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Research Article

Association of *NRAMP1* Polymorphisms with Immune Traits in Indonesian Native Chickens

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Abstract

Background: Natural resistance-associated macrophage protein-1 gene (*NRAMP1*) plays an important role in immune response against intracellular pathogens. **Objective:** This study was aimed at identifying *NRAMP1* polymorphisms and their association with immune traits in Indonesian native chickens. **Methodology:** Genetic polymorphism of chickens was investigated using polymerase chain reaction-restriction fragment polymorphism. The concentrations of leukocytes and differentiation (heterophile, lymphocyte and monocyte) were assessed by the Giemsa method and immune traits were detected by followed clearance test. **Results:** The results showed that *NRAMP1* was polymorphic in all native chickens. The CC genotype was significantly higher than CT and TT genotypes (p<0.05) in Sentul chickens resistant to *Salmonella pullorum*. Although, the concentrations of leukocytes and differentiation in chickens with all three of *NRAMP1* genotypes (CC, CT and TT) were not statistically different, there was a significant correlation between different *NRAMP1* genotypes and immune traits. Therfore, *NRAMP1* is proposed to be a disease resistance candidate gene. **Conclusion:** However, this study should be validated in other chicken populations to evaluate the potential exploitation of *NRAMP1* in selective breeding.

Key words: *NRAMP1* gene, PCR-RFLP, immune traits, Indonesian native chickens

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Various breeding programs have been developed and implemented in an effort to improve the productivity of livestock commodities and current breeding programs which improve resistance of livestock to disease have become increasingly popular as an alternative method for increasing productivity while reducing production costs. In addition to economic concern, the study of the genetics of disease resistance is also driven by a greater probability of success as a result of significant progress in the field of molecular technology. Genetic approaches to animal disease resistance are very important, especially for production systems in developing countries because general maintenance systems are still traditional or semi-intensive and small-scale since vaccinations and treatments are difficult to disseminate and expensive.

Previous study on chickens focusing on immune reactions to disease has shown a strong genetic component associated with resistance[1,2]. Differences in disease resistance are also a genetic basis for selection in breeding[3]. Candidate gene approaches have been successfully used to detect genes important for disease resistance in chickens including major histocompatibility complex, caspase-1, inducible nitric oxide synthase, toll-like receptor 4 and natural resistance-associated macrophage proteins-1 (*NRAMP1*) genes[2,4,5].

In chickens, a homologue of *NRAMP1* has been mapped on chromosome seven which consists of a promoter region, 15 exons, 14 introns and flanking regions 5760 bp in length[6]. The *NRAMP1* restricts microbial access to essential micronutrients such as Fe^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} within professional phagosomes[7-10] and has been identified as a candidate gene that controls resistance to *Salmonella enteritidis* in poultry[11]. The *NRAMP1* polymorphisms have been previously associated with certain immune traits in chickens[12]. For example, the CC genotype of *NRAMP1* had been associated with a higher *S. enteritidis* burden in the cecum and spleen of Malaysian native chickens[13]. The *NRAMP1* is mainly expressed in the liver, thymus and spleen in both female and male chickens[14].

Until now, variations in *NRAMP1* and their effect on disease have not been well investigated in Indonesian native chickens. Therefore, the objective of the present study was to identify the association of *NRAMP1* polymorphisms with immune traits in Indonesian native chickens in order to provide a reference for disease-resistant chicken breeding by marker-based selection.

MATERIALS AND METHODS

Blood samples: Blood samples were collected from seven breeds of Indonesian native chickens (n = 265 total) including Kampung (n = 57), Sentul (n = 96), Broiler (n = 10), Pelung (n = 10), Merawang (n = 23), F1 Kampung×Broiler (KB, n = 30) and F2 KB×KB (n = 39). The DNA was extracted from blood samples at the Animal Molecular Genetics Laboratory, Faculty of Animal Science, Bogor Agricultural University, Indonesia.

DNA extraction: Genomic DNA was extracted from blood samples using phenol-chloroform method followed by ethanol precipitation[15] and the DNA was dissolved in elution buffer. The quality of the total genomic extraction was assessed by 1% agarose gel electrophoresis.

DNA amplification: Polymerase Chain Reaction (PCR) was carried out using primers specific for a part of exon 11 (421 bp) of *NRAMP1* (GenBank Accession No. AY072001): Forward 5'-CAATGAGACGGTGTCTGTGG-3' and reverse 5'-CCCAGAAGAAATCTCCCTGC-3'. The PCR was carried out in a total reaction volume of 15 µL containing 0.5 µL of the genomic DNA template, 0.3 µL of each primer, 0.3 µL dNTPs, 1 µL $MgCl_2$, 0.05 µL of Taq polymerase, 1.5 µL 10X reaction buffer and 10.85 µL of distillated water. Amplification was carried out with a GeneAmp® PCR 9700 System (Applied Biosystems, USA). Thermal cycling conditions consisted of predenaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 30 sec the final extension step was at 72°C for 5 min. The DNA amplification products and a standard DNA ladder were separated on 1.5% agarose gels in 0.5X TBE buffer.

PCR-Restriction Fragment Length Polymorphism (RFLP) analysis: Genetic polymorphism of *NRAMP1* was conducted by PCR-RFLP using a *Sac*I restriction enzyme which recognizes and cleaves GAGCT|C sites. Amplification products were visualized on a 2% agoras gel containing 2.5 µL ethidium bromide and 0.5X TBE buffer (1 M tris-base, 0.9 M boric acid, 0.01 M EDTA, pH 8.0) with a DNA ladder as a standard size comparison. For enzymatic digestion and determination of RFLPs, PCR products (5 µL) were mixed with 0.3 µL of *Sac*I, 1 µL distillated water and 0.7 µL tango buffer and then incubated at 37°C for 16 h. The digestion products were separated by horizontal electrophoresis (100 V, 40 min) in 2% agoras gel in 0.5X TBE and 2.5 µL ethidium bromide visualized on an ultraviolet-transiluminator.

Concentration of leukocytes and differentiation: The concentrations of leukocytes and differentiation were assessed by the Giemsa method[16] as follows: 20 µL of chicken blood was dissolved in 380 µL of Turk solution (1 mL of 1% gentian violet in water, 1 mL glacial acetic acid and 100 mL distilled water) using a micropipet. The total number of leukocytes present was calculated by counting all viable cells present on four areas located in four corners of the room count under a light microscope (100X magnification) and then multiplying by 50 to determine the concentration of each millimeter cube[3].

Clearance test: Immune traits were detected in blood samples using the clearance test[17]. This method was used to look at normal bacterial population growth compared that of populations given specific treatment. The treatment impact on bacterial growth was measured after incubating for 24-48 h at 35±1°C.

Genotype and allele frequencies: Genotype and allele frequencies were calculated according to the formula by Nei and Kumar[18] using *NRAMP1* genotyping data. Genotype frequency was calculated with the following formula:

$$xi = \frac{\sum_{i=1}^{n} ni}{N}$$

Allele frequency was calculated with the following formula:

$$Xi = \frac{\left(2nii + \sum_{j\#1} nij\right)}{(2N)}$$

Where:
Xii = Genotype frequency
Xi = Allele frequency
nii = Number of samples with ii genotype
nij = Number of samples with ij genotype
N = Total number of samples

Statistical analysis: The *NRAMP1* polymorphism and immune trait data were analyzed using the GLM procedure of SAS 9.1.3 software (SAS Institute, Cary, NC, USA). The following model was used:

$$Y_{ij} = \mu + G_i + e_{ij}$$

where, Y_{ij} is the observation on immune traits, µ is the overall mean, G_i is the effect of the single nucleotide polymorphism genotypes and e_{ij} is the random residual effect.

RESULTS AND DISCUSSION

Genetic polymorphism of *NRAMP1*: All DNA samples from each chicken were amplified and the PCR products separated by agarose gel electrophoresis. Thus, the product was amplified with fine stability and specificity and could be cleavaged by the restriction enzyme directly. A 421 bp fragment of exon 11 from *NRAMP1* in Indonesian native chicken samples is shown in Fig. 1.

The *NRAMP1*|*Sac*I restriction analysis of the exon 11 region revealed three genotypic patterns in Indonesian native chickens and showed two alleles (C and T alleles) in all native chicken breeds examined. The restriction fragments included a single, uncut fragment of 421 bp (TT genotype), two fragments of 258 and 163 bp (CC genotype and three fragments of 421, 258 and 163 bp (CT genotype) (Fig. 2).

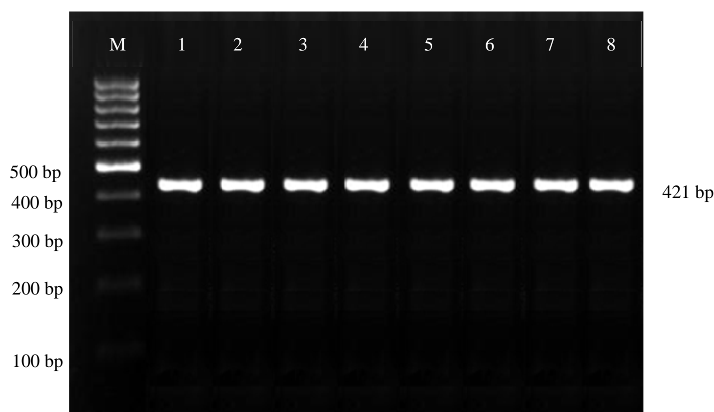

Fig. 1: *NRAMP1* PCR product, M: DNA ladder and Lanes 1-8: Individual PCR products

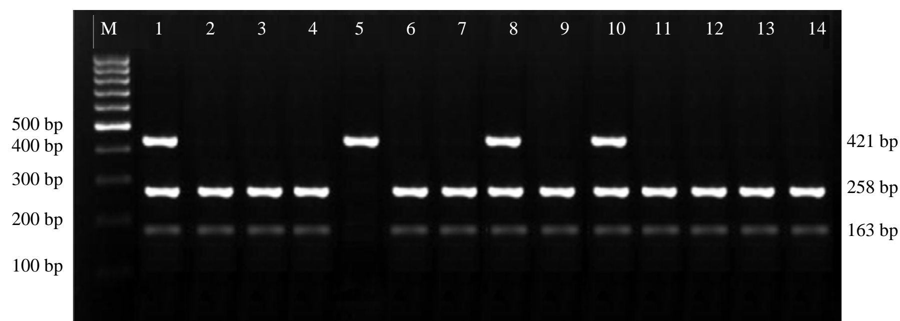

Fig. 2: *Sac*I restriction pattern of exon 11 fragment of *NRAMP1* by PCR-RFLP on 2% agarose gel, M: DNA ladder and Lanes 1-14: Individual PCR-RFLP products

Table 1: Genotypes and allele frequencies of polymerase chain reaction-restriction fragment length polymorphism products from part of exon 11 of *NRAMP1*

		Genotype frequency			Allele frequency	
Chicken breed	No. of samples	CC	CT	TT	C	T
Kampung	57	0.895	0.105	0	0.947	0.053
Sentul	96	0.896	0.073	0.031	0.932	0.068
Broiler	10	0.800	0.200	0	0.900	0.100
Pelung	10	0.800	0.100	0.100	0.850	0.150
Merawang	23	0.609	0.304	0.087	0.761	0.239
F1 Kampung×Broiler (KB)	30	0.700	0.300	0	0.850	0.150
F2 KB×KB	39	0.821	0.179	0	0.910	0.090
Total	265					

Table 2: Correlation analysis between *NRAMP1* genotypes and immune function in Sentul chickens

		Immune function			
Genotype	No. of samples	Leukocyte (10^3 mm^{-3})	Heterophile (%)	Lymphocyte (%)	Monocyte (%)
CC	15	18.82±2.18	44.73±3.88	52.33±3.79	2.33±0.30
CT	3	12.93±4.87	42.66±8.68	55.33±8.49	1.33±0.68
TT	2	19.00±5.97	50.00±10.64	47.00±10.40	2.00±0.84

The genotype and allele frequencies of exon 11 of *NRAMP1* in all Indonesian native chicken breeds are presented in Table 1. The homozygous CC genotype was present in the highest frequency across all breeds. The distribution of the *NRAMP1*|*Sac*I alleles of exon 11 is characterized by a higher frequency of the C allele compared to the T allele in most of the native chickens studied (Table 1).

The two genotypes found to in Kampung, Broiler, F1 KB and F2 KB×KB chickens were CC and CT (Table 1) CC, TT and CT genotypes were found in Sentul, Pelung and Merawang breeds. This genetic diversity can be caused by DNA sequence repeats sequences, insertions, deletions and/or recombinations between individuals, groups or populations[18]. The results show that the gene fragments of *NRAMP1*|*Sac*I exon 11 in Indonesian native chickens were polymorphic because there were three genotypes found in each of fragments obtained and an allele frequency around 0.900.

Concentration of leukocytes and differentiation: Leukocyte concentrations in Sentul chickens with CC, CT and TT genotypes were not statistically different (Table 2). Leukocytes (white blood cells) are mobile parts of the immune system. After formation, white blood cells enter the bloodstream and go to the necessary parts of the body. Granular leukocytes consist of heterophiles, eosinophils and basophils; no granular leukocytes are monocytes or lymphocytes[19]. These four cell types (leukocyte, heterophile, lymphocyte and monocyte) have four important immune functions and are presented in Table 2.

The concentration of leukocytes in chickens with CC, CT and TT genotype[20] were within the normal range 12-30×10^3 mm^{-3}. These results indicate that the Sentul chicken with the third *NRAMP1* genotype were not infected with the bacterium.

Resistance of Sentul chickens to *S. pullorum*: The CC genotype in Sentul chickens resistant to *S. pullorum* was

Table 3: Association of *NRAMP1* genotype in Sentul chickens resistant to *Salmonella pullorum*

Genotype	No. of samples	Early concentration (10^7 CFU mL^{-1})	Final concentration (10^5 CFU mL^{-1})	Death rate of bacteria (%)
CC	14	2.40	1.07 ± 4.27^a	55.05 ± 1.78^a
CT	3	2.40	1.73 ± 9.23^b	27.77 ± 3.84^b
TT	2	2.40	1.75 ± 11.31^b	27.08 ± 4.71^b

Different letters in the same column means difference significantly ($p<0.05$)

significantly higher ($p<0.05$) than the CT or TT genotypes (Table 3). According Tohidi *et al.*[13] the homozygous CC genotype was related to the highest *S. enteritidis* load in Malasyian native chickens and red jungle fowl. The death rate of *S. pullorum* in Sentul chickens with the three genotypes ranged from 27-55%. The association of *NRAMP1* genotype in Sentul chickens to *S. pullorum* resistance is presented in Table 3.

As a candidate disease resistance gene, *NRAMP1* has been studied by a number of researchers throughout the world. Numerous studies have revealed a correlation between *NRAMP1* and immune resistance to disease. Vidal *et al.*[21] found that in mice *NRAMP1* plays an important role during the early immune period when macrophages are activated by parasite antigens. Liu and Lamont[11] found that a single nucleotide polymorphism of highly conserved sequences of chicken *NRAMP1* is related to *S. enteritidis* vaccination and immune response to pathogenic attack.

CONCLUSION

The present study investigated the relationship between *NRAMP1* polymorphisms of Indonesia native chicken and demonstrated that genetic mutation exon 11 of *NRAMP1* effects the resistance of Sentul chickens to *S. pullorum*. Thus, *NRAMP1* is a good candidate gene for disease resistant breeding.

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