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Research paper

Expression pattern of porcine antimicrobial peptide PR-39 and its induction by enterotoxigenic *Escherichia coli* (ETEC) F4ac

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ABSTRACT

PR-39 is a gene-encoded, proline-arginine-rich porcine antimicrobial peptide with multiple biological functions. In the current study, the tissue-specific mRNA expression of PR-39 was investigated in Chinese Jinhua pigs, and the effect of enterotoxigenic *Escherichia coli* (ETEC) expressing F4ac (K88ac) fimbriae challenge on the mRNA expression of PR-39 in various tissues was compared between Jinhua and Landrace pigs. The three most stable expressed housekeeping genes were validated before evaluating PR-39 expression. PR-39 mRNA was predominantly expressed in the bone marrow compared with the spleen, thymus, MLN, liver and ileum. The ETEC F4ac challenge could up-regulate PR-39 mRNA expression in both Jinhua and Landrace pigs, but the changes were different between the two breeds. Jinhua pigs responded more strongly to ETEC F4ac challenge than did Landrace pigs, because the interaction between the breed and challenge significantly impact PR-39 mRNA in the thymus, liver and ileum. The PR-39 mRNA expression levels of challenged Jinhua pigs were significantly higher in the spleen, thymus, liver, ileum and MLN compared with challenged Landrace pigs. These differences in the mRNA expression of PR-39 could be a result of genetic differences in the resistance to ETEC F4ac infection between the two breeds, but this speculation requires further investigation.

1. Introduction

Antimicrobial peptides (AMPs) are a group of gene-encoded, cationic, small peptides that are fundamental components of the innate immunity in multiple organisms (Zasloff, 2002). Domesticated animals, including pigs, serve as abundant and valuable sources of AMPs (Brogden et al., 2003). To date, several AMPs have been identified in pigs, which can be classified into two major families: cathelicidin and defensin (Sang and Blecha, 2009). PR-39 is the first discovered porcine AMP, which was initially isolated from the small intestinal tissues of pigs (Agerberth et al., 1991). It is a linear peptide with 39 proline-arginine-rich amino-acid residues, exhibiting multiple biological functions including antimicrobial (Shi et al., 1996), antioxidant (Busch et al., 2002), anti-apoptotic (Ramanathan et al., 2004) and pro-angiogenic (Li et al., 2000).

Jinhua pigs are a native breed from Eastern China that has evolved greater adaptability to hot and humid weather

Abbreviations: AMPs, antimicrobial peptides; ETEC, enterotoxigenic *Escherichia coli*; MLN, mesenteric lymphoid node; mRNA, messenger RNA; qPCR, quantitative real-time PCR.

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Table 1
Primers, annealing temperatures (Tm), expected amplicon sizes, qPCR efficiency (E) and accession numbers or references.

Primer name	Primer sequence (5′–3′)	Tm (°C)	Amplicon size (bp)	E (%)	Accession number or reference
18S	CCCACGGAATCGAGAAAGAG TTGACGGAAGGGCACCA	60	122	97	Shan et al. (2010)
ACTB	TCTGGCACCACACCTTCT TGATCTGGGTCATCTTCTCAC	60	114	92	Erkens et al. (2006)
B2M	CAAGATAGTTAAGTGGGATCGAGAC TGGTAACATCAATACGATTTCTGA	58	161	98	Nygard et al. (2007)
GADPH	ACACTCACTCTTCTACCTTTG CAAATTCATTGTCGTACCAG	60	90	110	Nygard et al. (2007)
HPRT1	GGACTTGAATCATGTTTGTG CAGATGTTTCCAAACTCAAC	60	91	103	Nygard et al. (2007)
PR-39	CAAGGCCACCTCCGTTTT CCACTCCATCACCGTTTTCC	60	103	93	NM_214450.1
RPL4	CAAGAGTAACTACAACCTTC GAACTCTACGATGAATCTTC	60	122	106	Nygard et al. (2007)
RPL19	AACTCCCGTCAGCAGATCC AGTACCCTTCCGCTTACCG	60	147	91	Delgado-Ortega et al. (2011)
SDHA	GAACCGAAGATGGCAAGA CAGGAGATCCAAGGCAAA	58	191	94	Erkens et al. (2006)
TBP1	AACAGTTCAGTAGTTATGAGCCAGA AGATGTTCTCAAACGCTTCG	60	153	91	Nygard et al. (2007)

conditions as well as tough rearing environment (Cheng, 1984). However, little data are available on the exact immune characteristics of Jinhua pigs during normal and pathogen-infected conditions, particularly with respect to the expression of the antimicrobial peptide PR-39.

In the current study, we investigated the tissue-specific mRNA expression pattern of PR-39 in Jinhua pigs, and compared the effect of enterotoxigenic *Escherichia coli* (ETEC) F4ac (K88ac) challenge on the mRNA expression of PR-39 in various porcine tissues including the bone marrow, spleen, thymus, mesenteric lymph node (MLN), liver and ileum in two pig breeds – Chinese Jinhua pigs and European Landrace pigs. To obtain reliable results, we validated the stability of nine reference genes and chose the three most stably expressed genes to normalize PR-39 mRNA expression. Our results would be helpful to the breeding of disease resistance pigs and informative for selecting suitable reference genes for studies that evaluate gene expression using the ETEC F4ac challenge pig model.

2. Materials and methods

2.1. Animals and sample collection

All of the experimental pigs were provided by Zhejiang Jiahua Pig Breeding Co. Ltd. (Zhejiang, China). The Landrace pigs were of Danish origin. Two experiments were carried out. In experiment one, the tissue-specific expression pattern of PR-39 mRNA was investigated. The selected tissues, including the bone marrow, spleen, thymus, MLN, liver, lung and ileum, were taken from six 2-month-old healthy Jinhua pigs. In experiment two, the impact of ETEC F4ac challenge on the expression of PR-39 mRNA in Jinhua and Landrace pigs was investigated. Twelve Jinhua pigs and twelve Danish Landrace pigs were assigned to either the control or the ETEC F4ac challenge group. The pigs were of similar age and were individually penned under the same environmental conditions. The ETEC F4ac (K88ac) strain used was the standard strain C83907 (O149:K91B:F4ac) obtained from the China Institute of Veterinary Drug Control (Beijing, China). This strain was confirmed to be positive for heat-labile toxin (LT) and heat-stable enterotoxin b (STb), and expressed fimbriae F4ac, as determined by PCR genotyping (Supplementary Figs. S1 and S2). Challenged pigs were orally administered the ETEC F4ac at a concentration of 10^{11} CFU as previously described (Gao et al., 2013). At the end of the experiment, the pigs were narcotized, slaughtered and the afore-mentioned tissues were sampled. Samples were cut into small pieces, immediately snap-frozen in liquid nitrogen and stored at −80 °C. All of the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

2.2. Real-time PCR assays and validation of stable housekeeping genes

The mRNA expression of PR-39 was analyzed using quantitative real-time PCR (qPCR) with SYBR green. RNA was extracted from porcine tissues and cDNA was synthesized as previously described (Shan et al., 2010). The primers and their qPCR conditions are presented in Table 1. The qPCR was performed using the SYBR Premix Ex Taq™ kit (Takara, Dalian, China) on a StepOne Plus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The qPCR conditions were 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/elongation at Tm (Table 1) for 30 s. The specificity of the primers was determined by melting curve analysis and the size of amplicons was confirmed by agarose gel electrophoresis (Supplementary Fig. S3).

Prior to evaluating PR-39 mRNA expression, we validated the expression stability of nine housekeeping genes using the geNorm application (Vandesompele et al., 2002). These genes included 18S ribossomal RNA (18S), beta-actin (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L4