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Polymorphism of *Nramp1* Gene and Its Association with Diarrhea in Pigs

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ABSTRACT

Background: Natural resistance associated macrophage protein 1 (*Nramp1*) is a relatively conservative gene that plays a crucial role in swine immune response and disease resistance.

Methods: We identified the polymorphisms and gene variations in the exon 2 of *Nramp1* using polymerase chain reaction–restriction fragment length polymorphism and investigated the correlation between the polymorphisms and piglet diarrhea in four pig breeds (Bamei, Duroc, Landrace and Large White pigs).

Result: The results showed that two alleles (A and B) were identified in all the pig breeds, three genotypes (AA, BB, and AB) were detected in Bamei and Large White breeds and two genotypes (AA and AB) were detected in Landrace and Duroc breeds. Allele A and genotype AB were dominant in Bamei, Large White and Landrace breeds, whereas genotype AA was dominant in Duroc pigs. A moderate polymorphism was observed in Landrace and Large White pigs and polymorphism was abundant in Bamei pigs and low in Duroc pigs. The Chi-square test for Hardy-Weinberg equilibrium disclosed that the exon 2 of *Nramp1* in the four breeds of pigs did not deviate from the Hardy-Weinberg balance (P>0.05). The results of association analysis showed a significant correlation between breed and piglet diarrhea (P<0.05) and the diarrhea score of Bamei pigs was much lower than those of the other breeds. The study could supply theoretical references for further functional research on *Nramp1* gene and for screening genes related to disease-resistance breeding.

Key words: Diarrhea, *Nramp1*, Pig, Polymorphism.

INTRODUCTION

Natural resistance associated macrophage protein 1 (*Nramp1*) was discovered and cloned from inbred strain mice by Vidal *et al*. in 1995 and were named by Belouchi *et al*. (1995). Pig *Nramp1* genes have similar secondary structures and high amino acid sequence homology. *Nramp1* is a relatively conservative gene that encodes the phosphate glycoprotein of intact membrane. This gene is also a membrane integrin with 10-12 transmembrane regions, 1 cytoplasmic transporter feature and 1-2 cytoplasmic cyclic structures formed by glycogen-membrane integration protein. Cellier *et al*. (1994) cloned human *Nramp1* gene into the 2q35 position on chromosome 15 by screening the spleen cDNA library. Pig *Nramp1* gene consists of 15 exons and 14 introns; its exons encode 8-12 transmembrane regions each with a total length sequence of approximately 15 kb (Marquet *et al*., 2000). *Nramp1* has a cDNA sequence of 1617 bp and encodes 539 amino acids. Several studies on *Nramp1* gene in pigs have been conducted. Gu *et al*. (2011) studied the original promoter of the *Nramp1* gene specifically expressed in pigs and found that transcription factors in the core promoter region have specific transcriptional regulation effect. Zhao *et al*. (2012) used four pig breeds as research objects to carry out enzyme digestion reaction and found that the exon 2 of pig *Nramp1* gene has three genotypes. Zhou *et al*. (2015) found that the intron 6 of *Nramp1* gene has unique mutation sites, namely, 259 bp A→G mutation, 331 bp G→A mutation and the insertion of 1–2 T bases after 29 bp. Structural research on pig *Nramp1* gene is still in the primary stage and the research process needs long-term accumulation because the number of exons and introns is relatively large. The homologies of pig and sheep Nramp1 proteins with human Nramp1 protein are 85.7% and 85.3%, respectively (Zhao *et al*., 2012). This finding indicates that pig *Nramp1* gene is highly conservative.

Luo (2014) found polymorphic loci in the intron 6 of pig *Nramp1* gene. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) studies on three foreign pig breeds confirmed that the intron 6 of pig *Nramp1* gene is polymorphic (Yang *et al*., 2009). *Nramp1* gene polymorphism in pig is related to piglet diarrhea (Zhao *et al*., 2013). The function of porcine neutrophils and monocyte macrophages is related to the polymorphism of the *Nramp1* gene (Wu *et al*., 2005). Liu *et al*. (2009) studied the correlation between the intron 6 mutation of Xiang pig *Nramp1* gene and piglet diarrhea and found that A is the dominant gene. The different genotypes of the *Nramp1* gene are correlated with immune function in Holstein cattle; the AA genotype is the dominant genotype for mastitis resistance

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in Holstein cattle (Hu, 2008). Rakesh *et al*. (2015) used PCR–single-strand conformation polymorphism (SSCP) technology to identify the genetic variations in the 32 untranslated region of the *Nramp1* gene in Gaolao cattle (*Bos indicus*) and found four common SSCP patterns. Indrajit *et al*. (2016) found 321 C-to-A substitution mutations in three buffalo breeds; these mutations result in the loss of the potential N-glycosylation motif of the *SLC11A1* gene and may change the characteristics of the encoded protein. Zhang *et al*. (2017) found that the c.200C>G mutation of *Nramp1* gene in Chinese Holstein cows was significantly ($P<0.05$) or extremely significantly ($P<0.01$) correlated with milk fat percentage, daily milk yield and somatic cell score. Luo *et al*. (2017) conducted research on Large White piglets and found that the NdeI cleavage site of the sixth intron of *Nramp1* gene can be used as a molecular marker for the breeding of Large White piglets with diarrhea resistance before weaning. These series of studies have shown that *Nramp1* gene polymorphism is associated with diseases to some extent; there fore, studying the polymorphisms of this gene is necessary.

In this study, PCR–RFLP was used to detect and analyze the polymorphisms in the exon 2 of *Nramp1* gene in Bamei, Duroc, Large White and Landrace pigs. The relationship between the polymorphism in the exon 2 of *Nramp1* gene and piglet sex and their interaction with piglet diarrhea were clarified. The purpose of this study was to provide a comprehensive theoretical knowledge for the disease-resistance breeding research of local pig breeds and to identify candidate genetic markers associated with piglet diarrhea. Breeding diarrhea-resistant piglets would reduce the production costs generated in the breeding process, provide certain technical support for the pig industry and reduce the losses of farmers.

MATERIALS AND METHODS

Sample collection and genomic DNA extraction

The piglets randomly sampled in this experiment were obtained from Linze Xinghua Pig Breeding Farm (Zhangye, Gansu, China). Diarrhea was investigated on the piglets, which have a clear pedigree and unrelated relationship. A total of 344 purebred pigs, including 101 Bamei, 91 Duroc, 87 Large White and 65 Landrace pigs, were studied. All piglets were monitored two times a day during the entire suckling period (from birth to weaning, 0–28 d) and assigned a daily score based on the visual analysis of symptom traits as follows: 0 = normal, solid feces; 1 = slight diarrhea, soft and loose feces; 2 = moderate diarrhea, semiliquid feces; and 3 = severe diarrhea, liquid and unformed feces. The follow-up experiments of this study were all conducted in the School of Life Science and Engineering of Northwest Minzu University. The time is 2019.

Ear samples (~2 g) were collected from all the pigs, placed into Eppendorf tubes containing 75% alcohol, then transported back to the laboratory and stored at -20°C. The genomic DNA of the pigs were extracted from their ear tissue samples by standard phenol-chloroform extraction method and the concentration of the genomic DNA was detected by 1% agarose gel electrophoresis.

PCR primer design and amplification

This experiment was performed based on the *Nramp1* gene sequence of pigs published in the GenBank database (accession no. EF200585). Primer 5.0 software was used to design four primers for the exon 2 of pig *Nramp1* gene. The upstream primer sequence was 5′-GACTGAAGA AAGRAATCAAGGGC-3′ and the downstream primer sequence was 5′ -CTTGGCCAGAGAGATCCCAT-3′. The primers were synthesized by Suzhou Jinweizhi Biotechnology Company (China).

PCR analyses were carried out on the 20 µl sample solution containing 0.8 µl genomic DNA, 0.4 µl of each primer, 11 µl of 5 U/µl TaKaRa Ex Taq HS DNA polymerase and 7.4 µl of double-distilled water (ddH_2O).

The PCR conditions included an initial denaturing for 1.5 min at 94°C; then 30 cycles of a three-step process of denaturation at 94°C for 30 s, 54.2°C for 30 s and extension at 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were detected by 1% agarose gel electrophoresis.

PCR–RFLP analyses

All samples were screened using RFLP analysis to scan for polymorphisms in the amplified region. The restriction reaction system (20 µl) included 0.2 µl of restriction enzyme Ava-II (enzyme cleavage site: G^GWCC), 3 µl of PCR amplification product, 1 µl of CutSmart buffer and 5.8 µl of ddH_2O. The digestion reaction was performed at the constant temperature of 37°C. The 60 ml enzyme digestion reaction detection system contained 7.8 ml of 30% polyacrylamide gel electrophoresis, 4 ml of 5×Tris–borate–ethylene diaminetetra acetic acid, 25 µl of tetramethylethylenediamine and 140 µl of 10% ammonium persulfate. The enzyme digestion product was added to 3 µL of 6×loading buffer and shaken to homogenize. The enzyme digestion product was detected at room temperature by pre-electrophoresis for 30 min at 200 V and electrophoresis for 3.5 h at 150 V. The electrophoresis products were fixed for 10 min and stained with silver for 20 min and the genotypes of each product were photographed for analysis.

Statistical analysis

The nucleotide and amino acid sequences were statistically analyzed using MEGA6. Gene heterozygosity (He) was calculated using the POPGENE software (version 3.2). Polymorphism information content (PIC) was calculated by Botstein's method (1980).

The effects of genotype on piglet diarrhea were analyzed using the general linear model procedure in SPSS 20.0 according to the following statistical model:

$$Y_{ijkl} = \mu + B_i + S_j + G_k + e_{ijkl}$$

Where,

Y_{ijkl} is the observation value of piglet diarrhea score, µ is the overall population mean, B_i is the fixed effect of breed, S_j is

the fixed effect of gender, G_k is the effect of genotype and e_{ijkl} is the random residual effect. Preliminary analysis also included the fixed interaction effects of breed, sex and genotype; however, these interaction effects were subsequently removed because they did not have a substantial effect.

RESULTS AND DISCUSSION

DNA extraction result and PCR amplification results

The genomic DNA from pig ear tissues were observed by 0.8% agarose gel electrophoresis and the DNAs with clear and bright bands and without dragging were selected for amplification.

The electrophoretic fragment size of the PCR-amplified product was consistent with the expected fragment size and the bands were clear.

PCR–RFLP results

The results of the PCR–RFLP of the exon 2 of porcine *Nramp1* gene are shown in Fig 1.

PCR–RFLP genotype and allele frequency distribution

The distribution of the exon 2 genotypes and alleles of the four pig breeds is shown in Table 1. Genotypes AA and AB were detected in the four pig breeds, whereas genotype BB was only detected in Bamei and Large White breeds. Genotype AB was dominant in Bamei, Large White and Landrace pigs and genotype AA was the most common in Duroc pigs. Two alleles, namely, A and B, were found in this locus. Allele A was predominant in Duroc, Large White and Landrace pigs with frequencies of 0.95, 0.63 and 0.70, respectively. Allele B was predominant in Bamei pigs with a frequency of 0.64. The Chi-square (χ^2) test results showed that the *Nramp1* genes of the four pig breeds did not deviate from the Hardy-Weinberg equilibrium at this locus.

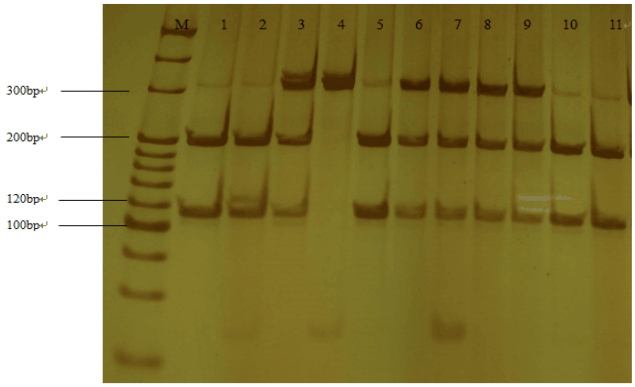

Fig 1: *PCR-RFLP* digestion of exon 2 of the *Nramp1* gene in pig. Note: M: 20bp DNA ladder; 1, 2, 5, 10, 11 genotype is BB; 4 genotype is AA; 6, 7, 8, 9 genotype is AB.

Analysis of *Nramp1* gene polymorphism

The analysis of the *Nramp1* gene polymorphism for each pig breed is shown in Table 2. The PIC of Bamei pigs was 0.65, which indicates that Bamei pigs have high polymorphism and a great selection potential. The PIC of Duroc pig was only 0.09, which indicates low polymorphism. The PICs of Large White and Landrace pigs were 0.36 and 0.33, respectively, which correspond to medium polymorphism. According to Vaiman *et al*. (1994), these loci show rich genetic diversity in different pig strains. The PIC and He in the exon 2 locus were lower in Duroc pigs than Bamei, Large White and Landrace pigs. However, this result may be caused by genetic background differences and selection pressures among the pig breeds (Table 2).

Correlation analysis between *Nramp1* gene and piglet diarrhea

Genotype and sex had little effect on piglet diarrhea ($P>0.05$), but a significant difference in the diarrhea scores of the different breeds of pig was observed ($P<0.05$). The diarrhea score of Bamei pigs was significantly lower than those of Duroc and Large White pigs ($P<0.05$) (Table 3,4,5).

Livestock and poultry diseases have attracted much attention with the development of science and technology. *Nramp1* gene plays an extremely important role in animal immune response and expresses specificity in phagocytes. Domestic and foreign research found that the *Nramp1* genes in cattle, sheep, rabbits, chickens and mice are related to disease resistance (Qin *et al*., 2013; Qiu *et al*., 2015; Liu *et al*., 2017). Wen *et al*. (2018) studied the structure and sequence of *Nramp1* in Tibetan, Gansu Black, Large White, Yorkshire and Duroc pigs. The sequence analysis of the *Nramp1* gene in these five pig breeds revealed 11 nucleotide variants in the intronic regions, 2 nucleotide variants in the control region, 10 nucleotide variants and one deletion in the 3′ non-coding region and 15 nucleotide variants in the exons (Wen *et al*., 2018). In the present study, PCR–RFLP was used to detect the polymorphisms in the exon 2 of the *Nramp1* gene in four breeding pigs. The results showed that the exon 2 of the *Nramp1* genes of Bamei, Large White and Landrace pigs had Ava-II locus. Large White and Landrace

Table 1: Exon 2 frequency and allele frequency of the *Nramp1* gene.

Breeds	Genotype frequency			Allelic frequency		χ^2 value
	AA	BB	AB	A	B	
Bamei pig	0.13(11)	0.41(39)	0.46(51)	0.36	0.64	21.14
Duroc pig	0.91(86)	-	0.09(9)	0.95	0.05	0.218
Large white pig	0.33(29)	0.05(5)	0.62(55)	0.63	0.37	9.427
Landrace	0.43(28)	-	0.57(37)	0.7	0.3	7.165

χ^2 (HWE) = Chi-square test for Hardy-Weinberg equilibrium.

Table 2: Analysis of *Nramp1* gene polymorphism.

Breeds	He	Ho	Ne	PIC
Bamei pig	0.46	0.54	1.86	0.65
Duroc pig	0.1	0.9	1.1	0.09
Large white pig	0.62	0.38	1.87	0.36
Landrace	0.55	0.45	1.71	0.33

He= Expected heterozygosity.
Ho= Observed heterozygosity.
Ne= Effective number of alleles.
PIC= Polymorphism information content.

Table 3: Effects of exon 2 of *Nramp1* gene on the diarrhea score of piglets in different breeds of pigs.

Breeds	Samples number	Least square means ± standard errors
Bamei pig	101	0.48 ±0.103a
Duroc pig	91	0.97 ±0.138b
Large white pig	87	0.82 ±0.118b
Landrace	65	0.77 ±0.136

Table 4: Genotype scores on diarrhea in piglets.

genotypes	Samples size	Diarrhea score
AA	149	0.77±0.094
BB	45	0.66±0.173
AB	150	0.85±0.087

Table 5: Correlation analysis of the genotypes of *Nramp1* exon2 in different gender and piglet diarrhea score.

Gender	Samples size	Diarrhea score
Male	177	0.70±0.085
Female	167	0.82±0.088

Note: The values without the same small letters in a column indicate the significant difference at P<0.05 level. Diarrhea score values are represented by least square means ± standard errors.

pigs had two alleles (A and B) and three genotypes (AA, AB and BB). This result is the same as Zhao *et al*.'s findings on the *Nramp1* gene's exon 2 at the HinfI digestion site. The exon 2 of the *Nramp1* gene in Duroc pigs had two alleles (A and B) and only two genotypes (AA and AB) at the Ava-II site. The BB genotype was not dominant possibly because of its inferiority or the number of samples used in the study was not enough. The frequencies of AA, AB and BB genotypes in Bamei pigs were 0.13, 0.46 and 0.41, respectively and the frequencies of alleles A and B in Bamei pigs were 0.36 and 0.64, respectively. The frequency distribution of this gene was unbalanced and the frequency of the AA genotype was low because the AA genotype is an unfavorable gene or the sample size in this study was too small. The frequencies of AA and AB in Duroc pigs were 0.91 and 0.09, respectively and the frequencies of alleles A and B were 0.95 and 0.05, respectively; thus, AA genotype was the dominant gene in Duroc pigs. The BB genotype, which may be an unfavorable gene, had the lowest frequency in Large White and Landrace pigs. He and PIC are often used to measure the degree of genetic variation in a population (Gu *et al*., 2017). The results of the homozygosity (Ho) and He analyses showed that the Ho was higher than the He of the *Nramp1* gene in the population. This result indicates that the degree of inbreeding was high; thus, the pig breeds have low disease resistance. The reason for the high Ho may be because some of the homozygote traits were higher than the heterozygote traits; hence, the frequency of homozygote genotypes was higher than that of heterozygotes.

We analyzed the PICs of the four pig breeds. The results showed that the PIC of Bamei pigs was 0.65. PIC>0.5 indicates high polymorphism. The PICs of Large White and Landrace pigs were 0.36 and 0.33, respectively. These breeds have medium polymorphism because 0.25<PIC<0.5. The PIC of Duroc pigs was 0.09, which indicates low polymorphism (PIC<0.25). Bamei pigs had the lowest diarrhea score and Duroc pigs had the highest diarrhea score among the four breeds. This difference in diarrhea score may be the result of a variety of changes and high selection pressure in the environment. Medium intensity selection can be implemented in subsequent breeding programs and differentiation breeding can also be implemented. Homozygous AA and BB individuals will be gradually selected to breed more disease-resistant breeding materials by mating with AB individuals.

The polymorphisms of the exon 2 of *Nramp1* gene in various pig breeds were studied. Bamei pigs have high polymorphism; Large White and Landrace pigs have medium polymorphism and Duroc pigs have low polymorphism. The χ^2 test results showed that the exon 2 of the *Nramp1* of the four pig breeds did not deviate from the Hardy-Weinberg balance. The results of correlation analysis showed that sex and the genotype of *Nramp1* gene in exon 2 were not related to piglet diarrhea (P>0.05), whereas pig breeds were significantly related to piglet diarrhea (P<0.05). The diarrhea score of Bamei pigs was far lower than those of the other pigs. Thus, Bamei pigs have the strongest resistance to diarrhea among the four pig breeds.

Conflict of Interest

The authors declare no conflicts of interest.

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