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White Blood Cell Differentiation Based on Toll-Like Receptor 4 (TLR4) Gene Polymorphism in Local Indonesian Chickens

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Abstract: Toll-like Receptors (TLRs) genes are a group of genes that transcribe TLRs proteins which act as receptors on the surface of phagocytic cells to recognize molecular patterns of pathogenic microbial components. This research aims to identify the variability of the TLR4 gene and its relationship with blood profiles in local Indonesian chickens. The samples used to identify TLR4 gene diversity in local Indonesian chickens were 135 samples. Research methods include DNA extraction, PCR amplification, RFLP analysis, and genotype determination. The genotyping results in the research were associated with a complete blood profile so that a genotype was obtained that could be used as a marker for resistance traits based on the blood profile. The results of this study showed that the TLR4 gene was polymorphic with three genotype types and an allele frequency of more than 0.01 in each KUB, Sensi, and Broiler CP 707 chicken. The frequency of the GG genotype is the highest compared to the AG and AA genotypes. White blood cell concentration values showed variations in the three genotypes of TLR4 gene and were still within normal standards, although statistically the differences were not significant.

Keywords: blood profile, local chickens, marker-assisted-selection, TLR4 gene.

INTRODUCTION

Treatment of infections in local Indonesian chickens is almost never done, but the population is increasing from year to year. Indonesia has experienced a high mortality rate in poultry due to repeated outbreaks of Avian Inluenza (AI), but naturally, chickens have the ability to resist virus attacks (Sulandari et al., 2009; Sartika et al., 2011). This proves that Indonesian chickens have the advantage of being relatively more resistant to several diseases. According to Maeda (2005), 63% of Indonesian chickens are in the resistant category and 37% are in the sensitive category to Avian influenza (AI) virus infection. Based on observations on purebred chickens, 100% were categorized as sensitive to AI infection.

Technological advances in the field of breeding make it possible to select chickens that are resistant to various infectious agents that attack the chicken's body. The association of candidate gene diversity can be a solution and strategic step to increase chicken resistance to infection. Various studies have shown sufficient evidence of the association of genetic diversity with resistance to important diseases in chickens, such as marek's, laryngitracheitis, newcastle disease, avian leukosis, pullorum, infectious bursal disease, avian infectious bronchitis, salmonellosis, rous sarcoma, fowl typhoid, coccidiosis, and ascaris (Bishop and Woolliams 2014). Several research results show that the TLR4 gene is a candidate gene for resistance to S. Enteritidis bacterial infection in chicken (Kramer et al., 2003; Lamont et al., 2002).

The TLR4 gene position is on chromosome 17 in chickens. Based on data from GenBank (with accession number: A Y064697.1), the TLR4 gene is 11698 bp in size. The structure of the TLR4 gene begins with the promoter region (2743 bp), exons 1, 2, and 3 (105, 167, and 3260 bp), introns 1 and 2 (934 and 984 bp), and ends with the flanking region (3505 bp). The TLR4 gene plays a role in activating non-specific immune responses. The TLR4 gene transcribes a protein that functions as a cell surface receptor for phagocytes. This receptor protein can recognize lipopolysaccharide (LPS) from gram-negative bacteria, including Salmonella, so the TLR4

gene can be activated by LPS (Kim et al., 2023; Emertcan et al., 2011; Akira and Takeda, 2004; Palsson and O'Neill, 2004; Akashi et al., 2001).

The TLR4 receptor protein on the surface of phagocytic cells influences the activity of these cells in the phagocytosis process against Salmonella sp bacteria. which infects (Kogut et al., 2005). This will directly impact the non-specific immune response that is generated. Indirectly, the TLR4 gene also influences specific immune responses mediated by T lymphocytes and B lymphocytes, as stated by Kabelitz (2007), that the TLR4 gene can also be expressed in T lymphocytes. This TLR4 gene has strong expression not only in chickens. Jie et al. (2013) reported that the TLR4 gene can be expressed very strongly in ducks in controlling their resistance to Salmonella sp. The TLR4 gene also indirectly has a positive influence on controlling coccidiosis in chickens and other poultry caused by Eimeria sp. (Zuoyong et al., 2013).

In humans and mice, mutations in the TLR4 gene have an impact on reducing the individual's ability to recognize LPS from Salmonella sp bacteria. These individuals become susceptible and are easily infected with Salmonella. Mutations cause the TLR4 gene to form several genotypes. Abasht et al., (2009) stated that variations in the genotype of the TLR4 gene in chickens influence their resistance to Salmonella sp bacterial infection. This research was conducted to identify the polymorphism of the TLR4 gene in local Indonesian chicken breeds using the PCR-RFLP method. It is hoped that the results of this research will be able to find local Indonesian chicken genotypes that are resistant to infection based on blood profiles so that they can be developed for breeding programs and benefit the wider community.

MATERIAL AND METHOD

Experimental animals and DNA isolation

A total of 135 local Indonesian chickens were used to identify the diversity of the TLR4 gene, consisting of 50 KUB chickens, 35 Sensi chickens, and 50 broiler CP 707 chickens. Blood sample was taken from each animal using a venoject tube with 0.5 M K2EDTA, and it was preserved for a few weeks at -25°C. Genomic DNA was extracted using the Genomic DNA Mini Kit Geneid procedure (ISO 9001:2008 QMS).

PCR amplification and Genotyping

DNA amplification was carried out on a Nexus Mastercycler PCR machine with a total volume of 15 µl consisting of 1 µl DNA, 0.5 µl Primer, 6.25 µl MyTaq HS Red Mix 2x, and 7.25 µl Water (dH2O). The PCR program is as follows: predenaturation (95^{o}C for 5 minutes); followed by 35 cycles including denaturation (95^{o}C for 10 seconds), annealing (60^{o}C for 20 seconds), and elongation (72^{o}C for 30 seconds); and one final extension cycle (72°C for 5 minutes). Primer sequences for TLR4 gene amplification were GCTCAAATTATTTTCATCACC (forward/F) and ATCTG GACTAAAGCTGCAC (reverse/R). Each PCR product was digested with 5 µl of Msc1 10 U at 37°C overnight. The DNA fragmen was electrophoresis on agarose gel 2.5% and viewed using an AlphaImager.

Determination of Leukocyte Concentration and Differentiation

Determination of the number of leukocytes using the Sastradipraja et al. (1989). Using a leukocyte thoma pipette, a 20 µl blood sample was pipetted to the 0.5 mark and diluted to the 11 mark using Turk's solution, then homogenized for 15-30 seconds. The unshaken liquid is discarded. The sample is dropped into the Neubeur hemocytometer counting chamber. The number of leukocytes was counted under a microscope with 100 times magnification. Leukocytes were counted in four fields located in the four corners of the counting room. The number of leukocytes is then multiplied by 50 to find out the concentration per mm3.

Leukocyte differentiation was observed in smear preparations using the method of Sastradipraja et al. (1989). The smear preparations were fixed with methanol for 5-10 minutes and then removed to air dry. The blood was soaked in Giemsa dye for 30 minutes, removed, and washed with running tap water to remove excess dye. The smear preparations were observed with a microscope at 1000 times magnification with the help of immersion oil. The number of lymphocytes, heterophils, monocytes, eosinophils, and basophils was counted in a zigzag pattern until the number of leukocytes counted was 100.

Data analysis

Genotype and allele frequencies were analyzed using Popgene software version 1.31. The MEGA-X program

(ENSCHIG00000024611) was used to correct SNP positions according to Ensembl's DNA sequence. SAS 9.1.3 software (SAS Institute, Cary, NC, USA) was used to analyze the relationship between genotype and white blood cell profile. The linear model applied was:

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

Where, Yij is the trait observation of the ij animals; μ is the overall mean; Gi is the genotype effect of the i animal, and ε_{ij} is the random error.

RESULT AND DISCUSSION

Polymorphism of **TLR4** gene in Indonesian local chicken

A total of 135 chicken samples consisting of KUB chickens (50), Sensi chickens (35), and CP 707 broiler chickens (50) have been successfully amplified with a success rate of 100%. Amplification of the TLR4 gene fragment in chickens resulted in a PCR product length of 220 bp (figure 1).

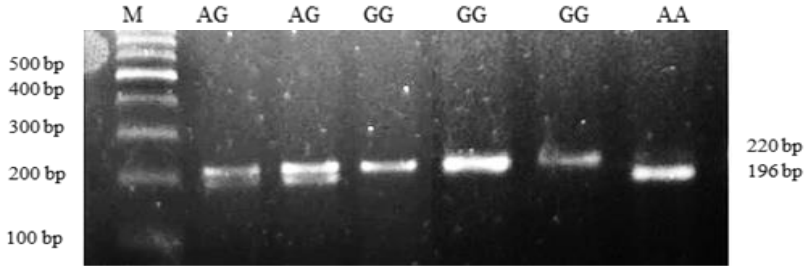

Figure 1. PCR-RFLP of the TLR4-Msc1 gene in KUB, Sensi, and Broiler CP 707 chickens. M = 100 bp DNA marker. The uncut allele is 220 bp (designated as G) and the cut alleles are 196 bp (designated as A) and 24 bp (not visible).

The TLR4 gene in chickens is on chromosome 17. Based on data obtained from GenBank (with accession number: A Y064697.1), the TLR4 gene is 11698 bp in size. The structure of the TLR4 gene begins with a promoter region measuring 2743 bp. The TLR4 gene has three exons measuring 105 bp, 167 bp, and 3260 bp and two introns of 934 bp and 984 bp. Genotyping of the TLR4 gene in local chickens was carried out on exon 2 (from bases 3898-4117), with a PCR product measuring 220 bp, as shown in Figure 2.

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3841 aaatcccaaa caccaccctg gacttggacc tcagtttcaa cagtctgaaa ttgctgagct
3901 caaattattt ttcatcagta cccgaactgc agtttctgga tctttcaagg taatgggcct
3961 ttttatgtgg ttcttggttt gtcttcacca tctgggcctc agcggttcct tattttggag
4021 tctcgtggct cttggctgtt tcctgtttca ctcttgcctc gatccctctg tctctctgca
4081 catgccatgg cagtgatgtg cagctttcag tccagatact gagtgcaaac tccgcagagc
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Figure 2. Amplified TLR4 gene sequence (GenBank, A Y064697.1). The primary sequence is the underlined nucleotides

Figure 2 is an amplified TLR4 gene fragment (220 bp in size) in exon 2. This fragment is then cut by the MscI restriction enzyme, at site 3924 with the TGG|CCA cut point position. Cutting by the MscI enzyme produces allele A (24 bp and 196 bp) and allele G (220 bp). AA, AG, and GG are the genotypes of the TLR4 gene from several identified local chicken breeds. From the results of this genotyping, a mutation was detected at site 3924. This mutation is a transition mutation where the base guanine changes to adenine (G --> A). This nucleotide change causes a change in the amino acid of the protein, namely glutamic acid (GAA) to lysine (AAA). Based on its effects, this mutation is a missense mutation, namely a mutation that causes changes in amino acids and can cause phenotypic changes. The findings of the present study are consistent with those published by Beaumont et al. (2003), who carried out genotyping on commercial brown laying hens.

Genotype and Allele Frequencies of TLR4 gene

The frequency of genotype and allele of the TLR4 gene in KUB chickens, Sensi chickens, and broiler CP 707 chickens are presented in Table 1. The genotype proportion of TLR4 gene in all chicken populations shows that the frequency of the GG genotype is the highest compared to the AG and AA genotypes. This causes the high frequency of the G allele in the entire Indonesian chicken population. The research of Tohidi et al. (2012) found that the frequency of the GG genotype of the TLR4 gene had a high frequency in local Malaysian and Red Jungle fowl chickens and the AA genotype was found to be low in both chickens.

Table 1. Genotype and Allele Frequencies of TLR4 gene in Indonesian local chicken

SNP Position	Chicken	N	Genotype Frequencies			Allele Frequencies	
			AA	AG	GG	A	G
g.3924 G>A	KUB	50	0,160	0,260	0,580	0,290	0,710
	Sensi	35	0,143	0,200	0,657	0,243	0,757
	Broiler CP 707	50	0,120	0,140	0,740	0,190	0,810
	Total	135					

N: number of samples, KUB: Kampung Unggul Balitbangtan, Sensi: Sentul terseleksi

The findings of this research revealed that the TLR4 gene was polymorphic with three genotype types and an allele frequency of more than 0.01 in each KUB, Sensi, and Broiler CP 707 chicken (Nei dan Kumar 2000; Allendorf et al., 2013:). This result was similar to those reported on the commercial brown laying hens (Beaumont et al., 2003) and on the kampung chicken population (Ulupi et.al., 2013). The frequency of the G allele is significantly higher than the A allele of the TLR4 gene in the entire chicken population. This is thought to be caused by the selection and mating management process resulting in the accumulation of certain alleles. Factors that influence gene frequency are selection, mutation, population mixing, internal crossing, external crossing, and genetic drift.

White blood cell profile and differentiation

The results of the descriptive analysis of leukocyte concentration and differentiation based on the TLR4 gene genotype are presented in Table 2. The leukocyte concentration values showed differences in the three genotypes of KUB, Sensi, and Broiler CV707 chickens, although not significant and still within the normal range. The highest leukocyte concentration values were found in the AG genotype of KUB (21.37±3.12), the AA genotype of Sensi (19.39±3.89), and the GG genotype of Broiler CV707 chickens (20.20±3.50). Leukocytes are cellular elements of the blood and part of the body's defense system that are capable of active movement. Based on their morphology, leukocytes are granular (granulocytes) and non-granulated (agranulocytes). Granulated leukocytes consist of heterophils, eosinophils, and basophils, while non-granulated leukocytes are monocytes and lymphocytes (Campbell and Ellis, 2012).

Table 2. Concentration and differentiations of leukocytes based on the genotype of TLR4 gene in Indonesian local chicken

Chicken Population	White blood cells	Genotype			Normal standard
		AA	AG	GG	
KUB	Leukocytes (10^3 mm^{-3})	14.26±4.77	**21.37±3.12**	16.68±2.61	13.00-32.00[1]
	Heterophils (%)	57.33±7.39	49.14±4.84	46.00±4.05	20.00-75.00[2]
	Lymphocyte (%)	39.66±7.41	48.28±4.85	51.20±4.05	20.00-65.00[2]
	Monocyte (%)	3.00±0.69	2.14±0.45	2.00±0.38	0.00-7.00[3]
	Rasio H/L	1.61±0.36	1.17±0.23	1.01±0.19	0.51-0.59[3]
Sensi	Leukocytes (10^3 mm^{-3})	**19.39±3.89**	17.46±3.55	17.51±2.90	13.00-32.00[1]
	Heterophils (%)	50.80±6.58	40.50±6.00	44.66±4.90	20.00-75.00[2]
	Lymphocyte (%)	46.60±6.45	57.00±5.89	51.77±4.80	20.00-65.00[2]
	Monocyte (%)	1.80±0.50	2.00±0.46	2.88±0.37	0.00-7.00[3]
	Rasio H/L	1.47±0.32	0.81±0.29	0.96±0.24	0.51-0.59 [3]
Broiler CP 707	Leukocytes (10^3 mm^{-3})	17.82±3.24	16.17±3.24	**20.20±3.50**	13.00-32.00[1]

Heterophils (%)	43.71±5.40	40.00±5.40	52.16±5.83	20.00-75.00[2]
Lymphocyte (%)	52.42±5.33	57.28±5.33	45.50±5.76	20.00-65.00[2]
Monocyte (%)	3.00±0.41	2.28±0.41	1.66±0.45	0.00-7.00[3]
Rasio H/L	0.94±0.56	0.78±0.42	1.47±1.06	0.51-0.59[3]

n: number of samples; H/L: Heterophils/ Lymphocyte; [1]Lokapirnasari *et al.* (2016), [2]Coles (2006), [3]Pollack *et al.* (2005).

The heterophil concentration values showed non-significant differences, but still within the normal range in the three genotypes of KUB, Sensi, and CP 707 broiler chickens. The highest heterophils concentration values were found in the AA genotype of KUB (57.33±7.39**)**, the AA genotype of Sensi (50.80±6.58**)**, and the GG genotype of Broiler CV707 chickens (52.16±5.83**)**. Heterophils are phagocytic cells and belong to the innate immune system in birds, which are produced in the bone marrow (Stabler et al., 1994: Tamiru et al., 2019). Heterophils have the same function as neutrophils in mammals (Campbell and Ellis, 2013; Ruhs et al., 2020), playing a role in acute inflammation, controlling bacterial, viral, and parasitic infections by phagocytosing and killing bacteria (Mortaz et al., 2018). Heterophil levels in the blood are influenced by: infections (bacteria, fungi, viruses, and parasites), inflammation, stress, certain toxicities, trauma, and leukemia. Heterophils have a short life span, where after destroying the disease agent they will die and release chemotactic factors to attract other Heterophils. The normal life span in blood circulation reaches 4-8 hours, in tissue, it reaches 4-5 hours (Tizard 1988).

Lymphocytes are a crucial component of the immune system, which produces antibodies in response to antigens. Lymphocytes consist of B cells and T cells, which are immune cells that play a pivotal role in specific immune responses and mediate the formation of humoral immunity and cellular immunity (Yalcinkaya et al., 2008). In this study, the number of lymphocytes was in the normal range which indicates that KUB, Sensi, and Broiler CV707 chickens have cellular and humoral immune responses. There were differences in lymphocyte concentrations in the three genotypes of KUB, Sensi, and Broiler CV707 chickens, although these differences were not significantly different.

Monocytes are the second line of defense after Heterophils which have phagocytic activity. When inflammation occurs Monocytes migrate to the tissue and turn into macrophages. These macrophages are potential phagocytic cells because their size is larger, their lifespan is longer, and their ability to swallow more bacteria than Heterophils (Kumar et al., 2018). According to Guyton and Hall (2006), macrophages are able to phagocytose 100 bacterial cells. The normal monocytes of KUB, Sensi, and CP 707 broiler chickens in this study indicate that there was no acute infection in these chickens. Similar to heterophils and lymphocytes, the monocyte concentration values showed non-significant differences in the three genotypes of TLR4 gene, and still within the normal range.

The ratio of Heterophils to Lymphocytes (H/L) is the main indicator of stress in poultry. The H/L ratio values of KUB, Sensi, and Broiler CP 707 chickens were outside the normal range, namely 1.01-1.61, 0.81-1.47, and 0.78-1.47 respectively. This shows the experimental chickens are under stress conditions. According to Pollack et al. (2005), the range of H/L ratios in poultry is around 0.51-0.59. The main cause of stress is high environmental temperature. High environmental temperatures will affect livestock behavior and the function of several body organs, such as the heart and respiratory organs, and indirectly influence the increase in the hormones corticosterone and cortisol, decrease the hormones adrenaline and thyroxine in the blood (Sohail et al. 2010), and increase rectal temperature (Tamzil et al. 2013).

Stress in livestock causes the neurogenic system to be directly activated (Virden and Kidd 2009), which is represented by an increase in blood pressure, muscle tone, nerve sensitivity, blood sugar, and respiration during the alarm phase. If these efforts fail to deal with stress, the hypothalamus pituitary-adrenal cortical system will be activated by the body and the hypothalamus immediately produces corticotropin-releasing factor (CRF), which in turn stimulates the pituitary to release adrenocorticotropin hormone (ACTH). When this system is activated, the hypothalamus produces corticotrophin-releasing factor (CRF), which in turn stimulates the pituitary to release adrenocorticotropin hormone (ACTH). ACTH secretion causes adrenal cortex tissue cells to proliferate and release corticosteroids. High

levels of corticosteroid hormones in the blood can inhibit lymphocyte formation and can reduce body immunity (Davis et al. 2008). A decrease in lymphocyte levels in the blood causes the percentage of heterophils to increase, so that the H/L percentage ratio value also increases.

CONCLUSIONS

The TLR4 gene in all chicken populations studied is polymorphic and can be used as a genetic marker. The white blood cell concentration values showed non-significant differences, but still within the normal range in the three TLR4 gene genotypes of KUB, Sensi, and CP 707 broiler chickens. The number of lymphocytes is in the normal range, indicating that KUB, Sensi, and Broiler CV707 chickens have cellular and humoral immune responses. Meanwhile, the heterophils and monocyte values indicate that the chickens are not infected by bacteria, viruses, and other parasites or are not in acute inflammation. The H/L ratio of KUB, Sensi, and CP 707 broiler chickens for the TLR4 gene has a value above normal, this indicates that the chickens studied were under stress.

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AUTHOR'S CONTRIBUTION

All authors contributed equally.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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