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# Study of Kampung Chicken Resistance Against *Salmonella enteritidis* Using TLR4 Gene as Marker

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**Abstract:** Kampung chicken eggs play an important role as substance in 'jamu preparation' in Indonesia, mostly provided and consumed without cooking. *Salmonella* free eggs become significant in producing the safe 'jamu preparation' and such eggs might be produced by chickens which have high resistance to this bacteria. One of excellent markers showing resistance of chicken against *Salmonella* is an active Toll-like Receptor 4 (TLR4) gene. TLR4 is a phagocytes cell surface receptor that plays a role to recognize lipopolysaccharide of gram negative bacteria including *Salmonella enteritidis.* It is transcribed by TLR4 gene and conserved in the activation of the non-specific immune system. The aim of the research is to prove resistance of Kampung chicken when was challenged with *S. enteritidis*, using TLR4 gene as marker. TLR4 gene was genotyped in 50 Kampung chickens with PCR-RFLP. Then all the Kampung chickens were challenged with *S. enteritidis* ($ID_{50}$:5x$10^5$ CFU/mL). Their expression on resistance against *S. enteritidis* as well as biological and molecular assays were measured. The genotyping result identified 3 genotypes of TLR4 gene: AA, AG and GG. All parameters including expression of TLR4 gene, concentration of leucocytes, differentiation of leucocytes, macrophages activity and capacity were not significantly different in AG and GG genotypes. There was no *S. enteritidis* finding in blood and eggs produced by AA, AG and GG chickens. There was found IgY specific to *S. enteritidis* in eggs yolk with very high concentration (2.94-3.89 mg/mL). The study proved that Kampung chicken resistant to *S. enteritidis* infection in all condition.

**Key words:** Kampung chicken, TLR4 gene, genotyping, non-specific immune

## INTRODUCTION

In the management of poultry rearing, *S. enteritidis* is a pathogen that is no considered important, because it is not lethal in poultry. In terms of food safety, these bacteria is very harmful, because it is zoonotic. As host of *S. enteritidis* is human and poultry, especially chickens (Bhunia, 2008). Chickens that were infected with *S. enteritidis* often did not show signs of illness, but the eggs produced by this chickens are contaminated with these bacteria. The isolation incidence of salmonellosis in humans due to consumed of chicken eggs mostly caused by *S. enteritidis* (Velge *et al*., 2005). Therefore it is recommended to consume eggs with cooked perfectly to avoid salmonellosis.

Kampung chicken is a kind of Indonesian local chicken. The Kampung chicken eggs was mostly used by Indonesian people as 'jamu' (a potion) or as an ingredient of potion that it was directly consumed without cooking. *S. enteritidis* free eggs become significant in producing the safe 'jamu preparation'. *S. enteritidis* free eggs might be produced by chickens which have high resistance to this bacteria.

One of excellent markers showing resistance of chicken against *S. enteritidis* is an active Toll-like Receptor 4 (TLR4) gene. This gene transcribes TLR4 protein and it has been known to associate strongly with the non-specific immune response. TLR4 protein is a phagocytes cell surface receptor that plays a role to recognize lipopolysaccharide (LPS) of gram negative bacteria including *S. enteritidis* (Akira dan Takeda, 2004; Emertcan *et al*., 2011).

To prove it, a series of studies have been carried out. The first study observed to resistance of the Kampung chicken against infection of S. *enteritidis* naturally using TLR4 gene as a marker. The result researched was the TLR4 gene on the exon 2 by PCR-RFLP method was found polymorphic with three kinds of genotypes were found (AA, AG, and GG). Kampung chicken can naturally infected *S. enteritdis* from environmental rearing, and the third genotype of TLR4 gene on the Kampung chicken was categorized resistant in these conditions. Eggs were produced by Kampung chicken contains high specific antibodies against *S. enteritidis* (0.76-0.92 mg/mL yolk egg) (Ulupi *et al*., 2013).

In normal conditions the Kampung chicken proved resistant to *S. enteritidis.* What if there was an outbreak of *S. enteritidis*, whether the Kampung chicken is still showing resistance as in naturally conditions. To answer this question, it is necessary further research.

The aim of the research is to prove resistance of Kampung chicken when challenged with *S. enteritidis*, using TLR4 gene as marker. The evidence was done by

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analyzing the association between genotype TLR4 gene with the factors that indicate the resistance to these bacteria that was obtained from molecular and biological assays.

## MATERIALS AND METHODS

The research was conducted from April to September 2013, at Laboratory of Poultry Production, Molecular of Animal Breeding and Genetics Laboratory, Faculty of Animal Science and Laboratory of Physiology, Laboratory of Medical Microbiology, Laboratory of Immunology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor.

**Animal experiments and rearing:** Fifty Kampung chicken female (8 months of age) were reared during four weeks (2-29 April 2013). They were randomly placed at cages. The cage size was 35x45x50 $cm^3$. All of the cages were placed in a pen (7x10$m^2$). The feed and water were placed in the front of cages. This pen was equipped with two light bulbs for lighting at night.

The feed was commercial feed containing 14-17% crude protein and 2850 kcal/kg metabolizable energy. Feed and water were given *ad libitum*. The weighing of feed and eggs, and recording of egg production were done per day.

**Genotyping of TLR4 gene:** At the beginning of the first week of Kampung chicken rearing, all of the Kampung chickens were genotyped using TLR4 gene as a marker. Blood sample was taken from the brachial vein in the wing area.

There was three phases: DNA extraction, PCR amplification and RFLP (*Restriction Fragment Length Polymorphism*). Genomic DNA extraction used phenol-chloroform method (Sambrook *et al*., 1989). PCR reactions was using primers designed (F): 5'-GCTCAAATTATTTTTCATCAGTggCC-3' and primer (R): 5'-ATCTGGACTAAAGCTGCAC-3'. PCR reaction was started with an initial denaturation at 95°C (5 minute). Then it was performed for 35 cycles of amplification, respectively at 95°C for 30 sec (denaturation), 60°C for 30 sec (annealing) and 60°C for 30 sec (extension). The final extension was for 5 min (72°C) (Muladno, 2010). RFLP method was used to determine the genotype TLR4 gene. PCR result of the TLR4 gene fragment was cut by *MscI* restriction enzyme. PCR products that have been cut with enzyme were electrophoresed using 2% agarose gel.

**Challenge test:** During 5 days on the end of the fourth week of the Kampung chicken rearing, challenge test was done. Every day (for 5 days), every chicken was infected by *S. enteritidis* via oral infection ($ID_{50}$:$10^5$ CFU/mL) (Harvey *et al*., 2007). On the final day of the infection, randomly (6 chickens of each genotype), the blood sample was taken for molecular and biological assays.

On genetic aspect, TLR4 gene expressions (mRNA copy number) in the intestine tissue (duodenum, yeyenum, ileum, ceca, and colon) and kidney tissue were analyzed by molecular assay. This test consists of three phases: RNA extraction, reverse transcriptase (used *GeneJET Purification Kit-Thermo Scientific*), and RT-PCR. RT-PCR was using the primers (*F)* : 5'-GTCCCTGCTGGCAGGAT-3', (R) : 5'-TGTCCTGTGCATCTGAAAGCT-3', and SYBR Green Super Mix. to determine curve standard of TLR4 gene, GAPDH control was used (MacKinnon *et al*., 2009; Anne *et al*., 2011).

Biological assays were done to analyze of resistance aspect. Concentration of leukocytes and differentiation of leucocytes were assayed with Giemsa method (Djokowoerjo *et al*., 1989). Activity and capacity of macrophages were measured. It consists of three phases: preparation of macrophages, preparation of *S. enteritidis*, and phagocytosis assay (Utama *et al*., 2000). Assay of *S. enteritidis* concentration on blood and eggs was refers to *Bacteriological Analitical Manual* (BAM, 2007). Concentration of IgY specific to *S. enteritidis* was measured by Indirect ELISA protocol (Abcam, 2013).

On production aspect was measured feed consumption, egg production and egg weight. Feed conversion ratio was calculated from feed consumption and total weight of eggs produced.

**Data analysis:** Data were analyzed with ANOVA using completely randomized design. TLR4 gene genotype was as treatment and observation data were as response. Statistical model was used $Y_{ij} = \mu + P_i + \varepsilon_{ij}$ (Mattjik and Sumertajaya, 2002).

## RESULTS AND DISCUSSION

**TLR4 gene genotype in Kampung chicken:** TLR4 gene in chicken was on chromosome 17. Based on the data from GenBank (A Y064697.1), the size of TLR4 gene was 11698 bp. Structure of this gene was begun by a promoter region (2743 bp), exon 1, 2 and 3 (105, 167 and 3260 bp), intron 1 and 2 (934 and 984 bp), and the end was the flanking region (3505 bp). TLR4 gene was genotyped on exon 2 (from base to 3898-4117), with a 220 bp PCR product. Result of this study was presented in Fig. 1.

Figure 1 was the result of PCR of TLR4 gene fragment (220 bp), which was cut by restriction enzyme *MscI*, in exon 2 site 3924 (TGG|CCA). In this figure, N was the TLR4 gene PCR amplification product in exon 2 by using primer (F): 5'-GCTCAAATATTTTCATCAGTggCC-3' and (R): 5'-ATCTGGACTGAAAG CTGCAC-3'. Cutting by restriction enzyme resulted allele A (with size 24 bp and 196 bp), and allele G (with size 220 bp). AA, AG and GG were TLR4 gene genotypes of Kampung chicken which was identified. Genotype of 50 Kampung chickens were AA (1), AG (18) and GG (31) (Ulupi *et al*., 2013).

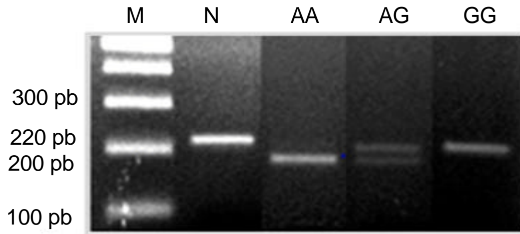


Fig. 1: PCR-RFLP amplification product of TLR4 gene at exon 2 that was cut by the *MscI.* N: fragment of TLR4 gene (220 bp). AA, AG and GG: TLR4 gene genotype of Kampung chicken (Ulupi *et al.*, 2013)

**Association of TLR4 gene genotype and the expression of resistance against *S. enteritidis* infection in Kampung chicken:** The association of TLR4 gene genotype with their phenotypic or their expression on Kampung chicken after infected by *S. enteritidis* ($ID_{50}$:5x$10^5$ CFU/mL) was analyzed. Their phenotypics were including genetic aspect, resistance aspect and production aspect.

**Genetic aspect:** On genetic aspect, TLR4 gene expression levels which was represented by the number of mRNA copies in this study was analyzed from intestinal and kidney tissues. Association of TLR4 gene genotype and the expression of resistance Kampung chicken after infected *S. enteritidis* on genetic aspect was presented in Table 1.

The value of average number of mRNA copies that was obtained from intestinal tissue was higher than kidney tissue. This result matches with Muhammad *et al.* (2005) which stated that the number of mRNA copies in intestinal tissue was highest, followed by liver and spleen, and the lowest was kidney, brain, heart and muscle. The value of the average number of mRNA showed that the TLR4 gene was strongly expressed. These result approached the research result of Jia *et al.* (2012), which stated that TLR4 gene on ducks were strongly expressed in the liver, kidney, spleen and intestine.

The number of mRNA copies of TLR4 gene in intestinal and kidney tissue in Kampung chicken with AG and GG genotypes was not statistically different. The number of mRNA copies from AA genotype was 3.92x$10^7$ (intestinal tissue) and 2.58x$10^7$ (kidney tissue). These value at least not lower than the average of mRNA copies number from AG and GG genotype. This result meant that all genotypes of TLR4 gene in Kampung chicken had the same potential on protein transcription. It was as phagocyte cell surface receptor. The function of these receptor was to recognize LPS.

No difference of the expression levels on genotype of TLR4 gene was caused by the rearing condition of the Kampung chicken generally in unhygienic. This condition allowed the continuous exposure naturally of *S. enteritidis.* The exposure will induce an increase of TLR4 gene activity. So the expression on all genotypes of TLR4 gene was shown in this study was also due to the interaction between factors of genetic and environment. This result was in accordance with Li *et al.* (2013), which stated that expression of genotype variation of TLR4 gene in Chinese local chicken were influenced by the interaction between genetic factor and the other factors.

Table 1: Association of TLR4 gene genotype and their expression on Kampung chicken after infected *S. enteritidis*

| | Genotypes of TLR4 gene | | |
|---|---|---|---|
| Phenotypic | AA*(1) | AG (6) | GG (6) |
| mRNA usus (x $10^7$) | 3.92 | 1.61±0.87 | 2.36±1.62 |
| mRNA ginjal (x $10^7$) | 2.58 | 1.12±0.88 | 1.43±0.91 |

Different letter in the same row was indicated significant differences (p<0.05). *AA genotype was not included in the statistical testing

Table 2: Association of TLR4 gene genotype and the expression of resistance Kampung chicken after infected *S. enteritidis* on resistance aspect

| | Genotypes of TLR4 gene | | |
|---|---|---|---|
| Phenotypic | AA*(1) | AG (6) | GG (6) |
| Leucocytes (103 cell/mm$^3$) | 22.16 | 19.50±3.83$^a$ | 22.69±9.11$^a$ |
| Heterophile (%) | 37 | 39.00±3.94$^a$ | 41.43±9.05$^a$ |
| Monocytes (%) | 5 | 5.20±1.79$^a$ | 4.71±1.11$^a$ |
| Lymphocytes (%) | 57 | 54.00±4.06$^a$ | 52.43±9.25$^a$ |
| Macrophages activity (%) | 74 | 74.40±4.39$^a$ | 71.86±3.72$^a$ |
| Macrophages capacity (bacteria/macrophage) | 40.96 | 40.33±1.26$^a$ | 42.13±3.29$^a$ |
| *S. enteritidis* in blood | negative | negative | negative |
| *S. enteritidis* in eggs | negative | negative | negative |
| IgY specific *S. enteritidis* in eggs yolk (mg/mL) | 2.94 | 3.69±0.68$^a$ | 3.89±0.83$^a$ |

Different letter in the same row was indicated significant differences (p<0.05). *AA genotype was not included in the statistical testing

**Resistance aspect:** The association of TLR4 gene genotype and their phenotypic on Kampung chicken after infected *S. enteritidis* in resistance aspect was presented in Table 2. The result of analysis of variance showed that all parameters were not significantly different in AG and GG genotype. There included concentration of leucocytes, percentage of leucocytes differentiation, phagocytic activity and capacity of macrophages, and concentration of IgY specific *S. enteritidis* on eggs yolk that were produced by Kampung chicken.

Leucocytes concentration of Kampung chicken (AA, AG, GG genotype), was included in normal category (12-30x$10^3$ cells/mm$^3$) (Jain, 1993). Percentage of leucocytes differentiation (heterophiles, monocytes and lymphocytes) almost equal to the result of Yusriani (2012) research, who reported that in 10 weeks aged of the Kampung chicken had percentage of heterophile, monocytes and lymphocytes, respectively 38.20, 5.67 and 56.80%. So treated like the challenge test with *S. enteritidis* on infectious dose ($ID_{50}$), did not cause physiological disorders on Kampung chickens.

Ratio of percentage of heterophile and lymphocytes (H/L) was an indicator of stress level for chicken. According to Swenson (1993), in chicken, the ideal value of H/L ratio was 0.45-0.50. Outside of this range, chicken is in stress conditions. In this study, ratio of H/L in the Kampung chicken was 0.65 (AA genotype), 0.72 (AG genotype) and 0.79 (GG genotype). These result showed that Kampung chicken was in stress condition due to the infection of *S. enteritdis*.

Beside that, this stress also can be caused due to the high ambient temperature of Kampung chicken rearing. The observations during this study was showed that in every day, just about 5 hours (at 24.00-05.00) Kampung chickens were on the thermal environment in accordance with the thermoneutral zone (20-23°C) (Bell and Weaver, 2002). During the next 19 h, the Kampung chickens were at an environment temperature that far from comfortable conditions (29-34.6°C). The high of environment temperature during rearing was one of the factors causing stress. The effect of it was very significant (Yousef, 1985). So the stress condition of Kampung chicken in this study was aggravated by the high of environment temperature during rearing.

In this study, Kampung chickens which were exposed with *S. enteritidis* produced macrophages activity 74.00% (AA), 74.40% (AG) and 71.86% (GG). Macrophages activity in this study was higher than the result of Afifudin (2009) research who reported that macrophages activity in normal conditions of laying-commercial chicken (16 weeks of age) was 46.1%. It meant that the phagocytic activity can be enhanced by giving certain treatment.

Capacity of macrophages was 40.96 bacteria/ macrophage (AA), 40.33 bacteria/macrophage (AG) and 42.13 bacteria/macrophage (GG). Okti *et al.* (2008) stated that the capacity of macrophages in laying commercial hens on period of production was 1.60 bacteria/macrophage. So the value of macophages capacity in this study was very high.

The high of phagocytic capacity was caused by the Kampung chickens that were being infected with *S. enteritidis*. This exposure led to increased the expression of TLR4 gene as shown in Table 1. It meant that all of the TLR4 gene genotypes had a high ability to transcribed of TLR4 protein. This protein was as a receptor on the phagocytes cell surface. Abundant TLR4 receptor protein on the surface of phagocytes cells will facilitate of the macrophages to capture *S. enteritidis,* because these receptors were able to recognize LPS from these bacteria. The increase of TLR4 gene expression can be increased the phagocytic capacity of phagocytes cell, especially macrophages cell. So the high phagocytic activity was positively correlated with TLR4 gene activity. The high activity of TLR4 gene was negatively correlated with the concentration of *S. enteritidis* in the blood (Zhongyong *et al.*, 2012).

Table 3: Association of TLR4 gene genotype and the expression of resistance Kampung chicken after infected *S. enteritidis* on production aspect

| | Genotypes of TLR4 gene | | |
|---|---|---|---|
| Production aspect | AA*(1) | AG (6) | GG (6) |
| Feed consumption (g/chicken/day) | 91.70 | 92.19±4.28[a] | 91.20±2.67[a] |
| Hen day production (%) | 52.00 | 54.88±19.72[a] | 39.08±19.86[b] |
| Egg weight (g/egg) | 41.00 | 40.47±1.94[a] | 40.55±2.46[a] |
| Feed conversion ratio | 4.41 | 4.22±1.37[a] | 9.61±5.21[b] |

Different letter in the same row was indicated significant differences ($p<0.05$). *AA genotype was not included in the statistical testing

It meant that the phagocytes cells can function very well, and it can eliminate (clearence) these bacteria. The impact of it was negative result on *S. enteritidis* assays in blood and eggs that produced by Kampung chickens as shown in Table 2. So in this study, the Kampung chickens were challenged with *S. enteritidis* also produced eggs with free of these bacteria.

The challenge test in this study increased the content of specific antibodies to *S. enteritidis* in eggs yolk that were produced by Kampung chickens. The concentration of specific IgY to *S. enteritidis* in eggs yolk were obtained at 2.94-3.89 mg/ml. The content of these specific IgY to *S. enteritidis* in eggs yolk of Kampung chicken was significantly higher than it was contained before challenged with this bacteria (0.76-0.92 mg/mL yolk egg) (Ulupi *et al.*, 2013).

**Production aspect:** Association of TLR4 genotype and the expression of resistance Kampung chicken after infected *S. enteritidis* on production aspect was presented in Table 3.

Feed consumption of Kampung chicken with AG and GG genotypes of TLR4 gene (92.19 and 91.20 g/chicken/day) were not different ($p>0.05$). The feed consumption of AA genotyped chicken (91.70 g/chicken/day) was similar to the other genotypes of Kampung chicken. All genotypes of TLR4 gene also produced eggs with average weight of nearly the same. The average of egg weight was ranged from 40.47-41.00 g/egg.

Hen day production of AA genotyped chicken (52.00%) was almost the same with AG (54.88%). Hen day production of AG genotyped chicken was significantly higher than GG genotyped chicken (39.08%) ($p<0.05$). When Kampung chickens was challenged with *S. enteritidis*, 22% of AG and 35% of GG showed symptoms of illness. So Kampung chicken with AG genotype was relatively healthier when was infected with these bacteria. This condition caused the rate of egg production of Kampung chicken with AG genotype was higher than GG. It influenced the feed conversion ratio. The value of feed conversion ratio of Kampung chicken with AG genotype of TLR4 gene (4.22) was significantly lower and more efficient than GG (9.61).

Feed conversion ratio of AA genotype was 4.41. This was similar to feed conversion ratio of AG genotype.
Liu *et al*. (2011) stated that variation of TLR4 gene genotype in Chinese local chicken does not show significant affect on any important commercial trait such as eggs production and chicken growth. This result was obtained during the chickens in state of normal and were not being challenged with *Salmonella*.

**Conclusion:** Kampung chickens (on all genotypes of TLR4 gene: AA, AG, and GG) are resistant against *S. enteritidis* infection in all conditions. Base on production aspect, AG genotyped chicken have higher resistance than GG.

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