g.640T>C Polymorphism of the TGF-β2 Gene is Associated with *Salmonella pullorum* Resistance in Indonesian Chicken

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Abstract. The objectives of this study were to identify polymorphism of transforming growth factor β2 (TGF-β2) gene associated with *Salmonella pullorum* resistance in Indonesian chickens. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays were used to identify polymorphism of Indonesian chickens. Analysis of polymorphism was conducted by using PopGen 3.2 software. The effect of genotype on *S. pullorum* resistance was analyzed using the SAS General Linear Model (GLM) procedure. Genotyping was performed on 278 chickens from 7 Indonesian chicken populations (Sentul, Merawang, Pelung, Kampung, Parent Cobb broiler, The F1 Crossbred of Kampung x Parent Cobb broiler (KB) and F2 KB x KB. The product of amplification was 284 bp. The TGF-β2| *Rsa*I locus was polymorphic in all populations, producing two alleles (T and C) and three genotypes (TT, CT, and CC). The result from the analysis of the allele and genotype frequency showed that the T allele had a higher frequency than the C allele in all populations. The χ^2 analysis showed that the 6 chicken populations were deviated from Hardy-Weinberg equilibrium, except the Parent Cobb broiler chicken. The association result showed that TT genotype was significantly associated with *S. pullorum* resistance in Sentul chicken. Although the leukocyte concentration, leukocyte differentiation and H/L ratio in Sentul chicken with three of TGF-β2 genotypes (TT, TC, and CC) were not statistically different. In conclusion, polymorphism in the TGF-β2 chicken gene can be used as a candidate marker to increase *S. pullorum* immune response.
Keywords: *TGF-β2 gene, PCR-RFLP, Salmonella pullorum, Indonesian chicken*

Abstrak. Penelitian ini bertujuan untuk mengidentifikasi keragaman gen transforming growth factor β2 (TGF-β2) yang berasosiasi dengan ketahanan terhadap *Salmonella pullorum* pada ayam Indonesia. Uji Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) digunakan untuk identifikasi keragaman ayam Indonesia. Analisis keragaman dilakukan menggunakan *software* Popgen 3.2. Pengaruh genotype terhadap ketahanan *S. pullorum* dianalisis menggunakan *software* SAS dengan prosedur General Linear Model (GLM). *Genotyping* telah dilakukan pada 278 ekor ayam dari 7 populasi ayam Indonesia (Sentul, Merawang, Pelung, Kampung, Parent Cobb broiler, F1 persilangan Kampung x Parent Cobb broiler (KB) and F2 KB x KB. Panjang produk amplifikasi sebesar 284 bp. Lokus TGF-β2| *Rsa*I bersifat polimorfik pada semua populasi, menghasilkan dua alel (T dan C) dan tiga genotipe (TT, TC dan CC). Hasil analisis frekuensi alel dan genotipe menunjukkan frekuensi alel T lebih besar daripada alel C pada semua populasi. Analisis χ2 menunjukkan 6 populasi ayam menyimpang dari keseimbangan Hardy-Weinberg, kecuali pada ayam Parent Cobb broiler. Hasil asosiasi ditemukan bahwa genotipe TT berasosiasi signifikan dengan ketahanan *S. pullorum* pada ayam sentul. Sedangkan konsentrasi leukosit, diferensiasi leukosit dan rasio H/L pada ayam sentul dengan ketiga genotipe gen TGF-β2 (TT, TC dan CC) tidak berbeda signifikan. Kesimpulan, keragaman gen TGF-β2 pada ayam dapat digunakan sebagai kandidat marker untuk meningkatkan respon imun terhadap *S. pullorum.*
Kata kunci: Gen TGF-β2, PCR-RFLP, *Salmonella pullorum*, Ayam Indonesia

Introduction

The *pullorum* disease also called bacillary white diarrhea (BWD), is caused by *Salmonella pullorum* bacteria. This disease is a severe septicemia disease that causes high morbidity and mortality in young birds, especially newly hatched chicks (Xie et al. 2017). In many developing countries, *S. pullorum* infection in poultry is common and *pullorum* disease remains a major disease threat in the poultry industry, causes severe economic losses (Guo et al. 2016). Therefore, there is a need for a

method that protects poultry against *S. pullorum* infections such as molecular approach.

The molecular approach has provided a tool for studying the genetic composition of individuals. In the selection program, using molecular data can improve the accuracy of selection. (Dekkers and Hospital, 2002). This approach offers a substantial way to detect molecular markers using only candidate genes. One of the candidate genes that play an important role in increasing the immune response is transforming growth factor β gene (TGF-β). The TGF-β gene superfamily is composed of multifunctional cytokines that promote cell growth and extracellular matrix synthesis and mediate the immune response (Massague, 2012). The TGF-β gene is recognized not only as a growth factor but also immune modulation cytokines (Li et al. 2006) also affect the function and phenotype of lymphocytes, dendritic cells, and macrophages (Li et al. 2006; Tran, 2012; Chistiakov et al. 2015). From differentiation to activation and proliferation, T lymphocytes are affected by the TGFβ gene (Han et al. 2009).

The TGF-β gene superfamily contains four different isoforms (TGF-β1, TGF-β2, TGF-β3, and TGF-β4) and many other signaling proteins produced by all white blood cell lineages (Johnson and Newfeld, 2002; Huminiecki et al. 2009). The chicken TGF- β2 gene is located on chromosome 3 and is approximately 70 kb long with seven exons (Burt and Paton, 1991). Recently, a new single nucleotide polymorphism (SNP) has been identified in the TGF-β2 promoter region which has a positive effect on chicken growth (Tang et al. 2011). However, a few studies have also reported an association between this chicken TGF-β2 polymorphism and resistance to bacteria such as *S. enteritidis* (Kramer et al. 2003; Malek and Lamont 2003; Tohidi et al. 2012) and chicken antibody response (Zhou et al. 2002).

Therefore, based on these biological functions, the objective of the current experiment was to study associations of TGF-β2 gene polymorphisms with the resistance of Indonesian chickens to *S. pullorum*. This information can be used to recommend Single Nucleotide Polymorphisms (SNPs) as candidates for Marker Assisted Selection (MAS). These findings can be applied not only in the chicken selection strategy to obtain better performance but can relatively be used in related studies in other poultry.

Materials and Methods

Experimental animal

Samples collected from wing veins into EDTA tubes, blood samples were obtained from 278 chickens from 7 different populations: Sentul (n = 96), Merawang (n = 23), Pelung (n = 10), Kampung (n = 57), Parent Cobb broiler (n = 10), F1 crossbreed of Kampung x Parent Cobb broiler (KB; n = 30) and F2 KB x KB (n = 52). All chickens bred in the Animal Breeding and Genetics Division, Department of Animal Production and Technology, Faculty of Animal Science, Bogor Agricultural University (IPB), Indonesia. All chickens used in the current study were maintained according to the principle of animal welfare. The chicken was maintained and fed under the same conditions to minimize the effect of environment. For association analysis, in this study was associated with Sentul chicken.

DNA extraction

The DNA extraction protocol was modified from Sambrook and Russel (2001) with the following procedures: each 20 µL of chicken blood was added to 1000 µL of 0.2% NaCl and then homogenized and centrifuged (8000 rpm) for 5 min and the supernatant part was removed. The precipitate was added to 40 µL of 10% SDS, 10 µL proteinase-K (5 mg/mL) and 1 x STE up to 400 µL. Then, the mixture was slowly shaken in the incubator at 55 ° C for 2 h. Phenol

solution (400 µL), CIAA (400 µL) and NaCl 5M (40 µL) were added to the mixture and slowly shaken at room temperature for 1 h. DNA molecules were separated from phenol using a centrifuge (12000 rpm) for 5 min. DNA phases were moved and added to 800 µL EtOH absolute and 40 µL NaCl 5M. Then, samples were frozen overnight. DNA molecules were centrifuged (12000 rpm) for 5 min and supernatant part was removed. DNA precipitate was air dried and dissolved in 100 µL 80% TE.

Amplification and genotyping of TGF-β2 gene

Polymerase chain reaction (PCR) was carried out using primers specific for a part of exon 1 (284 bp) of TGF-β2 gene (GenBank Accession No. X58071.1): forward 5'- GCC ATA GGT TCA GTG CAA G -3'; reverse 5' TGA CAG AAG CTC TCA AGC C -3'. 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 MgCl2, 0.05 µL of Taq polymerase, 1.5 µL 10X reaction buffer, and 10.85 µL of distilled water. Amplification was performed with a GeneAmp® PCR 9700 System (Applied Biosystems, USA). The thermal cycling conditions consisted of predenaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s; the final extension step was at 72 °C for 5 min. DNA amplification products and standard DNA ladder were separated on 1.5% agarose gels on 0.5X TBE buffer.

Genetic polymorphism of TGF-β2 gene was conducted by Restriction Fragment Length Polymorphism (RFLP) using a *Rsa*I restriction enzyme, which recognizes and cleaves GT|AC sites. Primer annealing position and *Rsa*I restriction site are shown in Figure 1.

Amplification products were visualized on a 2% agarose 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 RsaI, 1 µL distilled water, and 0.7 µL Tango buffer and then incubated at 37 oC for 16 h. The digestion products were separated by horizontal electrophoresis (100 V, 40 min) in 2.5 % agarose gel in 0.5X TBE and 2.5 µL ethidium bromide visualized on an ultraviolet transilluminator.

DNA Sequencing

Sequencing was performed for a different genotype of TGF-β2 gene in exon 1. Forward and reverse primer fragments were sequenced using sequencer machine (ABI Prims 3100-Avant Genetic Analyzer) in 1st Base Selangor, Malaysia.

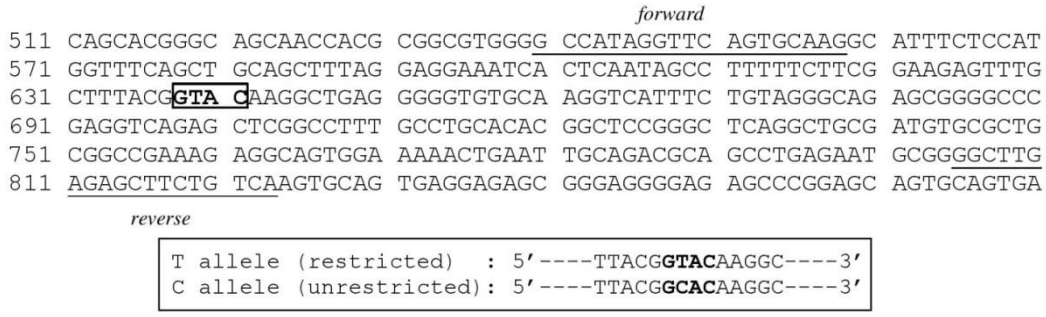

Figure 1. Forward and reverse primer annealing positions (underline), bold shows *Rsa*I restriction sites, and box shows the SNP position (GeneBank accession number: X58071.1)

Heterozygosity

Polymorphism of population can be analyzed by using heterozygosity number. Observed heterozygosity (Ho) (Weir, 1996) and expected heterozygosity (He) (Nei and Kumar, 2000) were computed by using PopGene software version 3.2 and were tested by the following formula:

$$\mathrm{Ho} = \sum_{i \neq j} \frac{n_{ij}}{N}$$

$$\mathrm{He} = 1 - \sum_{i=1}^{q} x_i^2$$

Description:

Ho = Observed heterozygosity
n_{ij} = Number of heterozygous animals
N = Number of observed animal
He = Expected heterozygosity
x_i = Frequency of allele
q = Total alleles

Concentration of Leukocytes

The concentrations of leukocytes were measured using counting chamber method (Fischbach and Marshall, 2008) 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 micropipette. 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 mm^3.

Leukocyte Differentiation

The value of leukocyte differentiation was calculated using the Rapid method (Fischbach and Marshall, 2008) as follows: 5 µL of chicken blood was dropped on the object glass with a horizontal position and another object glass placed with an angle of 45°. Object glass was air dried and fixed with methanol for 5 min. After that, the object glass was placed into 10% Giemsa for 30 min. Then, the object glass was washed with running water and dried air. The leukocyte differentiation was calculated by counting under a microscope (100x magnification).

Phagocytosis assay

The phagocytosis assay was modified from Wang et al. (2009) with the following procedures: (1) Macrophage cell preparation. Macrophages were taken from peritoneal fluid of chicken. Collect peritoneal fluid of chicken by injecting 5 mL of NaCl in peritoneal cavity by following a gentle massage of the abdomen. The number of macrophages in peritoneal fluid was calculated using Neubauer hemocytometer. (2) Bacteria (*Staphylococcus aureus*) preparation and Phagocytosis assay. Peritoneal exudate cell suspension (as a source of macrophages) used for in vitro phagocytosis assays (Ulupi et al. 2014). Calculate percent phagocytosis by counting the number of 50 macrophages. The activity and capacity of macrophages are calculated by the following formula (Wibawan and Laemmler, 1994):

$$\text{Macrophage activity} = \frac{\sum \text{Macrophage Active}}{\sum \text{Macrophage Total}} \times 100\%$$

$$\text{Macrophage capacity} = \frac{\sum \text{bacteria Ingested}}{\text{Macrophage}}$$

Clearance Test

Immune traits were detected in blood samples using the clearance test (Jackson et al. 1998). This method is used to identify the normal growth of bacterial populations (*S. pullorum*) as compared to the specially treated population. The effect of treatment on bacterial growth was measured after incubation for 24-48 h at 35 ± 1 °C. Preparation of bacterial culture begins with the rejuvenation of culture on nutrient media at 36 ± 1 °C for 18-24 h and subculture on Brain Heart Broth media at 36 ± 1 °C for 18-24 h.

Data analysis

Determination of SNP (single nucleotide polymorphism) and alignment sequence were performed with Molecular Evolutionary

Genetics Analysis 5 (MEGA5) (Tamura et al. 2011). The sequencing results were analyzed by BioEdit (Hall, 2011). The BLAST (Basic Local Alignment Search Tool) program was used to search the NCBI GenBank databases for reference and homologous sequences. Genotype frequency, allele frequency, heterozygosity and the Hardy-Weinberg Equilibrium (HWE) were analyzed using PopGen version 3.2 (Raymond and Rousset, 2001). Test of HWE was conducted with chi-square test (χ^2) (Kaps and Lamberson, 2004). The number of degrees of freedom (df) is equal to the number of possible genotypes minus the number of alleles (Allendorf et al. 2013). The association of TGF-β2| *Rsa*I genotype with *S. pullorum* disease resistance was analyzed using the GLM procedure of SAS 9.1.3 software (SAS Institute, Cary, NC, USA). Duncan's multiple range test was used to identify significant differences between means. The genetic effects were analyzed using the following model:

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

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 Discussions

Polymorphism of TGF-β2 gene

A total of 278 samples were successfully amplified and resulted in a 284 bp fragment of the partial TGF-β2 gene exon 1 through PCR (Figure 2). The 284 bp fragment of exon 1 of the TGF-β2 gene was digested with *Rsa*I restriction enzyme. The allele designated by T shows the pattern of 2 fragments (184 and 100 bp), and C allele shows only 1 fragment (284 bp). The two alleles produce three genotypes, TT, TC and CC (Figure 3).

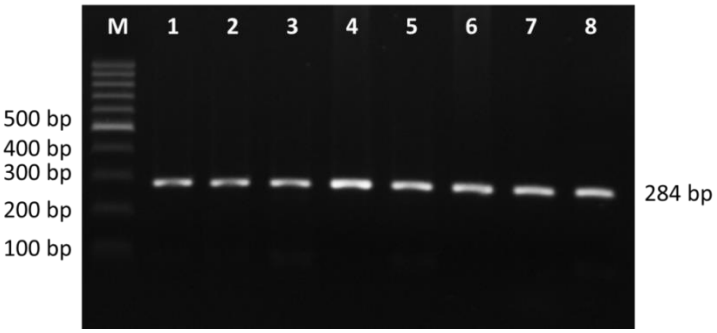

Figure 2. Amplification TGF-β2 gene exon 1 in 1.5% agarose gel (line 1-8: samples; M: 100 bp ladder)

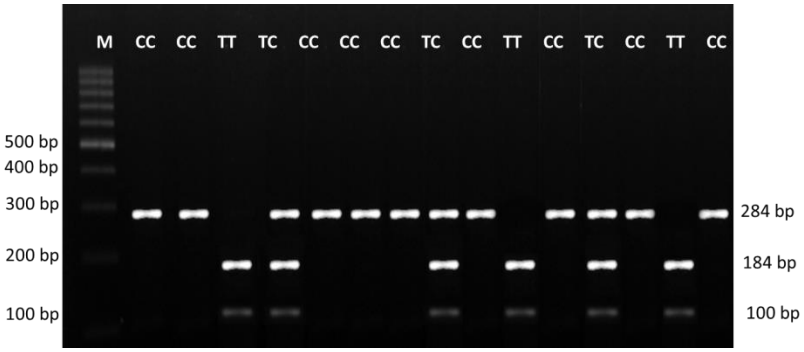

Figure 3. *Rsa*I restriction pattern of exon 1 fragment of TGF-β2 by PCR-RFLP on 2.5 % agarose gel. M= 100 bp ladder; TT, TC, CC = genotype.

This SNP characterized by PCR-RFLP method was compatible with previous reports (Kramer et al. 2003; Malek and Lamont 2003; Tohidi et al. 2012). The calculated genotype and allele frequencies of Indonesian chickens TGF-β2 gene are shown in Table 1. The homozygous TT genotype was present in the highest frequency across all the population. The distribution of the TGF-β2| *Rsa*I alleles of the exon 1 is characterized by a higher frequency of the T allele compared to the C allele in all Indonesian chickens population studied.

Indonesian chickens populations were polymorphic with two alleles found (T and C). Nei and Kumar (2000) and Allendroft et al. (2013) proposes that populations may be classified as polymorphic if there are more than one allele at the locus and if the most common allele frequencies are less than 0.99. The observed heterozygosity (Ho) number in Indonesian chickens was lower than the expected heterozygosity (He) number (Table 2). According to Allendroft et al. (2013), this result indicated that the occurrence of inbreeding is a result of intensive selection of TGF-β2 gene in Indonesian chickens.

Table 1. Genotype and allele frequency of TGF-β2| *Rsa*I locus in Indonesian Chickens

Chicken population	n	Genotype frequency			Allele frequency	
		TT	TC	CC	T	C
Sentul	96	0.479	0.250	0.271	0.604	0.396
Merawang	23	0.609	0.130	0.261	0.674	0.326
Pelung	10	0.600	0.100	0.300	0.650	0.350
Kampung	57	0.491	0.281	0.228	0.632	0.368
Parent Cobb Broiler	10	0.600	0.200	0.200	0.700	0.300
F1 Kampung x Parent Cobb Broiler (KB)	30	0.467	0.300	0.233	0.617	0.383
F2 KB x KB	52	0.731	0.192	0.077	0.827	0.173
Overall population	278					

n: Number of samples

Table 2. Heterozygosity and Hardy-Weinberg equilibrium of TGF-β2| *Rsa*I locus in Indonesian Chickens

Chicken breed	n	Ho	He	χ^2 value for test of HWE
Sentul	96	0.250	0.478	22.376*
Merawang	23	0.130	0.439	12.267*
Pelung	10	0.100	0.455	7.120*
Kampung	57	0.281	0.465	9.406*
Parent Cobb Broiler	10	0.200	0.420	3.487
F1 Kampung x Parent Cobb Broiler (KB)	30	0.300	0.472	4.405*
F2 KB x KB	52	0.192	0.286	6.117*
Overall population	278			

n: Number of samples, Ho: Observed heterozygosity, He : Expected heterozygosity, χ^2: Chi-square, *: Significantly different (χ^2 0.05= 3.841), HWE: Hardy-Weinberg equilibrium

The χ^2 analysis showed that all chicken populations were deviated from Hardy-Weinberg equilibrium, with the exception of the Parent Cobb broiler chicken. Allendroft et al. (2013) explained that χ^2 is in Hardy-Weinberg equilibrium if the genetic variation, allele and genotype frequencies in a population remain constant from one generation to the next in the absence of disturbing factors.

Sequence Analysis of TGF-β2 Gene

Sequence analysis using forward and reverse primer revealed that PCR product of TGF-β2 gene exon 1 in this study was similar with the 284 bp in length based on previously reported by Tohidi et al. (2012). The results of sequence alignment compared with *Gallus gallus* (chicken) GenBank (accession number X58071.1) showed that a mutation occurred in exon 1 of TGF-β2 gene (Figure 4) that caused a lack of *Rsa*I restriction site (GT|AC).

Malek and Lamont (2003) reported that there was a T-C transition mutation found at position 640 bp in the exon 1 of chicken TGF-β2. Transition mutation between thymine (T) base with cytosine (C) base in the chickens with CC genotype causing an alteration in *Rsa*I restriction site sequences GT|AC became GC|AC so that the *Rsa*I enzyme couldn't recognize its restriction site. IUPAC single-letter codes were used to facilitate the definition of nucleotide sequences, which are Y symbol for pyrimidine (C or T/U), R symbol for purine (A or G), W symbol for weak (A or T) and K symbol for G or T. This sequence analysis results confirmed that TGF-β2| RsaI locus in all Indonesian chicken population studied was polymorphic.

Concentration of leukocytes, Leukocytes differentiation, and H/L ratio

The concentration of leukocytes, leukocytes differentiation and H/L ratio in Sentul chickens with TT, TC, and CC genotypes were not statistically different (Table 3). The concentration of leukocytes in chickens with TT, TC, and CC genotype were within the normal range 17-32 x 10^3 mm^{-3} (Davis et al. 2008).

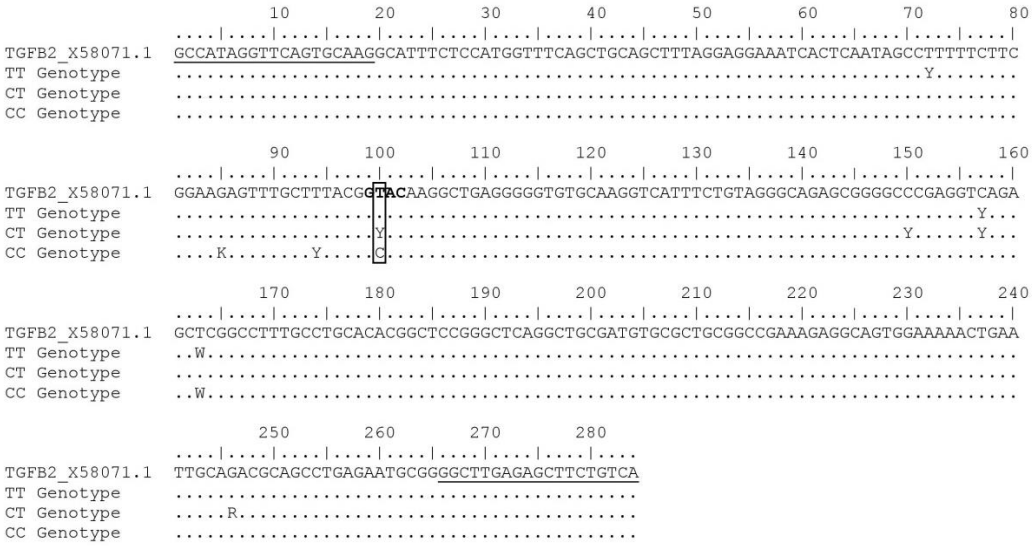

Figure 4. Nucleotide sequence alignment of TGF-β2 gene partial exon 1. Underline shows forward and reverse primer annealing positions; bold shows *Rsa*I restriction sites; boxes show g.640T>C SNP target (GenBank accession number: X58071.1)

These results indicate that the Sentul chicken with the third TGF-β2 genotype was not infected with the bacterium. Muhsinin et al. (2016) reported values of leukocyte differentiation are Sentul chicken is heterophile (45.80%), lymphocytes (51.55%) and monocytes (1.89%). Whereas Nurfaizin et al. (2014) have reported values for leukocyte differentiation in broiler chickens based on the density of the cage that is heterophile (27.57%), lymphocytes (56.44%) and monocytes (13.56%). The ratio of heterophils and lymphocytes (H/L) is used by ornithologists as a tool to monitor immune function in birds (Davis et al. 2008). In chicken, heterophils and monocytes are capable of phagocytosis. In acute inflammatory responses to infectious as well as non-infectious causes, heterophils are one of the first phagocytes to accumulate in the affected tissue (Potter et al. 2016).

Association of TGF-β2 gene polymorphisms with *S. pullorum*

The analysis of the association between the TGF-β2 gene genotype with *S. aureus* and *S. pullorum* in Sentul chicken is shown in Table 4 and Table 5. Chicken with TT genotype showed higher resistant to *S. aureus* and *S. pullorum* ($p<0.05$) compared to that of the TC and CC genotypes.

Table 3. Association of TGF-β2 gene genotypes on concentrations of leukocytes, leukocytes differentiation, and H/L ratio in Sentul chicken

Trait	Genotype		
	TT (n=7)	TC (n=7)	CC (n=6)
Leukocyte (10^3 mm^{-3})	17.82±3.24	16.17±3.24	20.20±3.50
Heterophile (%)	43.71±5.40	40.00±5.40	52.16±5.83
Lymphocyte (%)	52.42±5.33	57.28±5.33	45.50±5.76
Monocyte (%)	3.00±0.41	2.28±0.41	1.66±0.45
H/L ratio	0.94±0.56	0.78±0.42	1.47±1.06

n: Number of samples

Table 4. Association of TGF-β2 gene genotype with macrophage activity and capacity in Sentul chickens when challenged to *Staphylococcus aureus*

Trait	Genotype		
	TT (n=7)	TC (n=7)	CC (n=6)
Macrophage activity (%)	92.12±1.52^a	84.90±1.52^b	82.29±1.65^b
Macrophage capacity (bacteria macrophages^{-1})	2220.66±43.97	2153.81±43.97	2187.56±47.50
Total of bacteria ingested (10^5)	102.33±3.02^a	91.46±3.02^b	90.21±3.26^b

n: Number of samples, the different superscript within the same row shows significant ($P<0.05$) difference

Table 5. Association of TGF-β2 gene genotype on resistant to *Salmonella pullorum* in Sentul chickens

Trait	Genotype		
	TT (n=7)	TC (n=7)	CC (n=6)
Early concentration (10^7cfu mL^{-1})	2.40	2.40	2.40
Final concentration (10^5cfu mL^{-1})	97±6.69^a	141±6.69^b	160±7.52^b
Death rate of bacteria (%)	59.40±2.90^a	41.07±2.90^b	33.33±3.13^b

n: Number of samples, the different superscript within the same row shows significant ($P<0.05$) difference

In the previous study, the use of SNP of innate immune genes, such as *NRAMP1* (Beaumont et al. 2003; Muhsinin et al. 2016), *TLR4* (Li et al. 2010), *MyD88* (Liu et al. 2015), *CD28,* and *MD-2* (Malek et al. 2004), leads to enhancement of *S. pullorum* resistance in chicken. Tohidi et al. (2012) reported that the single nucleotide polymorphism (SNP) within the same region in TGF-β2 gene maintained a significant association with resistant to salmonellosis in Malaysian indigenous chicken. The allele T in the study by Tohidi et al. (2012) showed a positive effect on immune traits.

Through the analysis, the polymorphisms of the TGF-β2 gene and the correlation with resistance to *S. pullorum* suggested that TGF-β2 gene may be one of the *S. pullorum* resistant genes in the innate immune system.

Conclusions

Indonesian chickens are polymorphic in the TGF-β2| *Rsa*I locus. The T allele frequency is higher in all populations. The TT genotypes in Sentul chicken having positive effects on the resistant to *S. pullorum* could be considered for selection strategies of the birds in the population.

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