*s gene expression between the two breeds was compared. The study showed that β-defensin genes have higher expression levels in most tissues of Min pigs compared to Landrace pigs. The higher expression of *pBD*s might be one of the mechanisms explaining Min pigs' higher immunity, adaptability, and disease resistance; yet, this hypothesis needs confirmation in future studies.

Acknowledgement. Our thanks are due to Neil Wu for language corrections.

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Received: 2015–11–19
Accepted after correction: 2016–11–04