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Research Article
Association of the Toll-like Receptor 4 (TLR4) and Myxovirus (Mx) Genes With Resistance to *Salmonella* and Newcastle Disease in Selected Sentul Chickens

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Abstract

Background and Objective: Toll-like receptor 4 (TLR4) and myxovirus (Mx) genes have been demonstrated to confer resistance to *Salmonella* and Newcastle disease (ND), respectively. These genes have not yet been investigated in Sentul chickens. Therefore, the aim of this study was to determine the polymorphisms and association of the TLR4 and Mx genes as candidate genes underlying resistance to *Salmonella* and ND in selected Sentul chickens. **Methodology:** One hundred and eighty-five Sentul chickens were genotyped using PCR-RFLP. The genotype and allele frequencies, polymorphic information contents and *Hardy-weinberg* equilibrium (HWE) statuses were analysed. The genotypes were associated with immunoglobulin Y (IgY), *Salmonella*-specific antibodies and Newcastle disease-specific antibodies. These parameters were determined using indirect ELISA, clearance tests and haemagglutination inhibition, respectively. The immune traits were further grouped into high, medium and low categories. The data were analysed using the GLM and t-test. **Results:** Polymorphisms in TLR4|MscI and Mx|Hpy8I were observed. The population was in HWE for both genes. The frequencies of the TLR4 allele G and genotype GG were significantly high ($p<0.05$) in chickens with high immune traits. The genotype GG of TLR4 gene recorded significantly higher immune traits than genotypes AG and AA. For the Mx gene, the frequency of allele A was higher than of allele G at a high IgY titre. **Conclusion:** TLR4|MscI and Mx|Hpy8I are potential markers for selection against *Salmonella* and Newcastle disease in Sentul chickens. However, further investigations are recommended.

Key words: Disease resistance, Mx gene, Newcastle disease, polymorphism, *Salmonella*, sentul chickens, TLR4 gene

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

There are approximately 30 distinct native chicken breeds in Indonesia and studies have reported that amongst them, Sentul chickens have considerable potential to be selected as a dual-purpose breed. Furthermore, the raw eggs are used to prepare the herbal drink 'Jamu'. However, the breed is at risk of *Salmonella* infection, which can contaminate the poultry products. In addition, significant losses are reported in the population due to Newcastle disease infection[1]. Concerted effort is therefore needed to alleviate these diseases in the population.

Salmonella is a zoonotic disease that has been reported to be responsible for most food-borne disease outbreaks worldwide. The disease is caused by gram-negative *enterobacteria* (S. *enterica*) of different serotypes, which reside in the chicken internal tracts. The most common serotypes leading to human illness are *S. enteritidis* and *S. typhimurium*. These serotypes can infect chickens without causing any visible symptoms. Other serotypes, such as *S. gallinarum* and *S. polluram*, are host-specific and infect a single species. These serotypes can cause severe typhoid-like symptoms, which can lead to death. Consequently, they lead to disease outbreaks and severe economic losses in the poultry industry[2,3]. On the other hand, Newcastle disease (ND) is a viral disease caused by a virulent strain of Newcastle disease virus (NDV) known as Avian paramyxovirus serotype-1 (AMPV-1). It is the most common disease that strikes the poultry industry and is very contagious. Severe neurological and respiratory signs as well as a reduction in egg quality and production are attributes of infected birds[4].

Prophylactic measures, vaccination and the use of antibiotics are insufficient to eradicate *Salmonella* and ND in poultry stocks. Furthermore, usage of the listed measures is criticized due to the development of antibiotic-resistant bacteria and antibiotic residues in food products[3]. In this context, Lamont *et al.*[5] recommended selection of more resistant chickens using the candidate gene approach. This approach is a very effective and applicable method in complex traits. Candidate genes that have been previously identified as being associated with resistance to *Salmonella* and ND in chickens are the Toll-like receptor 4 (TLR4) gene and myxovirus (Mx) gene, respectively.

Toll-like receptor 4 belongs to a family of innate immune receptors (Toll-like receptors) and recognizes lipopolysaccharide (LPS) on the surface of gram-negative bacteria. For effective LPS recognition, TLR4 requires an additional molecule, MD-2. LPS interacts with CD14 on the macrophage surface to trigger the formation of the TLR4/MD-2 complex. This pathway leads to the transcription of immune response genes against *Salmonella*[6]. Myxovirus proteins are antiviral GTPase enzymes that are induced by interferon (IFN). Mx proteins inhibit viral replication by blocking the initiation of virus transcription by blocking the RNA primer and mRNA synthesis[7,8]. The variation in the antiviral properties of the Mx gene is determined by an amino acid substitution at position 631. Asparagine (Asn) corresponds to the positive antiviral property and serine (Ser) corresponds to the negative antiviral property[1].

Polymorphic mutations of the TLR4 gene have been investigated in different breeds and several SNPs associated with resistance to *Salmonella* have been identified[6,9-13]. The Mx gene has been mostly studied in several experimental populations. Polymorphism was reported to be associated with antiviral activity in Tolaki chickens[14], several Asian local chickens[15] and Kenyan indigenous breeds[16].

Sentul chickens are among the Indonesian breeds for which little to no information regarding the candidacy of the TLR4 and Mx genes is available. Ulupi *et al.*[17] reported that the TLR4 gene was polymorphic in Sentul chickens but the information provided by the study was not sufficient for understanding the function of the TLR4 gene as a marker in the population. On the other hand, no studies on the Mx gene in Sentul chickens have been conducted. Therefore, this study aimed to identify polymorphisms and associations of the TLR4 and Mx genes with resistance to *Salmonella* infection and Newcastle disease in selected Sentul chickens. The study provides additional information important for selecting *Salmonella* and Newcastle-resistant strains whilst using the TLR4 and Mx genes as markers.

MATERIALS AND METHODS

This study was conducted from July 2017 to January 2018 at the Animal Breeding and Genetics Laboratory, Faculty of Animal Science and Physiology, Medical Microbiology Laboratory, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor.

TLR4 and Mx genotyping: In this study, 185 blood samples were collected from the brachial veins of unvaccinated and unchallenged Sentul chickens of 6 months of age. The TLR4 and Mx genes were genotyped using four methods: DNA extraction, DNA amplification, PCR-restriction fragment length polymorphism (PCR-RFLP) analysis and DNA fragment visualization[18].

The SNPs and primers for TLR4 and Mx were used according to Ulupi *et al.*[17] and Pagala *et al.*[19], respectively. The primers for the TLR4 gene (F: 5′-CTCAAATTATTTTTCAT CAGTGGCC-3′, R: 5′-ATCTGGACTAAAGCTGCAC-3′) and Mx gene (F: 5′-GCACTGTCACCTCTTAATAGA-3′, R: 5′-GTATTGG TAGGCTTTGTTGA-3′) were annealed at 61°C for 1 min and 60°C for 20 sec, respectively. The SNP at position 3924 bp (TGG|CCA) on chromosome 17 (exon 2) was differentiated into different allelic transcripts of the TLR4 gene using the MscI enzyme, whilst the SNP at position 2032 bp (GTN|NAC) of chromosome 1 (exon 13) was differentiated into allelic transcripts of the Mx gene using the Hpy8I enzyme. The respective GenBank accession numbers for the TLR4 and Mx genes are AY064697.1 and DQ788615. The PCR-RFLP products were visualized using agarose gel electrophoresis at concentrations of 2.0% for the TLR4 gene and 2.5% for the Mx gene.

Biological assays: Biological assays were used to analyses the resistance factors in chickens. Concentrations of immunoglobulin Y (IgY), *Salmonella*-specific antibodies (*Salmonella* antibodies) and ND-specific antibodies (HI antibodies) were measured using the respective assays indirect ELISA[20], clearance test[21] and haemagglutination inhibition[22].

Data analysis: The allelic and genotypic frequencies, Hardy-Weinberg equilibrium values, polymorphic information contents (PICs) and observed and expected heterozygosities of the genotypes obtained via the PCR-RFLP method were calculated[23]. The association was analysed using the General Linear Model (PROC GLM) (SAS Institute Inc., Cary, USA) and descriptive analysis with individual and categorized immune traits. The categories were determined using the descriptive statistics. Significant differences were evaluated using the t-test.

The association model used was as follows:

$$Y_{ijk} = \mu + G_i + S_j + \varepsilon_{ijk}$$

Where

Y_{ij} = Observations of immune traits,
μ = Population mean
G_i = Treatment effect of the single-nucleotide polymorphism genotype
S_j = Gender effect
ε_{ij} = Residual error

The differences in the genotypes of each gene were compared using Tukey's test at the 5% level. To determine the level of significance, the t-test was used, where $p<0.05$ indicated statistically significant differences[21]. The immune parameters were further categorized into 3 groups of low, average and high titres. The criteria used for the titre ranges for the respective groups of IgY were <5, $5 \leq \times \leq 8$ and >815 μg mL^{-1}, whereas the *Salmonella* antibody titres (Log10) were <4, $4 \leq \times \leq 6$ and >6 CFU ml^{-1} and the HI antibody titres (Log2) were <3, $3 \leq \times \leq 5$ and >5 CFU mL^{-1} (Schade and Hlinak[24], Ulupi *et al.*[13]). The genotype and allele frequencies of the categorized immune traits for TLR4 and Mx genes were calculated according to the method described by Nei and Kumar[23]. The genotypes of the categories of each gene were compared and t-test was used to determine significant difference ($p<0.05$)[21].

RESULTS

Phenotype of Immune traits: The descriptive statistics for the immune trait data for the TLR4 and Mx genes are shown in Table 1. The parameters that were assayed were IgY, *Salmonella* antibody and HI antibody titres. The average titres of the assayed IgY, *Salmonella* antibody and HI antibody were 7.89 ± 3.96 μg mL^{-1}, $3.5 \times 10^9 \pm 7.4 \times 10^9$ CFU mL^{-1} and 3.11 ± 2.19 μg mL^{-1}, respectively (Table 1).

Genotype and allele frequency analysis of the TLR4 and Mx genes: TLR4|MscI and Mx|Hpy8I were genotyped using 220 and 299 bp PCR products, respectively. The results are presented in Fig. 1 and 2, respectively. The distributions of the genotype and allele frequencies are shown in Table 2. The restriction fragments of the TLR4 gene resulted in allele A (196 and 24 bp) and allele G (220 bp). The digestion of the Mx gene PCR product resulted in allele A (299 bp) and allele G (200 and 99 bp). The distributions of genotype and allele frequencies are as follows: TLR4|MscI (AA = 0.011, AG = 0.319, GG = 0.670 and A = 0.170, G = 0.830) and Mx|Hpy8I (AA = 0.342, AG = 0.500, GG = 0.158 and A = 0.592 and G = 0.408).

Heterozygosity, polymorphic information content and hardy-weinberg equilibrium: Analyses of Ho, He, PIC and χ^2 were used to determine the genetic variabilities of specific genes in a population. The results of these analyses are presented in Table 2. Based on the results (Table 2), the respective values of H_0, He and PIC recorded for the TLR4 were 0.319, 0.283, 0.243 and those for the and Mx gene were

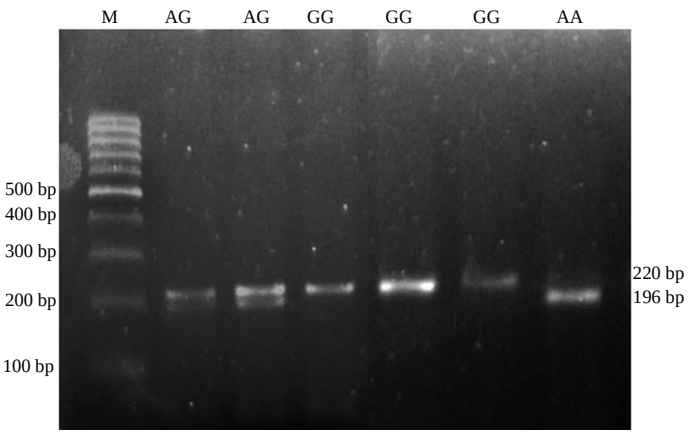

Fig. 1: RFLP products for TLR4|MscI, 2.5% agarose gel

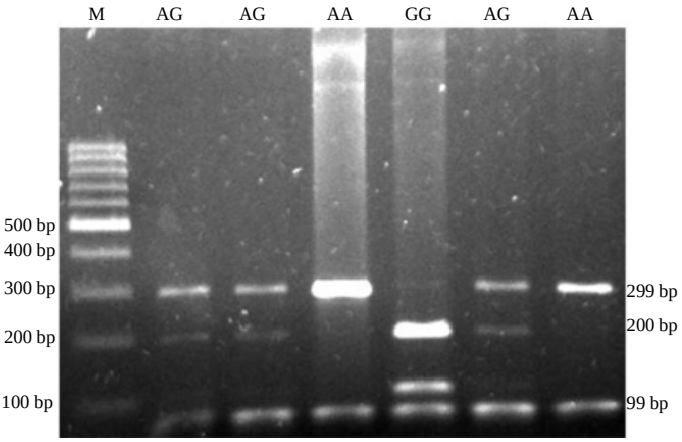

Fig. 2: RFLP products for Mx|Hpy8I, 2.0% agarose gel

Table 1: Descriptive statistics of the immune traits in all the investigated Sentul chickens

Variable	Mean	Standard deviation	Minimum	Maximum
IgY titre (μg mL^{-1})	7.89	3.96	0.96	14.95
Salmonella antibody titre (CFU mL^{-1})	3.5×10^{9}	7.4×10^{9}	4.4×10^{3}	3.7×10^{10}
HI antibody titre (Log_2 μg mL^{-1})	3.11	2.19	0	8

Table 2: Genotype and allele frequencies of TLR4|MscI and Mx|Hpy8I in selected Sentul chickens

		Genotype frequency			Allele frequency	
Gene	N	AA	AG	GG	A	G
TLR4	185	0.011 (2)	0.319 (59)	0.670 (124)	0.170	0.830
Mx	184	0.342 (63)	0.500 (92)	0.158 (29)	0.592	0.408

N: Total number of samples

0.500, 0.483 and 0.366. The calculated Chi-square values for the TLR4 and Mx genes (3.064 and 0.230, respectively) were significantly smaller than the critical value (3.84).

Association of TLR4|MscI and Mx|Hpy8I with individual immune traits in selected Sentul chickens: The association analyses of the TLR4 and Mx genes with individual immune traits in selected Sentul chickens are presented in Table 4. The immune traits presented in Table 4 were not significantly different between any of the genotypes (GG and AG) of the TLR4 gene. The association of Mx|Hpy8I with individual immune traits (Table 4) also showed no significant differences between any of the genotypes (AA, AG and GG) for both immune traits, i.e., IgY and HI antibody titres.

Association of TLR4/MscI and Mx/Hpy8I with categorized immune traits in selected Sentul chickens: The association results of the genotype and allele frequencies of TLR4|MscI and Mx|Hpy8I with high, medium and low antibody titres are shown in Table 5, 7, 9 and 11. The association of the categorized immune traits of TLR4|MscI and Mx|Hpy8I are shown in Table 6, 8, 10 and 12. The GG genotype and allele G frequencies were significantly high among the high immune traits, i.e., IgY titres (0.70 and 0.85) (Table 5) and *Salmonella* antibody titres (0.68 and 0.83) (Table 7). However, there was no significant difference between the observed and expected frequencies of the genotypes for low and medium immune

Table 3: Heterozygosity and polymorphic information content of TLR4|MscI and Mx|Hpy8I in selected Sentul chickens

Gene	χ^2	He	Ho	PIC
TLR4	3.064ns	0.283	0.319	0.243
Mx	0.230ns	0.483	0.500	0.366

ns: Not significant, χ^2 count<χ^2 table, 0.05, 1=3.84

Table 4: Association of the TLR4|MscI and Mx|Hpy8I genotypes with individual immune traits

	Genotype			
Gene	Immune traits	AA (2)	AG (48)	GG (101)
TLR4	IgY titre (μg mL^{-1})	7.97±8.42	7.25±4.271	8.28±3.877
	S. antibody titre (CFU mL^{-1})	$1.7\times10^9\pm1.6\times10^9$	$4.4\times10^9\pm1.4\times10^9$	$3.1\times10^9\pm6.5\times10^8$
		AA (53)	AG (82)	GG (27)
Mx	IgY titre (μg mL^{-1})	7.82±4.062	8.13±3.926	7.31±3.964
	HI antibody titre (log_2 μg mL^{-1})	2.91±1.730	3.02±2.304	3.7±2.599

IgY titre: Immunoglobulin Y titre, *Salmonella* antibody titre: *Salmonella*-specific antibody titre, HI antibody titre: Haemagglutinin inhibition antibody titre (Newcastle disease-specific)

Table 5: Genotype and allele frequency of TLR4|MscI in the IgY titre category

		Genotype			Genotype frequency			Allele frequency	
IgY titre	Total No.	AA	AG	GG	AA	AG	GG	A	G
Low	40	1	14	25	0.03	0.35	0.62	0.20	0.80
Medium	13	0	3	10	0.00	0.23	0.77	0.12	0.88
High	100	1	29	70	0.01	0.29	0.70	0.15	0.85
Total	153	2	46	105					

Table 6: Association of the TLR4|MscI with categorized IgY titre

	TLR4 genotype		
IgY titre (μg mL^{-1})	AA	AG	GG
Low	0.960±0.859^a	1.271±0.302^a	2.923±0.172^b
Medium	-	5.560±0.247^a	6.724±0.135^b
High	7.590±1.493^a	8.783±0.277^a	11.430±0.180^b

The different superscripts within the same row show significant (p<0.05) difference, -shows that there are no samples

Table 7: Genotype and allele frequency of TLR4|MscI in the *Salmonella* antibody titre category

		Genotype			Genotype frequency			Allele frequency	
Salmonella antibody titre	Total No.	AA	AG	GG	AA	AG	GG	A	G
Low	2	0	2	0	0.00	1.00	0.00	0.50	0.50
Medium	32	0	11	21	0.00	0.34	0.66	0.17	0.83
High	117	2	35	80	0.02	0.30	0.68	0.17	0.83
Total	151	2	48	101					

Table 8: Association of the TLR4|*MscI* with categorized S. antibody titre

	TLR4 genotype		
S. antibody titre (CFU mL^{-1})	AA	AG	GG
Low	-	$5.9\times10^3\pm2.0\times10^3$	-
Medium	-	$1.1\times10^5\pm6.6\times10^{5a}$	$4.2\times10^6\pm4.8\times10^{5b}$
High	$1.0\times10^7\pm5.3\times10^9$	$5.0\times10^7\pm1.3\times10^9$	$6.3\times10^9\pm8.5\times10^8$

The different superscripts within the same row show significant (p<0.05) difference, -shows that there are no samples

Table 9: Genotype and allele frequency of Mx|Hpy8I in the IgY titre category

		Genotype			Genotype frequency			Allele frequency	
IgY titre	Total No.	AA	AG	GG	AA	AG	GG	A	G
Low	44	15	21	8	0.34	0.48	0.18	0.58	0.42
Medium	15	4	7	4	0.27	0.46	0.27	0.50	0.50
High	103	34	54	15	0.33	0.52	0.15	0.59	0.41
Total	162	53	82	27					

Table 10: Association of the Mx|Hpy8I with categorized IgY titre

	Mx genotype		
IgY titre (CFU mL^{-1})	AA	AG	GG
Low	1.215 ± 0.129^{a}	2.392 ± 0.109^{b}	4.289 ± 0.177^{c}
Medium	5.458 ± 0.171^{a}	6.383 ± 0.129^{b}	7.090 ± 0.171^{c}
High	8.703 ± 0.154^{a}	10.646 ± 0.122^{b}	13.975 ± 0.231^{c}

The different superscripts within the same row show significant ($p<0.05$) difference, -shows that there are no samples

Table 11: Genotype and allele frequency of Mx|Hpy8I in the HI antibody titre category

		Genotype			Genotype frequency			Allele frequency	
HI antibody titre	Total No.	AA	AG	GG	AA	AG	GG	A	G
Low	83	28	43	12	0.34	0.52	0.14	0.60	0.40
Medium	54	20	25	9	0.37	0.46	0.17	0.60	0.40
High	25	5	14	6	0.20	0.56	0.24	0.48	0.52
Total	162	53	82	27					

Table 12: Association of the Mx/Hpy8I with categorized HI titre

	Mx genotype		
HI titre (Log2 CFU mL^{-1})	AA	AG	GG
Low	0.679 ± 0.092^{a}	1.698 ± 0.074^{b}	1.833 ± 0.140^{b}
Medium	3.000 ± 0.110^{a}	4.320 ± 0.098^{b}	5.111 ± 0.164^{c}
High	6.000 ± 0.285^{a}	6.929 ± 0.170^{b}	8.000 ± 0.260^{c}

The different superscripts within the same row show significant ($p<0.05$) difference, -shows that there are no samples

traits for the TLR4 gene. Furthermore, a high frequency of Mx gene allele A was determined in the high IgY titre group, i.e., 0.59 (Table 9). Other frequencies did not show associations with the categorized immune traits (Table 11). The association of the TLR4 gene with IgY titre showed significantly higher IgY concentrations in genotype GG than genotypes AG and AA in medium and high categories (Table 6). The medium category of S. antibody titre had significantly higher concentration of S. antibody in genotype GG than genotypes AG and AA (Table 8). The concentrations of IgY and S. antibody that had genotypes AA and AG of TLR4 gene did not show statistical difference in all the categories (Table 6 and 8). The association of Mx gene with IgY and HI titres showed statistical differences between AA, AG and GG genotypes where genotype AA had the lowest concentrations and genotype GG had the highest concentrations of the antibodies (Table 10 and 12).

DISCUSSION

The phenotypes of the Sentul chickens that were recorded in the current study illustrated high IgY and *Salmonella* antibody concentrations/titres, which was similar to the results reported by Ulupi *et al.*[12,13] for Kampung chickens. A relatively low average HI antibody concentration was observed in this study. These results were in contrast with the concentrations that were reported by Pagala *et al.*[14] for ND virus-challenged Tolaki chickens. The differences were due to the unchallenged population that was used in the present study. Challenging the chickens with virus induces the production of antibodies against the virus[14]. Therefore, the results obtained in this study suggest a very high immune response against Salmonella infection and a relatively low immune response against ND in unvaccinated and unchallenged Sentul chickens. The respective categorizations of the antibodies into low, moderate and high titre groups were as follows: 0-5, 5-8 and 8-15 μg mL^{-1} for IgY, <4, 4-6 and >6 (Log10) CFU mL^{-1} for the *Salmonella* antibody and <3, 3-5, >5 (Log2) μg mL^{-1} for the HI antibody[12,24].

In the polymorphism study, the fragments of the TLR4 and Mx genes were amplified using PCR. This study concluded that both genes were polymorphic because both alleles of each gene had frequencies > 0.01[23]. The genotype frequencies of the TLR4 gene reported in the present study were

inconsistent with the results reported for other Indonesian chickens[17]. The frequency of resistant allele A of the Mx gene reported in the current study was similar to that reported in a study conducted by Sulandari *et al.*[25] These researchers reported a range from 0.35-0.89 for allele A, which was resistant to AI/ND in an Indonesian local chicken population. However, previous studies conducted by Maeda[15] in local populations in ASEAN countries and Pagala *et al.*[14] in Tolaki chickens reported higher frequencies of the resistant allele and genotype. These authors reported frequencies of 63% for resistant allele A, 37% for allele G,61.17% for genotype AA and 0.74% for allele A[14,15]. The lower frequency of the resistant allele reported here was probably due to the selection for growth in the studied population[14]. In the present study, the results indicated genetic variation of both genes because Ho values were more than 0.25. However, the PIC values showed low (PIC<0.25) polymorphism in the TLR4 gene, whereas moderate (0.25<PIC>0.50) polymorphism in the Mx gene was reported[25]. These results are in accordance with data from Ulupi *et al.*[12], Okumu *et al.*[16,] Sambrook and Russell[18] and Ulupi *et al.*[13], Schade and Hlinak[24]. Furthermore, the calculated Chi-square (X^2) values for the TLR4 and Mx genes were significantly smaller than the critical value (3.84). The present study, therefore, concluded that the sampled population was in Hardy-Weinberg equilibrium ($p<0.05$) for both genes[23,26]. This conclusion implies that the impact of selection in the population was low and did not have a significant effect on the distribution of the TLR4 and Mx genotypes.

The association of TLR4 genotypes with individual traits showed no significant difference among the 3 genotypes (GG, AG and AA). This observation was in agreement with the results reported by Ulupi *et al.*[12,13]. The authors demonstrated that none of the parameters were significantly different between the AG and GG genotypes. The parameters included leucocyte concentration, percentage of leucocyte differentiation, phagocytic activity and capacity of macrophages and concentration of IgY specific to *S. enteritidis* in egg yolk in Kampung chicken. The insignificant difference among the genotypes of high immune traits of the 3 genotypes obtained in this study may imply that all genotypes in the population are resistant to *Salmonella* infection[12,13]. Therefore, the results showed that TLR4|MscI can prove resistance against *Salmonella* infection in the population. It is widely recognized that Toll-like receptors (TLRs) are part of a family of genes whose proteins are the main sensors used by the innate immune system to detect invading pathogens. The predicted structure of TLR4 is identical to that of all TLR genes and is thus significant for the differential resistance or susceptibility of chickens to *Salmonella* infection[11]. Alternatively, the results obtained in this study could also be specific to the *In vitro* model that was used. Previous studies reported that allelic variation in the TLR4 gene is linked to *Salmonella* infection in chickens[2,3,6,9,11,27-28]. In addition, Keestra and van Putten[29] did not observe the TLR4 gene polymorphism in vitro. Thus, it is likely that factors other than LPS, such as alternative TLR4 agonists, may play roles *In vivo*[11]. In addition, according to Leveque *et al.*[9], the bioassays used in the present study (indirect ELISA and clearance test) may not be the most appropriate for revealing the innate response to LPS. Yang *et al.*[30] measured the concentrations of the proinflammatory cytokines IL-1b, IL-6 and TNFa and the chemokine KC 6 and suggested measures of the production of superoxide, nitric oxide, phagocytosis, apoptosis and survival. The listed parameters are the final outcomes of the TLR4 gene pathway. Calenge and Beaumont[2] also indicated that the effect of TLR4 was studied in several independent studies, which failed to identify the major and stable effects of this gene. These inconsistent results for different SNPs of the TLR4 gene seem to indicate the unique roles of the different loci in resistance to *Salmonella* infection[11]. Therefore, the TLR4 SNP investigated in this study requires further studies to prove its association with *Salmonella* infection in the breed. The association of Mx genotypes with individual immune traits showed no significant difference among the 3 genotypes analysed (AA, AG and GG). These results were in contrast with those of previous studies that were conducted in challenged flocks. This discrepancy implies that the production of antibodies specific to ND in chickens is triggered by the invasion of the ND virus enteritidis. The exposure induces an increase in Mx gene activity[14]. Pagala *et al.*[14] reported resistance to viral attacks in Tolaki chickens with allele A, thus elucidating that this allele possesses the resistance aspect to ND viral attacks and the G allele is associated with ND susceptibility. Sartika *et al.*[1] established that allele A is highly dominant in resistance to viral diseases. Maeda[15] also conducted a similar study in local Indonesian chickens and reported a 63% resistance to infection in chickens with the resistant allele A. Several studies in Native Kenyan chickens also reported the resistance of allele A of the Mx gene to viral diseases[16,31,32]. The G/A polymorphism at position 2032 of chicken Mx cDNA, resulting in the substitution of serine with asparagine at position 631 of the Mx protein, seems to influence its antiviral activity[9].

The TLR4 genotype GG and the frequency of allele G were significantly associated with IgY and *Salmonella* antibody titres categorized as high and the association of the observed frequency of allele A with high IgY and *Salmonella* antibody titres was significantly lower. Furthermore, the highest IgY and HI titres were associated with genotype GG. These results imply that allele G of the TLR4 gene is associated with resistance to *Salmonella* infection and that allele A is associated with susceptibility to *Salmonella* infection. This postulation is consistent with the data from Ulupi *et al.*[12,13] reporting that the resistance aspect was associated with allele G of the TLR4 gene in Kampung chickens. High antibody titres were attributed to the presence of the resistant allele (G). The frequency of allele A of the Mx gene was associated with a high IgY titre. This result was in accordance with those of previous studies reporting that allele A of the Mx gene was resistant to viral attacks in avians[16,31,32]. However, there was no significant association between the dominant and recessive alleles and the HI antibody titre. Furthermore, the genotype GG of Mx gene recorded the highest antibody concentrations and genotype AA recorded the lowest antibody concentrations in all the categories. This observation was as a result of the unchallenged flock. The immune response is activated by virus invasion, which was not the case in the present study[14]. These results were similar to results of the association of the Mx gene with individual immune traits reported in the present study, indicating that no significant difference existed among the immune traits of all 3 genotypes. This was also reported to be an attribute of the unchallenged flock that was investigated in the present study[14].

This study showed that the TLR4 and Mx genes can be used to select Sentul chickens that are resistant to *Salmonella* and Newcastle disease. It also suggests that further studies should be conducted in challenged chickens and other different phenotypes should be assayed to provide more information about the TLR4|MscI gene in Sentul chickens. The Mx|Hpy8I locus should also be investigated in challenged and selected vaccinated Sentul chickens to provide further information on the gene as a marker in the population.

CONCLUSION

TLR4|MscI and Mx|Hpy8I are polymorphic. Association analysis revealed that TLR4 genotypes with the allele G were related to high IgY and *Salmonella* antibody titres. This study demonstrated that allele A of the Mx gene is associated with a high IgY titre and has the potential to be used to select Newcastle disease-resistant Sentul chickens. The present findings will improve and enrich current understanding of the role of the avian TLR4 and Mx genes in the resistance of selected Sentul chickens to *Salmonella* infection and Newcastle disease.

SIGNIFICANCE STATEMENT

This study discovers the associations of TLR4 and Mx polymorphisms and immune traits in Sentul chickens that can be beneficial for the selection of birds with high immunity against *Salmonella* and Newcastle disease using the TLR4 and Mx genes. The results of the current study will assist researchers in uncovering the potential of the TLR4 and Mx genes as selection markers for high immunity traits in Sentul chickens.

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