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Variation and Association of Avian β-Defensin 2 Gene with the Concentration of Immunoglobulin Y and the Titer of Newcastle-Disease Antibody in IPB-D1 Chicken

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ABSTRACT

Defensins play roles in innate immunity by exhibiting antimicrobial activity against microbes such as gram-negative and -positive bacteria, viruses, and fungi. This study aimed to identify variants of the Avian β-Defensin 2 *(AvBD2)* and determine their associations with the concentration of immunoglobulin Y and the titer of Newcastle disease (ND) antibody in IPB-1 chicken. The chicken population used in this study was 21-week-old IPB-D1 chickens (n=90). Variations in *AvBD2* were analyzed by direct DNA sequencing. IgY concentration was measured by indirect ELISA, and the titer of ND antibody was measured by the hemagglutination-inhibition test. The *AvBD2* association was analyzed by the general linear model procedure and Duncan's multiple range test. The results revealed 10 SNPs located in intron 1 (3 SNPs), exon 2 (3 SNPs), and intron 2 (4 SNPs). Six of these SNPs were associated with IgY concentration. The CC genotype of g.5002 C>T was associated with IgY concentration and produced the highest mean IgY concentration. This g.5002 C>T mutation results in alanine-to-valine substitutions. The CC genotype of g.5002 C>T could be considered as a criterion for selecting chickens with high IgY concentrations.

Keywords: AvBD2; IPB-D1 chicken; IgY concentration; Newcastle disease antibody titer

INTRODUCTION

Innate immunity is aided by defensins, which suppress the activities of microbes such as gram-negative and gram-positive bacteria, viruses, and fungi (Terada *et al.*, 2018). Alpha, beta, and theta-defensins are the three subfamilies of defensins, of which only beta-defensins are present in chicken (Sugiarto & Yu, 2004). According to Hasenstein & Lamont (2007), beta-defensins are linked to various diseases in humans, cows, and poultry. In chicken, 14 different forms of avian β-defensin (AvBD1 to AvBD14) have been reported (Lynn *et al.*, 2007). A study by Hong *et al.* (2012) found that the AvBD genes are expressed in the chicken intestine and up-regulated upon *Salmonella* infection. In addition, Terada *et al.* (2018) reported that AvBD1, 2, 4, 6, and 7 show decreased expressions in the ileum and cecum after hatching. Moreover, *AvBD1*, 2, and 7 were found to be expressed in the chicken bone-marrow tissue (Derache *et al.*, 2009), indicating that leukocytes from the bone marrow are involved in the synthesis of AvBDs.

The disease can cause very large problems such as high morbidity and mortality. Newcastle disease (ND; also known as *tetelo* in Indonesia) is a viral disease that causes mortality in poultry, including local chicken breeds. This disease is caused by Paramyxovirus type-I (PMV-1) from the Paramyxoviridae family. The ND virus has negative-stranded RNA that encodes six proteins: fusion protein, hemagglutinin-neuraminidase, nucleoprotein, phosphoprotein, matrix, and polymerase RNA. The F and HN proteins are involved in adhesion to the surface of cells via antibodies and both proteins produced by the ND virus (Indriani & Dharmayanti, 2016). Antibodies play roles in neutralizing the ND virus by binding and preventing it from attaching to the host cells. Antibodies were detected on the host cells and in the blood from 6 days after ND-virus infection. In chicken, there are three antibody types, IgM, IgY, and IgA, produced as parts of the immune response. Hen plasma contains approximately 30% IgY and 1% IgM and IgA, which are transferred passively to the offspring; if the antibody level is sufficiently high, it can protect against ND virus (Kapczynski *et al.*, 2013).

Antibodies or immunoglobulins are the main product of humoral immunity and are secreted by the plasma cells in response to antigen exposure. In birds, mammals, and lungfish, immunoglobulin (Ig) Y is the main antibody type. IgY is primarily present in the blood and the fluid fraction of the egg in birds, protecting the offspring (Munhoz *et al.,* 2014). According to Kowalczyk *et al.* (1985), the levels of IgY in the bird serum are higher (5–15 mg mL^{-1}) than those of IgM (1-3 mg mL^{-1}) and IgA (0.3–0.5 mg mL^{-1}).

Several studies have reported that β-defensins can promote local/systemic and innate/adaptive immune responses. In sheep, β-defensin-1 was found to be increased after infection with parainfluenza type 3 virus. In addition, in mice, the influenza virus can cause the upregulations of β-defensins-1, -2, and -3 in the lungs. AvBD has shown a clear antiviral activity against pigeon Paramyxovirus type 1, in addition to its antibacterial activity (Liu *et al.,* 2018). A study by Rengaraj *et al.* (2018) showed that chicken AvBD8 protein was strongly expressed in the White Leghorn intestine and in the macrophages and that the expression of AvBD8 gene was highly upregulated in the macrophages; it also showed that the expression and regulation of chicken AvBD8 protein in the immune tissues and cells play crucial roles in innate immunity. AvBDs play crucial roles in host defense as antimicrobial peptides and as immunomodulators by activating the mitogen-activated protein kinase signaling pathway and inducing the expression of proinflammatory cytokines and chemokines (Hong *et al.,* 2020).

In 2019, the Minister of Agriculture of the Republic of Indonesia released the IPB-D1 chicken, produced by the cross of Pelung × Sentul (male) and Kampung × Cobb broiler (female) (Sumantri *et al.*, 2020). IPB-D1 has various superior characteristics, such as faster growth (Al Habib *et al.,* 2020) and resistance to *Salmonella pullorum* (Ulupi *et al.,* 2016), and ND (Sumantri *et al.,* 2020). The information about the genetic variation of the AvBDs genes in Indonesia has been previously reported, especially in IPB-D1 chicken population (Masruroh *et al.,* 2021). However, the effects of this genetic variation on IgY concentration and ND-antibody titer have never been reported. Given the antiviral activities of AvBDs and their important responses to viral infection, this study was established to determine the polymorphism of the *AvBD2* and its association with IgY concentrations and ND-antibody titers in IPB-D1 chickens.

MATERIALS AND METHODS

Experimental Chickens and Blood Collection

A total of 90 IPB-D1 chickens (23 males and 67 females) were used for this study. The IPB-D1 chickens were reared in an intensive system with facilities for feeding, drinking water, and laying eggs. The experimental chickens were fed twice a day, in the morning and in the evening. The feed given was 100% commercial feed for DOC up to 4 weeks old, a 70:30 ratio of commercial feed and rice bran from 4 to 12 weeks old, and a 60:40 ratio of commercial feed and rice bran from 12 to 21 weeks old. The experimental chickens were given ND vaccine twice, i.e., at 3 days old and 3 weeks old. Blood samples were collected at 21 weeks. This study was approved by the Institutional Animal Care and Use Committee of IPB University (approval number 163-2019).

Measurement of IgY Concentration

IgY concentration was measured using indirect ELISA. The microplates were coated with IgG goat anti-IgY (SAB3700195; Sigma-Aldrich), which were added and diluted with bicarbonate buffer (Na_2NO_3, pH 9.6) then incubated overnight at 4°C. The microplates were then washed three times using PBST-20 (pH 7.4) and blocked with 100 µL per well of 2% BSA, followed by incubation for 1 h at 37 °C.

The microplates were subsequently washed three times with 0.05% PBST. One hundred microliters of serum sample at 1:100 dilution were added to each well, followed by incubation for 1 h at 37 °C. After incubation and washing three times with 0.05% PBST, 100 µL of secondary antibody IgG rabbit anti-IgY (A9046; Sigma-Aldrich) was conjugated with the peroxidase enzyme, was added to each well and incubated for 1 h at 37 °C. The microplate was then washed with PBST three times, and 100 µL of TMB substrate was added to each well. Optical density was observed at a wavelength of 450 nm using an ELISA reader.

Analysis of ND-Antibody Titer

The antibody-titer analysis consisted of two steps, hemagglutination test (HA test) for determination of 4 HA units of ND virus and hemagglutination-inhibition test (HI test) to determine the ND-antibody titer in the serum (Rahman *et al.,* 2017). For the HA test, 25 µL of PBS was added to each well of a U-bottomed microplate. A total of 25 µL of the ND-virus suspension was placed into the first well and serially diluted twofold by transferring 25 µL of fluid from each well to the next, and finally discarding 25 µL from the last well. Then, 25 µL of PBS was inserted into each well, followed by 25 µL of 1% chicken RBCs, and incubated at room temperature for 30 min. The results were considered positive when agglutinations occurred.

The HI test started upon adding 25 µL of PBS into each well of a U-bottomed microplate. Twofold serial dilution of field serum (25 µL) was performed up to the 11^{th} well. Standard virus (4 HAU units) was added to each well and then incubated for 30 min. After 30 minutes of incubation, 25 µL of 1% chicken RBCs were added to all wells and incubated for 30 min. The results of HI test were obtained after the hemagglutination-inhibition reactions occurred in the positive-control wells and the final limit of a complete-agglutination inhibition was the result of antibody titer.

Polymorphism Analysis

A total of 90 DNA samples were extracted from the fresh blood samples using the genomic DNA

extraction mini kit (Geneaid™, Taiwan) according to the manufacturer's instructions. Amplification of the *AvBD2* fragment was carried out using a PCR machine (GeneAmp PCR System 9700; Applied Bio Systems). The target of *AvBD2* amplification is a 411-bp DNA sequence covering a part of intron 1 to intron 2 (AY621317.1). The primer sequences are as follows: F: 5′-CCCACAGAGCATCCATGA-3′ and R: 5′-TTGCTGTTGTTGCAGGGTTG-3′. The primers were designed using the Primer Designing Tool application (http://www/ncbi.nlm.nih.gov/tools/primer-blast/). PCR products were visualized in 1.5% agarose gel and observed using a UV Transilluminator (Alpha Imager). The sequencings of PCR products were outsourced to 1st Base in Selangor, Malaysia. The results of DNA sequencing were analyzed using FinchTV, MegaX, and PopGen32.

Data Analysis

Genetic polymorphism was analyzed based on allele and genotype frequencies, Hardy–Weinberg equilibrium test, and determination of heterozygosity using PopGen32. The associations of the SNPs with IgY concentration and ND-antibody titer were analyzed using the general linear model (GLM) procedure with SAS 9.2 software (SAS Institute, Cary, NC, USA), while the least mean square values for genotypes were compared by Duncan's multiple range test (Harter, 1960). Associations were considered significant at $p<0.05$. The statistical model for GLM is as follows:

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

where Y_{ij} is the dependent variable (IgY concentration and ND antibody titer), μ is the mean square value, G_i is the effect of the I genotype, and e_{ij} is the random error.

RESULTS

AvBD2 Mutations in IPB-D1 Chicken

The *AvBD2* in IPB-D1 chicken was successfully amplified at an annealing temperature of 62 °C and produced a PCR product of 411 bp. A total of 10 SNPs were found in three locations: 3 SNPs in intron 1, 3 SNPs in exon 2, and 4 SNPs in intron 2 (Table 1). The SNP g.5002 C>T was also reported by Morammazi & Habibi (2017) in local Iranian chickens, while the other SNPs have not been reported previously. The SNPs g.4841 T>A, g.5111 T>G, g.5116 G>T, and g.5177 G>T were categorized as transversion mutations, while the SNPs g.4853 G>A, g.4859 T>C, g.4881 A>G, g.4889 G>A, g.5002 C>T, and g.5075 C>T were categorized as transition mutations (Figure 1). Only the SNP g.5002 C>T is associated with an amino acid substitution (Figure 2).

Polymorphism of *AvBD2* in IPB-D1 Chicken

Polymorphism of the *AvBD2* in IPB-D1 chicken is presented as genotype frequency, allele frequency, and heterozygosity in Table 2. Each SNP has three genotypes and two alleles, with the exceptions of g.4843 T>A and g.5116 G>T, which have only two genotypes and two alleles (A and T). All SNPs have an allele frequency higher than 1%, indicating that all SNPs in the *AvBD2* in IPB-D1 chicken can be defined as polymorphisms. The H_o value being lower than the H_e value at the SNP g.5075 C>T may indicate the occurrence of inbreeding in the IPB-D1 chicken population.

Association of *AvBD2* Genotypes with IgY Concentration and ND-Antibody Titer

Six SNPs are associated with IgY concentration (Table 3), namely, g.4843 T>A, g.4853 G>A, g.4859 T>A, g.4881 A>G, g.4889 G>A, and g.5002 C>T. The IgY concentration of chickens with the CC genotype of g.5002 C>T was significantly different ($p<0.05$) from chickens with the CT and TT genotypes. In addition, the CC genotype of g.5002 C>T was the genotype with the highest mean IgY-concentration among the SNPs and other genotypes. Meanwhile, SNPs of the *AvBD2* did not show a significant association with ND-antibody titer in IPB-D1 chicken.

DISCUSSION

Identifying genetic variation in genes encoding components of the immune system is one approach to characterize the immune system in chicken, including in the local Indonesian breed IPB-D1. In this study, 10 SNPs were found in three locations of the *AvBD2*: g.4843 T>A, g.4853 G>A, and g.4859 T>C in intron 1; g.4881 A>G, g.4889 G>A, and g.5002 C>T in exon 2; and g.5075 C>T, g.5111 T>G, g.5116 G>T, and g.5177 G>T in intron 2. The SNP g.5002 C>T was also previously reported by Morammazi & Habibi (2017). Six of the ten SNPs are classified as transition mutations (g.4853 G>A, g.4859 T>C, g.4881 A>G, g.4889 G>A, g.5002 C>T, and g.5075 C>T). All SNPs in exon 2 were categorized as transition mutations, according to the report by Guo *et al.* (2017), stating that transition mutations are more common in protein-coding regions. On the other hand, transversions are mutations involving the conversion of purine to pyrimidine or pyrimidine to purine. Transversion mutations have a greater impact on the function of regu-

Table 1. SNP positions and amino acid changes of AvBD2 gene in IPB-D1 chicken

No.	SNPs	Location	Amino acid change
1	g.4843 T>A	Intron 1	-
2	g.4853 G>A	Intron 1	-
3	g.4859 T>C	Intron 1	-
4	g.4881 A>G	Exon 2	-
5	g.4889 G>A	Exon 2	-
6	g.5002 C>T*	Exon 2	Ala to Val
7	g.5075 C>T	Intron 2	-
8	g.5111 T>G	Intron 2	-
9	g.5116 G>T	Intron 2	-
10	g.5177 G>T	Intron 2	-

Note: Also reported by Morammazi & Habibi (2017).

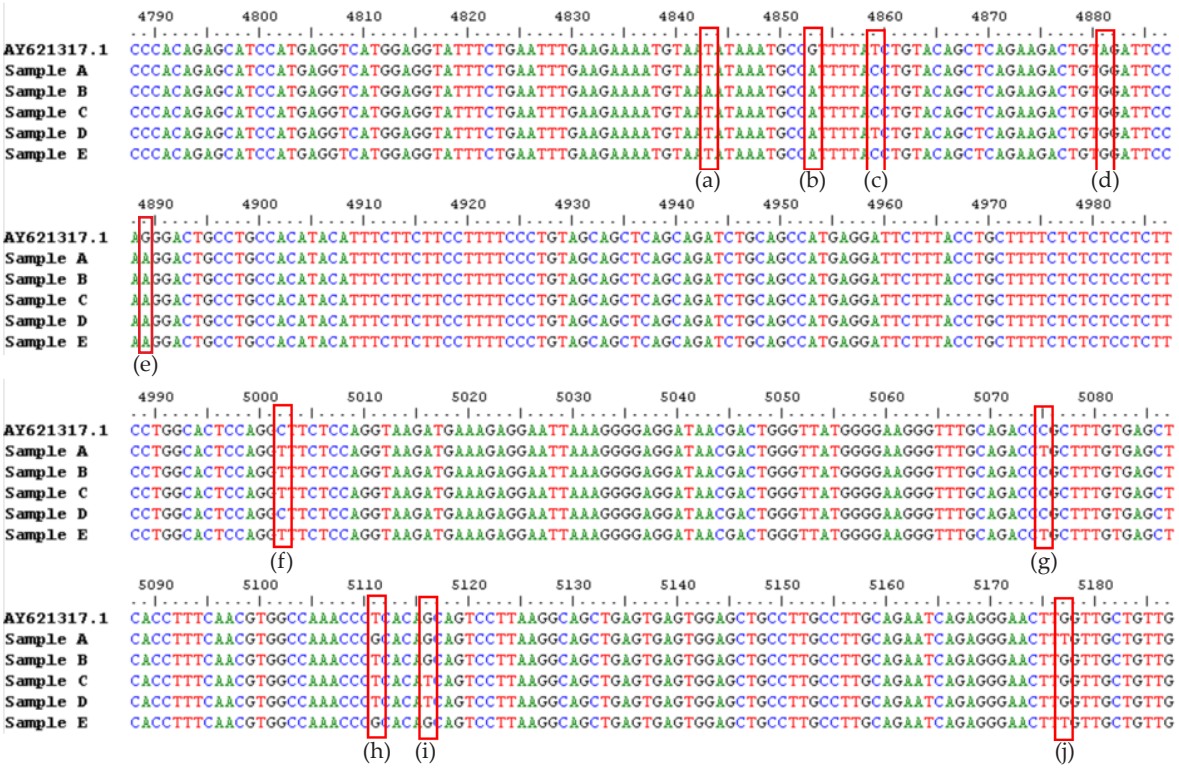

Figure 1. SNPs position of AvBD2 gene in IPB-D1 chicken, i.e g.4843T>A (a), g.4853 G>A (b), g.4859 T>C (c), g.4881 A>G (d), g.4889 G>A (e), g.5002 C>T (f), g.5075 C>T (g), g.5111 T>G (h), g.5116 G>T (i), and g.5117 G>T (j).

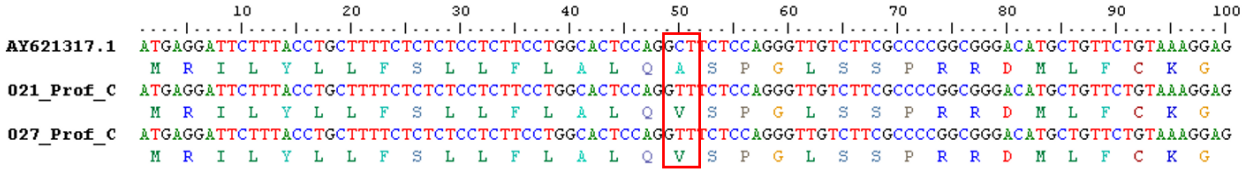

Figure 2. Amino acid changes alanine to valine of SNP g.5002 C>T

latory elements, according to Guo *et al.* (2017), and non-coding variants are predicted to contribute significantly to human traits and diseases.

g.5002 C>T in exon 2 causes an amino acid change because it is located in a coding region, while g.4881 A>G and g.4889 G>A do not involve changes in amino acid sequence because they are located in non-coding regions. An amino acid change from alanine to valine is occurred due to g.5002 C>T. This amino acid change was also reported by Morammazi & Habibi (2017). Valine is a component of BCAAs (branched-chain amino acids), besides leucine and isoleucine. In humans and animals, BCAAs play an important role in maintaining immune function (Cruzat *et al.*, 2014). Li *et al.* (2007) also stated that BCAAs play a major role in regulating protein synthesis and activating cytokine and antibody production through mTOR signaling.

In this study, all SNPs had two alleles and three genotypes, except the SNPs g.4843 T>A and g.5116 G>T, which had only two alleles and two genotypes. Each allele in each SNP had an allele frequency higher than 0.01, so all SNPs could be formally categorized as involving polymorphisms. Allendorf & Luikart (2007) stated that SNPs could be categorized as polymorphic if the allele frequency is ≤ 0.99 in a large population and ≤ 0.95 in a small population. Each SNP was shown to have three genotypes, with the exceptions of g.4843 T>A and g.5116 G>T, which had only two genotypes, namely, TT and TA, with the TT genotype having the highest genotype frequency (0.94), and GG and TT, with the GG genotype having the highest genotype frequency (0.69).

All H_o and H_e values range from 0 to 1. All SNPs had Ho higher than He in this study, except for g.5075 C>T. If H_o is less than H_e, this is suggestive of inbreeding in the population (Mukhopadhyay & Bhattacharjee, 2016), so the findings may indicate the occurrence of inbreeding in the IPB-D1 chicken population.

The IgY concentrations in IPB-D1 chickens were observed to be in the range of 3–15 mg mL^{-1}. Kowalczyk *et al.* (1985) stated that IgY is present at a higher level (5 to 15 mg mL^{-1}) than IgM (1 to 3 mg mL^{-1}) and IgA (0.3 to 0.5 mg mL^{-1}) in the bird serum. The results of this study showed that 6 out of the 10 SNPs were associated with IgY concentration. Specifically, there were significant differences in the IgY concentrations ($p<0.05$) between a) the TT genotype of g.4843 T>A and the AA genotype, b)

Table 2. Genotype frequency, allele frequency, and heterozygosity of AvBD2 gene in IPB-D1 chicken

SNP	N	Genotype frequency			Allele frequency		H_o	H_e
g.4843 T>A	90	TT	TA	AA	T	A	0.05	0.05
		0.94	0.06	0.00	0.98	0.02		
g.4853 G>A	90	GG	GA	AA	G	A	0.50	0.40
		0.02	0.51	0.47	0.27	0.73		
g.4859 T>C	90	TT	TC	CC	T	C	0.53	0.43
		0.04	0.50	0.46	0.31	0.69		
g.4881 A>G	90	AA	AG	GG	A	G	0.58	0.44
		0.04	0.58	0.38	0.32	0.68		
g.4889 G>A	90	GG	GA	AA	G	A	0.52	0.44
		0.05	0.53	0.42	0.33	0.67		
g.5002 C>T	90	CC	CT	TT	C	T	0.57	0.42
		0.02	0.57	0.41	0.30	0.70		
g.5075 C>T	90	CC	CT	TT	C	T	0.36	0.47
		0.19	0.36	0.45	0.38	0.62		
g.5111 T>G	90	TT	TG	GG	T	G	0.36	0.33
		0.6	0.37	0.03	0.79	0.21		
g.5116 G>T	90	GG	GT	TT	G	T	0.30	0.26
		0.69	0.31	0.00	0.85	0.15		
g.5177 G>T	90	GG	GT	TT	G	T	0.50	0.48
		0.33	0.51	0.16	0.59	0.41		

Note: N=total sample; Ho=observed heterozygosity; He=expected heterozygosity.

Table 3. Association of AvBD2 gene genotypes with IgY concentration and ND-antibody titer in IPB-D1 chicken

SNPs	Parameter	Genotype (n)		
g.4843 T>A		TT	TA	AA
	IgY (mg mL^{-1})	12.25 ± 2.98^{a} (5)	9.28 ± 2.94^{b} (85)	0
	ND-antibody titer (log 2 HI unit)	3.35±2.27 (77)	2.20±2.04 (5)	0
g.4853 G>A		GG	GA	AA
	IgY (mg mL^{-1})	6.12 ± 1.72^{b} (2)	9.17 ± 2.55^{ab} (48)	9.85 ± 3.39^{a} (42)
	ND-antibody titer (log 2 HI unit)	5.00±0.00 (1)	3.43±3.01 (41)	3.07±2.36 (40)
g.4859 T>C		TT	TC	CC
	IgY (mg mL^{-1})	11.99 ± 4.19^{a} (4)	9.89 ± 3.27^{ab} (48)	8.56 ± 2.21^{b} (38)
	ND-antibody titer (log 2 HI unit)	3.33±2.88 (3)	3.51±2.97 (43)	3.00±2.35(34)
g.4881 A>G		AA	AG	GG
	IgY (mg mL^{-1})	14.87 ± 2.09^{a} (3)	9.78 ± 3.10^{b} (52)	8.42 ± 2.23^{b} (35)
	ND-antibody titer (log 2 HI unit)	4.50±0.70 (2)	3.41±2.94 (46)	3.02±2.41 (34)
g.4889 G>A		GG	GA	AA
	IgY (mg mL^{-1})	11.72 ± 3.59^{a} (6)	9.58 ± 3.09^{ab} (47)	8.85 ± 2.63^{b} (37)
	ND-antibody titer (log 2 HI unit)	3.20±2.16 (5)	3.39±2.95 (43)	3.14±2.47 (34)
g.5002 C>T		CC	CT	TT
	IgY (mg mL^{-1})	15.16 ± 2.87^{a} (2)	9.84 ± 3.19^{b} (51)	8.53 ± 2.23^{b} (37)
	ND-antibody titer (log 2 HI unit)	5.00±0.00 (1)	3.45±2.93 (46)	3.00±2.38 (35)
g.5075 C>T		CC	CT	TT
	IgY (mg mL^{-1})	10.37±3.58 (17)	9.40±2.99 (33)	9.04±2.72 (40)
	ND-antibody titer (log 2 HI unit)	3.75±2.59 (16)	3.20±3.29 (29)	3.13±2.22 (37)
g.5111 T>G		TT	TG	GG
	IgY (mg mL^{-1})	9.69±3.29 (24)	9.00±2.56 (33)	9.17±1.46 (3)
	ND-antibody titer (log 2 HI unit)	3.48±2.64 (49)	2.80±2.74 (30)	4.66±3.05 (3)
g.5116 G>T		GG	GT	TT
	IgY (mg mL^{-1})	9.50±2.83 (63)	9.24±3.40 (27)	0
	ND-antibody titer (log 2 HI unit)	3.30±2.86 (56)	3.23±2.33 (26)	0
g.5177 G>T		GG	GT	TT
	IgY (mg mL^{-1})	9.67±3.87 (29)	9.44±2.71 (46)	8.90±1.77 (15)
	ND-antibody titer (log 2 HI unit)	3.80±2.36 (26)	2.92±2.88 (42)	3.35±2.70 (14)

Note: Means in the same row with different superscripts differ significantly between genotypes ($p<0.05$). n= total sample.

the AA genotype of g.4853 G>A and the GG genotype, c) the TT genotype of g.4859 T>C and the CC genotype, d) the AA genotype of g.4881 A>G and the GG genotype, e) the GG genotype of g.4889 G>A and the AA genotype, and f) the CC genotype of g.5002 C>T and the CT and TT genotypes, with the CC genotype having the highest mean IgY being 15.16±2.87 mg mL^{-1}.

Antimicrobial peptides (AMPs) are essential components of the innate immune system. The AMP gene developed the same peptide that responds to pathogens in general, but the peptide can function on various pathogens. β-Defensins are AMPs that protect against various pathogens, including fungi, bacteria, viruses, and even protozoa (van Dijk *et al.*, 2008). Some defensins can promote local-innate and systemic-adaptive immune responses in addition to their direct antimicrobial activities (Cuperus *et al.*, 2013). In the chicken genome, 14 distinct avian β-defensin (AvBD) genes have been discovered (Lynn *et al.*, 2004). According to Liu *et al.* (2018), the *AvBD2* plays a critical role in the host's innate immune response to Newcastle Disease Virus (NDV) infection and is regulated by p38-MAPK.

This study showed that the titer of ND antibody did not differ significantly among the genotypes and SNPs. The insignificant differences in the titer of ND antibody mean that the body does not overreact in producing specific antibodies to ND virus. Chickens infected by ND viruses and those unexposed were reported to have the same antibody (Al Habib *et al.*, 2020). The titers of ND antibody in IPB-D1 chickens were reported to be between 2.2 and 5 log 2 HI units. Hossain (2010) stated that the antibody titer level for ND < 3 log 2 HI units is low, while > 3 log 2 HI units are categorized as high and generally accepted as indicating positivity for specific immunity. The results of this study are expected to be helpful for the genetic selection of disease resistance in local Indonesian chickens, especially IPB-D1 chickens.

CONCLUSION

A total of 10 SNPs of the *AvBD2* were found in intron 1, exon 2, and intron 2 in IPB-sD1 chicken. All SNPs are formally defined as polymorphic. Six of these SNPs were found to be associated with IgY concentration. In addition, the CC genotype of g.5002 C>T was shown to have the potential for use in the selection of chickens with high levels of IgY. No SNPs were associated with the titer of ND antibody in this study.

CONFLICT OF INTEREST

Asep Gunawan and Cece Sumantri serve as editor of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the materials discussed in this manuscript.

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