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NRAMP1 Gene Polymorphism: A Pathogenic Bacterial Resistance Trait in Indonesia Cattle

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The pathogenic bacterial infection is the main cause of decreased cow productivity. This study aimed to investigate the relationship of *Natural Resistance Associated Macrophage Protein 1* (*NRAMP1*) gene polymorphisms with pathogenic bacterial resistance traits in Indonesian cattle. A total of seventeen (17) animals of mixed-breed cattle comprising two categories healthy (7 heads) and infected with bacteria (10 heads) were used in this study. Forward sequencing analysis was performed to detect single nucleotide polymorphisms (SNPs) in the partial *NRAMP1* gene (954 bp) using polymerase chain reaction (PCR). Results showed that a novel SNP of g.109Y (intron 5) and two common SNPs of g.221K (intron 5) and g.642R (intron 6) were detected in the NRAMP gene in Indonesian native cattle. In addition, these SNPs have a moderate polymorphic information content (PIC) value (0.30 - 0.50). Therefore, eight (8) haplotypes of the *NRAMP1* gene were determined in the present study. Thus, the highest bacterial infection frequency was shown in the TT/GT/AG haplotype. In conclusion, three (3) nonsense SNP in the *NRAMP1* gene have the potency as a genetic marker for bacterial resistance trait in Indonesian cattle.
Keywords: Cattle, haplotype, NRAMP1 gene, pathogenic bacterial, SNP.

INTRODUCTION

Detection of disease resistance in beef cattle is the main criterion in selecting superior breeds. NRAMP1 detection in Indonesian local cattle has never been done and is a novelty in this research. The NRAMP gene in many previous studies was polymorphic and associated with pathogenic bacterial resistance traits. Infections by pathogenic bacteria have been reported as main factor of economic losses and lower productivity of livestock (Davies *et al*., 2009). Several diseases in cattle that are caused by pathogenic bacteria are Mastitis and Leptospirosis. Mastitis is caused by infection of pathogenic bacteria in the intramammary and can evolve into subclinical mastitis (SCM) or clinical mastitis (CM) if the udder is not cleared by the farmer (Narayana *et al*., 2022). Leptospirosis is one of the worldwide distribution zoonotic diseases, caused by spirochetal pathogens of the genus *Leptospira* (Ibrahim *et al*., 2022). The infection of pathogenic bacteria is still prevalent worldwide health issues, good biosecurity, mass vaccination, and slaughtering policies are often promoted against the disease (Mittal *et al*., 2018; Akhtar *et al*., 2019; Glass *et al*., 2011).

A new solution is needed to reduce the transmission of infectious diseases, such as breeding livestock that inherently possess greater resistance or natural resistance to pathogens (Glass et al., 2011). Natural resistance proves to be more efficient in defending against specific pathogens (Sponner *et al*., 1975; Barger, 1989; Paixao et al., 2005). One gene related to natural resistance in cattle is the Bovine Natural Resistance–resistance-associated macrophage Protein 1 (bNRAMP1) gene. This gene, initially identified in mice, plays a crucial role in countering intracellular pathogens like Mycobacterium sp., Salmonella sp., and Leishmania. Its function involves preventing bacterial growth during the macrophage stage and contributing to innate immunity (Paixao et al., 2007; Pinedo et al., 2009; Akhtar et al., 2019). The NRAMP1 gene consisted of 14 introns and 15 exons was located in chromosome 2q43-44(Putra, W.P.B 2019). The NRAMP1 gene polymorphism provides significant genetic variation in cattle resistance to tuberculosis infection (Cheng *et al*., 2015; Baqir *et al*., 2016; Liu *et al*., 2017; Holder *et al*., 2020), mastitis (Joo *et al*., 2003; Hu *et al*., 2009) and brucellosis resistance (Kumar *et al*., 2005). Genetic factor plays an important part in the specific traits of genetic segregation (Akhtar *et al*., 2019; Rahella *et al*., 2019) and has

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been related to differences in host susceptibility (Pinedo *et al*., 2009) based on that information, the genetic selection method can be used for disease resistance trait to control infection by pathogenetic bacteria. This study aimed to investigate the relationship of *NRAMP1* gene polymorphisms with pathogenic bacterial resistance traits in Indonesia native cattle.

MATERIALS AND METHODS

***Ethical Clearance*:** All experiments were approved by the animal ethics committee of the Indonesian Agency for Agricultural Research and Development (Balitbangtan /Lolitsapi /Rm/11/2021).

***Animal and DNA extraction*:** A total 17 mixed-breed cattle comprised of 10 Madura (*Bos indicus*), 2 Bali (*Bos javanicus*) and 5 Friesian Holstein (*Bos taurus*) were used in this study and classified into healthy groups (7 heads) and bacterial infected group (10 heads). The blood samples (±3 mL) were collected from the *jugular vein of* each animal. DNA extraction was performed using a Genomic DNA Isolation Kit (Geneaid New Taipei City, Taiwan) following the manufacturer protocol.

***Pathogenic bacterial examination*:** Pathogenic bacterial examination was prioritized for Leptospirosis and Mastitis disease. The testing was conducted at the Veterinary Research Institute Laboratory in Bogor. Leptospirosis examination uses blood samples collected from bacterial-infected cows and serological tests are performed. The blood samples were extracted to obtain serum. The serological tests were performed using ELISA (Enzyme-linked Immunosorbent Assay) or Immunofluorescence tests to detect the presence of IgM and IgG antibodies to Leptospira in the serum. A confirmation test was then conducted using the MAT (Microscopic Agglutination Test) to determine the titer of the antibodies to Leptospira.

Mastitis disease examination using milk samples. The indicator for infected milk was done using the CMT method. Milk samples were taken from cows infected with Mastitis, about 10-15 ml, and placed on a CMT plate and mixed with a CMT solution in a 1:1 ratio, then observed changes in milk consistency. Milk consistency was classified into five groups: a. Clean (negative = less than 250,000 cells/ml); b. (+1) Positive 1 (250,000 - 400,000 cells/ml); c. (+2) Positive 2 (400,000 - 1,500,000 cells/ml); d. (+3) Positive 3 (1,500,000 - 5,000,000 cells/ml); e. (+4) Positive 4 (over 5,000,000 cells/ml).

***PCR and sequencing*:** PCR analysis was performed on each sample with a total volume of 30 µL per sample consisting of 3 µL extracted DNA, 0.6 µL forward primer, 0.6 µL reverse primer, 15 µL PCR mix and 10.8 µL DDW. NRAMP1 gene was amplified with the primer pairs designed (GenBank: AY338470) i.e., forward primer 5'-TCCGACATGCAGGAAGTCAT-3' and reverse primer 5' GCCGAAGGTCAAGGCCATTATGG-3' with amplicon size of 954bp (Kumar et al. 2011). The thermocycler (Veriti Applied Biosystem, USA) was set for pre-denaturation temperature at 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), 72°C for 20 seconds (extension), and final extension 72°C for 10 minutes. PCR visualization product (amplification) was carried out in the gel documentation (Infinity VX2, France) using 1% agarose gel (Thermo Scientific, USA). The DNA fragment size was compared with a 100 bp DNA marker (Thermo Scientific, USA). Therefore, sequencing analysis was performed with 25 µL of PCR product from each sample through a commercial laboratory service (1st Base Laboratory, Malaysia).

***Data analysis*:** The DNA sequences from sequencing analysis were used for alignment analysis with BioEdit software (Hall, 2011) to detect the mutation points (SNPs). The genetic diversity parameters include allelic frequency, genotypic frequency, polymorphic informative content (PIC), number of effective alleles (n_e), and Chi-square (χ^2) values were calculated using a formula referring to Nei & Kumar (2000). Thus, the association study of *NRAMP1* gene polymorphisms with pathogenic bacterial resistance was analyzed descriptively.

RESULTS

The NRAMP1 gene fragments in this study were amplified at an annealing temperature of 58°C with a PCR product of 954 bp (Fig. 1). The result of genotype and allele frequencies of NRAMP1 in Indonesian native cattle is shown in Table 1. In this study were found two mutation sites in intron 5 (g.109Y and g.221K) and one mutation site in intron 6 (g.642R) of the NRAMP1 gene. All SNP were detected to have three genotypes.

Table 1. Genetic diversity of three nonsense mutation sites in *NRAMP1* gene of Indonesian native cattle.

Mutation	Genotype frequency			Allelic frequency		H_o	H_e	n_e	PIC	χ^2
g.109Y	TT(0.53)	TC(0.41)	CC(0.06)	T(0.74)	C(0.26)	0.41	0.39	1.64	0.31	0.06
g.221K	GG(0.29)	GT(0.65)	TT(0.06)	G(0.62)	T(0.38)	0.65	0.47	1.90	0.36	2.33
g.642R	AA(0.29)	AG(0.59)	GG(0.12)	A(0.59)	G(0.41)	0.59	0.48	1.94	0.37	0.78

H_o: observed heterozygosity; H_e: expected heterozygosity; n_e: number of effective allele; PIC: polymorphic informative content; χ^2: Chi-square value

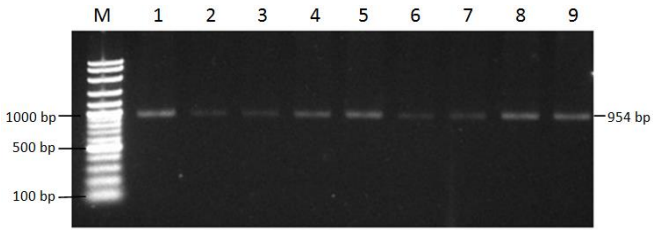

Figure 1. The amplification of *bovine NRAMP1* gene along 954 bp. M: DNA ladder 100 bp; Lane 1-9: DNA sample.

The genotypes of SNP g.109Y were found TT, TC, and CC with the TT and TC genotypes frequencies were 53% and 41%, while genotype CC was found in lower frequencies was 6%. In SNP g.109Y, the T allele is common. The SNP g.221K has genotypes GG, GT, and TT with genotypes frequency were 29%, 65%, and 6%, respectively and the G allele is a common allele. The SNP g.642R was found in AA, AG, and GG genotypes. The allele frequency of A and G were 59% and 41%. These three mutation sites were polymorphic and had a moderate PIC value (0.31-0.37). Among the three of SNPs, SNP g.109Y was detected as the novel mutation of the bovine NRAMP1 gene. The association of the haplotype bovine NRAMP1 gene with the pathogenic bacterial resistance trait is shown in Figure 2. There is no association with Brucellosis resistance trait in cattle. The infected animals were detected on animals with TT/GT/AG haplotype.

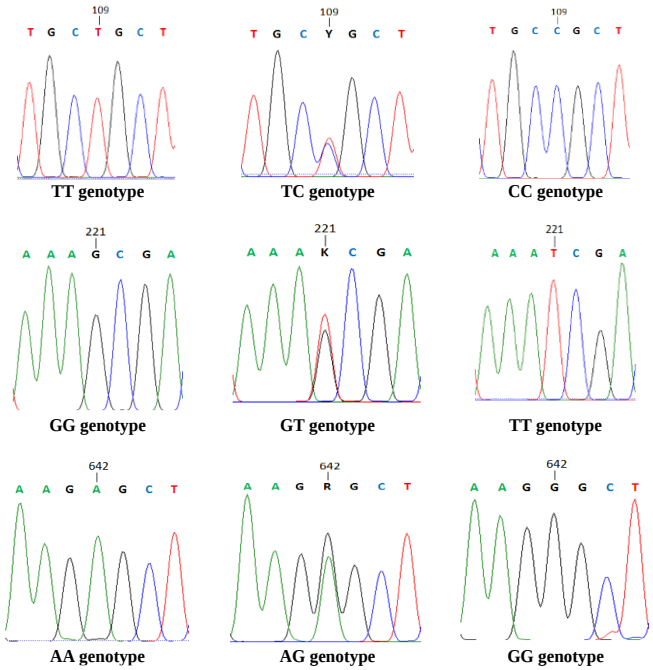

Figure 2. Three nonsense mutations of g.109Y (top); g.221K (center) and g.642R (bottom) were detected *inbovineNRAMP1* gene (GenBank: AY338470.2)

DISCUSSION

Along with 954 bp of *NRAMP1* gene fragments were successfully amplified on 1% agarose gel as shown in Figure 1. Therefore, three (3) nonsense mutations of g.109Y (intron 5); g.221K (intron 5), and g.642R (intron 6) as shown in Figure 2. However, a mutation point of g.109Y was confirmed as the novel mutation in the *NRAMP1* gene of Madura and Bali cattle. Meanwhile, two mutation points of g.221K and g.642R are the common mutations in the *bovine NRAMP1* gene that are able to detect by using the PCR-RFLP method with *Taq*I (T*CGA) and *Alu*I (AG*CT) restriction site, respectively (Kumar *et al.*, 2011). Mostly the PIC and n_e values in three observed SNPs were>0.30 and >1.50, respectively (Table 1). In addition, SNP g.221K was low in Friesian cross (0.06) and Hariana (0.07) cows. Meanwhile, SNP g.642R was moderated in a Friesian cross (0.32) and Hariana (0.27) cows (Kumar *et al.*, 2011).

The PIC value can be classified into low (0.10 - 0.30); moderate (0.31 - 0.50) and high (>0.50) categories (Nei and Kumar, 2000). The moderate PIC value indicated that the observed locus gene has moderate genetic diversity. Hence, molecular selection in Indonesian native cattle can use three SNPs in the *NRAMP1*. The n_e value of more than 1.50 indicated that the observed locus has two common alleles. The genetic diversity in livestock can be affected by selection, migration, inbreeding, and crossbreeding. The χ^2 value in all observed SNPs indicated that the genetic diversity in the *NRAMP1* gene of Indonesian native cattle is under genetic equilibrium. Hence, it can be suggested that there are no selection programs for pathogenic bacterial resistance traits in Indonesia native cattle. Selecting cows with resistance to pathogenic bacteria can be achieved through an initial screening of milk samples using the CMT (California Mastitis Test) method, which is still considered accurate and fast. A milk curdling limit of ≥2 indicates bacterial infection (Kaki et al., 2019). In their report, Bagheri and Zahmatkesh (2019) stated that the NRAMP1 gene can reduce susceptibility to intracellular pathogens. This gene plays a significant role in the innate immune system by enabling macrophages to kill bacteria.

Despite intron 5 and intron 6 regions, many SNP's of *bovine NRAMP1* gene (GenBank: KR002421) such as c.6955Y (exon 9); c.8698S (exon 11); g.6950R (intron 9); and g.9106W (intron 12) can be detected with PCR-RFLP method (Baqir *et al.*,2016; Liu *et al.*, 2016). Previously, there were four SNPs observed in the intron 5 region of the *bovine NRAMP1* gene (GenBank: AY338470.2) *i.e.*, g.161Y; g.221K; g.223K; g.235R (Ables *et al.*, 2002; Schutta, 2006). In the present study, eight (8) haplotypes of the *bovine NRAMP1* gene were determined according to three detected SNPs (Figure 3). According to Figure 3, most of the infected animals were detected on cows with TT/GT/AG haplotype. Kumar *et al.* (2011) reported no association of g. 221K and

g.642R with *Brucellosis* resistance trait in cattle. However, many previous studies reported that *bovine Tuberculosis* resistance can be associated by many SNP's of *NRAMP1* gene at 3'UTR (Kadarmideen *et al.*, 2011); exon 4, intron 4 (Cheng *et al.*, 2015); exon 9, exon 11 (Baqir *et al.*, 2016), intron 9, intron 12 (Liu *et al.*, 2016), exon 10 and exon 11 (Paixao *et al.*, 2012) regions. Nonetheless, Joo *et al.* (2003) reported monomorphism in the exon 5 of NRAMP gene in healthy and infected *Mastitis* cows.

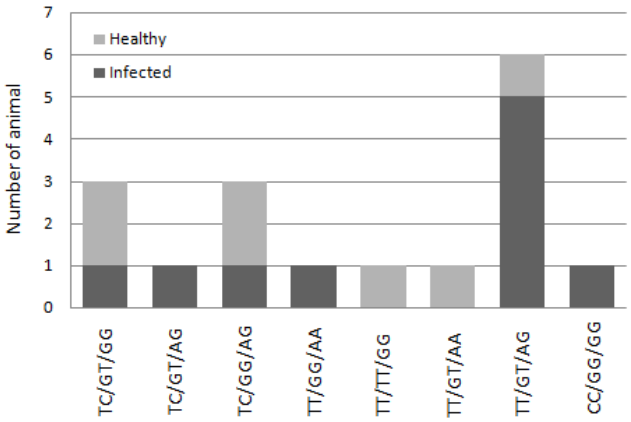

Figure 3. Association of haplotype *bovine NRAMP1* gene with the pathogenic bacterial resistance trait in native cattle of Indonesia

***Conclusion*:** The NRAMP gene in native cattle of Indonesia was polymorphic with three SNPs at the intronic region *i.e.* g.109Y (intron 5); g.221K (intron 5) and g.642R (intron6). These SNPs have a moderate PIC value and can be used for molecular selection. However, SNP g.109Y was detected as the novel mutation of the *bovine* NRAMP gene. In this study, mostly the bacterial infected animals were detected in cows with TT/GT/AG haplotype. In the future, a depth study with large samples and health records is important to confirm these findings.

***Conflict of interest*:** The authors declare that they have no conflict of interest.

***Authors' Contribution Statements*:** HH and WPBP conceived, designed the research, and executed the experiment. NDY, SI, and DKR analyzed all samples. All authors analyzed and interpreted the data, critically revised the manuscript for important intellectual contents, and approved the final version.

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