



## Association of IFN- $\gamma$ Gene Polymorphisms with Avian Influenza-Related Immune Traits in Indonesian Local Chickens

W. Kuswandi<sup>1</sup>, C. Sumantri<sup>1\*,#</sup>, K. Listyarini<sup>1</sup>, S. Darwati<sup>1</sup>, R. H. Mulyono<sup>1</sup>, & S. Murtini<sup>2\*,#</sup>

<sup>1</sup>Department of Animal Production and Technology, Faculty of Animal Science, IPB University

<sup>2</sup>Medical Microbiology Division, School of Veterinary Medicine and Biomedical Sciences, IPB University,  
Jl. Agatis, Kampus IPB Dramaga, Bogor, West Java, Indonesia

\*Corresponding authors: [ceces@apps.ipb.ac.id](mailto:ceces@apps.ipb.ac.id); [srilmurtini\\_fkh@apps.ipb.ac.id](mailto:srilmurtini_fkh@apps.ipb.ac.id)

<sup>#</sup>Both corresponding authors have equal contributions

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### ABSTRACT

Avian influenza (AI) remains a major threat to poultry production, and genetic improvement for disease resistance has emerged as a promising complementary strategy alongside vaccination and biosecurity measures. Interferon-gamma (IFN- $\gamma$ ) is a key cytokine involved in antiviral immunity; however, information regarding its genetic diversity and association with AI-related immune responses in Indonesian local chickens remains limited. This study aimed to identify single-nucleotide polymorphisms (SNPs) in the chicken IFN- $\gamma$  gene and evaluate their associations with AI-related immune response traits. A total of 94 chickens, comprising IPB D1 (n = 60) and Merawangarab chickens (n = 34), were used. Blood samples were collected for DNA extraction, hematological analysis, quantification of immunoglobulin Y (IgY), and determination of avian influenza antibody titers. A 410 bp fragment of the IFN- $\gamma$  gene was amplified and sequenced, revealing two SNPs located in exon 4, namely c.304C>G and c.390G>A. Genotype and allele frequencies were estimated, and association analyses were performed using a General Linear Model (GLM). Significant genotype effects were further compared using Tukey's multiple comparison test. The C allele of c.304C>G and the G allele of c.390G>A were predominant in both chicken populations. Comparison of genotype distributions between populations revealed a significant difference for SNP c.304C>G ( $\chi^2 = 8.713$ ,  $p = 0.013$ ), whereas no significant difference was observed for SNP c.390G>A ( $\chi^2 = 3.784$ ,  $p = 0.151$ ). SNP c.304C>G was significantly associated with AI antibody titer, total leukocyte count, and eosinophil percentage, whereas SNP c.390G>A was significantly associated with AI antibody titer, total leukocyte count, heterophil percentage, and heterophil-to-lymphocyte ratio ( $p < 0.05$ ). These findings indicate that IFN- $\gamma$  gene polymorphisms are associated with variation in AI-related immune responses in Indonesian local chickens and highlight IFN- $\gamma$  as a candidate gene for marker-assisted selection to improve immune competence and disease resilience in poultry.

**Keywords:** Avian influenza; IFN- $\gamma$  gene; immune-related traits; Indonesian local chickens; single nucleotide polymorphism (SNP)

### INTRODUCTION

Avian influenza (AI) remains one of the most economically important viral diseases affecting the global poultry industry, particularly highly pathogenic avian influenza (HPAI) H5N1, which poses a major threat to poultry production and public health by causing severe disease and mortality in chickens (Ayuti *et al.*, 2024; Simancas-Racines *et al.*, 2025). AI infection can cause respiratory distress, cyanosis, hemorrhage, sudden death, and damage to multiple organs (Kim *et al.*, 2025). In Indonesia, the human AI case fatality rate (CFR) has reached approximately 84%, exceeding the regional average of 66.3% (Pawestri *et al.*, 2020), while HPAI H5N1 outbreaks reported in several provinces in 2026 resulted in 14,125 poultry deaths (Ministry of Agriculture of the Republic of Indonesia, 2026). Despite

vaccination, biosecurity, and molecular surveillance (Sajjadi *et al.*, 2025), including strengthened vaccination programs, PCR-based detection, and One Health coordination during 2025–2026, AI control remains challenging due to viral mutation and antigenic drift. These challenges highlight the need for complementary and sustainable strategies, including marker-assisted selection (MAS) to identify favorable alleles associated with enhanced immune responsiveness and disease resistance. In this context, the genetic diversity and adaptability of Indonesian chickens provide valuable resources for identifying immune-related genetic markers to support breeding programs aimed at improving disease resistance.

The application of genetic approaches to disease resistance requires chicken populations with diverse genetic backgrounds and well-characterized phenotypic

traits. In this regard, IPB D1 and Merawangarab chickens represent relevant Indonesian genetic resources for investigating variation in immune-related traits. IPB D1 is a composite breed derived from F1 PS (Pelung × Sentul) males and F1 KM (Kampung × Cobb) females, comprising approximately 75% indigenous germplasm and 25% commercial broiler ancestry, and was officially recognized under Ministry of Agriculture Decree No. 693/KPTS/PK.230/M/9/2019. IPB D1 chickens reach approximately 1.2–1.3 kg body weight at 12 weeks of age (Prawira *et al.*, 2026) and have demonstrated protective antibody titers against Newcastle disease ( $\geq 3.5 \log_2$  HI) (Putri *et al.*, 2022). Merawangarab chickens, derived from crosses between Merawang and Arabian chickens (Irmaya *et al.*, 2024), attain an average body weight of  $1,014.49 \pm 35.98$  g at 12 weeks of age and  $1,154.11 \pm 94.42$  g at 16 weeks of age, with an average egg weight of  $42.68 \pm 1.16$  g (Depison *et al.*, 2025).

Despite their distinct genetic backgrounds and favorable production characteristics, the genetic basis underlying immune responses to avian influenza (AI) in IPB D1 and Merawangarab chickens remains poorly understood. Interferon-gamma (IFN- $\gamma$ ), primarily produced by T helper 1 (Th1) and natural killer (NK) cells, plays a central role in antiviral immunity by regulating macrophage activation, antigen presentation, and T-cell and NK-cell activity (Frank & Paust, 2020). Experimental evidence supports its antiviral activity against avian influenza viruses. Chicken IFN- $\gamma$  (chIFN- $\gamma$ ) has been shown to inhibit viral RNA replication and enhance interferon-stimulated genes and type I interferons (Yuk *et al.*, 2016), while exerting stronger inhibitory effects on H9N2 replication than chicken IFN- $\alpha$  and IFN- $\beta$  (Song *et al.*, 2022). These findings support IFN- $\gamma$  as a candidate gene for investigating genetic variation associated with antiviral immune responses.

Genetic variation in the chicken IFN- $\gamma$  gene has been characterized through single-nucleotide polymorphisms (SNPs). According to Ji *et al.* (2015), 52 SNPs were identified within a 1,137-bp region spanning the promoter and all four exons, including the -316A/G polymorphism associated with antibody responses following H5N1 vaccination in Red Junglefowl, with the AA genotype exhibiting higher antibody titers than the GG genotype. Several coding-region polymorphisms, including a novel missense mutation, were also identified, although their associations with immune-related traits remain insufficiently

characterized. The chicken IFN- $\gamma$  gene is located on chromosome 1, spans approximately 4,154 bp, and comprises four exons and three introns, with exons 2–4 encoding most of the mature protein (Kaiser *et al.*, 1998). Exon 4 encodes the C-terminal region of mature IFN- $\gamma$  and contains the translation termination codon, followed by the 3' untranslated region (3'UTR). Because nucleotide variation within exon 4 may affect protein-coding or regulatory functions, this region represents a biologically relevant target for investigating polymorphisms associated with immune response traits.

However, information on coding-region polymorphisms of the IFN- $\gamma$  gene and their associations with immune-related traits remains limited, particularly in Indonesian chicken populations. To the best of our knowledge, the association of IFN- $\gamma$  polymorphisms with avian influenza (AI)-related hematological and immune response traits has not been investigated in IPB D1 and Merawangarab chickens. Therefore, this study aimed to identify single-nucleotide polymorphisms (SNPs) within exon 4 of the IFN- $\gamma$  gene and evaluate their associations with hematological parameters and AI-related immune response traits. The findings may provide insights into the genetic basis of antiviral immune responsiveness and facilitate the identification of potential molecular markers for marker-assisted selection toward improved disease resistance in Indonesian chicken breeding programs.

## MATERIALS AND METHODS

### Ethical Approval

All procedures in this study were conducted in accordance with animal welfare principles and were approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University, under approval number 209/KEH/SKE/IV/2024.

### Experimental Animals and Management

A total of 94 chickens, approximately 2 years of age, were included in this study, comprising 60 IPB D1 chickens (15 males and 45 females) and 34 Merawangarab chickens (10 males and 24 females) (Figure 1). All birds were reared under intensive management at the IPB D1 Chicken Breeding Center, IPB University, Indonesia, in well-ventilated  $3 \times 3$  m



Figure 1. Phenotypic appearance of female IPB D1 (A), male IPB D1 (B), female Merawangarab (C), and male Merawangarab (D)

litter-floor pens at a stocking density of 15 birds per pen (approximately 0.6 m<sup>2</sup> per bird). Standard management and biosecurity practices, including routine pen sanitation, restricted access to the farm area, and regular health monitoring, were maintained throughout the study. The chickens were fed a commercial layer diet (324 KJ, PT Charoen Pokphand Indonesia Tbk, Indonesia) twice daily, containing 17.5%–18.5% crude protein, ≥3.0% crude fat, ≤7.0% crude fiber, ≤14.0% ash, 3.25%–4.25% calcium, and ≥0.55% phosphorus. None of the chickens had received avian influenza or other vaccinations before sampling, nor were they experimentally challenged with avian influenza virus. Therefore, the antibody titers measured in this study were considered to reflect naturally acquired immune responses under routine farm conditions.

### Sample Collection

Blood samples were collected from the brachial vein (wing vein) using sterile 3 mL syringes fitted with 23-gauge needles. Approximately 2 mL of blood was collected from each bird without anesthesia following disinfection of the venipuncture area with 70% alcohol. Blood samples were transferred into two types of tubes: EDTA-containing tubes for hematological analysis and DNA extraction, and non-anticoagulant tubes for serum collection used in Immunoglobulin Y (IgY) analysis and avian influenza antibody titers.

Blood samples collected in EDTA tubes were gently homogenized to ensure thorough mixing with the anticoagulant, then stored at approximately 4°C until further analysis. Hematological analyses were performed according to standard laboratory procedures.

### Hematological Analysis

**Erythrocyte count.** Erythrocyte counts were determined using an Improved Double Neubauer counting chamber (Germany). Blood samples collected in EDTA tubes were diluted with Hayem's solution (France) and gently homogenized using a figure-eight motion. The diluted samples were subsequently loaded into a hemocytometer and observed under a microscope at 10× magnification, as described by Pujiastuti *et al.* (2017).

**Hemoglobin and hematocrit measurement.** Hemoglobin concentration and hematocrit values were measured using a Mindray BC-2800 Vet Auto Hematology Analyzer (Mindray, China). Blood samples were introduced into the analyzer via the designated nozzle, and the measurement results were automatically recorded.

**Erythrocyte indices.** Erythrocyte indices were calculated to evaluate red blood cell (RBC) characteristics and to assist in the detection of anemia-related conditions. The calculated indices included mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), following the method described by Sarma (1990) using the following formulas:

$$\text{MCV} = \text{Hematocrit (\%}\times 10) / \text{Erythrocyte (million}/\mu\text{L)}$$

$$\text{MCH} = \text{Hemoglobin (g/dL)} / \text{Erythrocyte count (million}/\mu\text{L)}$$

$$\text{MCHC} = \text{Hemoglobin (g/dL)} / \text{Hematocrit (\%}\times 10)$$

**Total leukocyte count.** Total leukocyte counts were determined using an Improved Neubauer hemocytometer following the method of Maheshwari *et al.* (2017). Blood samples were diluted with Rees and Ecker solution, homogenized, and loaded into the counting chamber. Leukocytes were counted manually under a microscope at 400× magnification in four large squares of the chamber. The total leukocyte concentration was calculated using a standard dilution factor.

**Differential leukocyte count.** Differential leukocyte counts were performed using Giemsa-stained blood smears. Blood smears were fixed with methanol, stained with Giemsa solution, and examined under a microscope at 100× magnification. Leukocyte types, including lymphocytes, monocytes, heterophils, eosinophils, and basophils, were identified and counted using the battlement method. At least 100 leukocytes were counted per slide, and the percentage of each leukocyte type was calculated as described by Thiam *et al.* (2021).

**Heterophil-to-Lymphocyte (H/L) ratio.** The heterophil-to-lymphocyte (H/L) ratio was calculated by comparing the number of heterophils to the number of lymphocytes obtained from the differential leukocyte count. The H/L ratio was calculated using the following formula:

$$\text{H/L} = \text{Heterophils (\%)} / \text{Lymphocyte count (\%)}$$

**Total serum IgY concentration.** Total serum immunoglobulin Y (IgY) concentration was measured using an indirect enzyme-linked immunosorbent assay (ELISA) adapted from Indriyani *et al.* (2024). Anti-IgY was diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to a final concentration of 2.5 µg/mL, and 100 µL was added to each well and incubated overnight at 4 °C. The plates were washed four times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 5% skim milk in PBS at 37 °C for 2 h. Serum samples diluted 1:100 were then added and incubated at 37 °C for 1 h, followed by incubation with 100 µL of HRP-conjugated anti-IgY diluted 1:25,000 at 37 °C for 1 h. After washing, 100 µL of tetramethylbenzidine (TMB) substrate was added, and the reaction was incubated for 20–30 min. Optical density was measured at 650 nm using an ELISA reader, and IgY concentrations were determined using an IgY control standard.

### Hemagglutination Inhibition (HI) Assay for Avian Influenza

Avian Influenza (AI) antibody titers were measured using the hemagglutination inhibition (HI) assay, as described by Novitasari and Anwar (2020). Briefly, 50 µL of phosphate-buffered saline (PBS) and

50 µL of the serum sample were added to a 96-well microplate, and the sample was serially diluted up to the 11th well. Subsequently, 50 µL of AI antigen containing 4 HAU was added and incubated at room temperature for 30 min, followed by the addition of 50 µL of 0.5%–1% chicken red blood cell (RBC) suspension. Hemagglutination inhibition was observed after incubation at room temperature for 30–45 min, and antibody titers were expressed as log<sub>2</sub> HI values.

### DNA Extraction

Genomic DNA was extracted from blood samples using the Promega Wizard® Genomic DNA Purification Kit following the manufacturer's protocol with minor modifications. Briefly, 50 µL of fresh blood was transferred to a 1.5 mL microtube and mixed with 250 µL of phosphate-buffered saline (PBS). Samples were lysed with cell lysis solution, then with nucleus lysis solution, and finally treated with RNase. Protein precipitation was performed with protein precipitation solution, and DNA was precipitated with isopropanol, washed with 70% ethanol, and air-dried at room temperature. The DNA pellet was rehydrated in DNA rehydration solution, incubated overnight at 4 °C, and stored at –20 °C until further analysis.

### Amplification and Sequencing

Amplification of the chicken Interferon-Gamma (IFN-γ) gene was performed using a self-designed primer pair generated with Primer3 (<https://primer3.ut.ee/>) and evaluated using Primer Stats ([https://www.bioinformatics.org/sms2/pcrprimer\\_stats.html](https://www.bioinformatics.org/sms2/pcrprimer_stats.html)) based on the reference sequence GenBank accession Y07922.1. The primer pair was designed to amplify a 410 bp fragment spanning exon 4 of the IFN-γ gene. The forward primer sequence was 5'-GGCATCTCAAACCAGCAATG-3', and the reverse primer sequence was 5'-ATACTTCACCACACCACCTC-3'. The PCR reaction was prepared in a total volume of 26 µL, consisting of 2 µL of genomic DNA template, 0.3 µL of forward primer, 0.3 µL of reverse primer, 12.5 µL of Red Master Mix, and 10.9 µL of nuclease-free water. The PCR program consisted of a pre-denaturation step at 95 °C for 1 minute, followed by denaturation at 95 °C for 15 seconds, annealing at 64 °C for 15 seconds, and extension at 72 °C for 10 seconds, for 35 cycles, with a final extension at 72 °C for 5 minutes. The amplification products were visualized on a 1.5% agarose gel by electrophoresis, stained with a DNA stain, and observed under a UV transilluminator. Successfully amplified PCR products were subsequently sequenced using the Sanger sequencing method by MacroGen (Singapore).

### Data Analysis

Single-nucleotide polymorphisms (SNPs) were identified from sequencing chromatograms using FinchTV version 1.4.0 (<http://www.geospiza.com/>), with double peaks considered heterozygous. The IFN-γ gene sequences were aligned with the reference

sequence (GenBank accession no. Y07922.1) using Molecular Evolutionary Genetics Analysis (MEGA) version X software. Descriptive analyses of immune-related and hematological parameters were conducted to summarize the general characteristics of the study data. Allele and genotype frequencies were calculated using the following formulas, following Nei and Kumar (2000):

$$X_i = \frac{(2n_{ii} + \sum j \neq i n_{ij})}{2N} \quad X_{ii} = \frac{n_{ii}}{N}$$

where  $X_i$  represents the allele frequency,  $X_{ii}$  represents the genotype frequency,  $n_{ii}$  is the number of individuals with genotype  $ii$ ,  $n_{ij}$  is the number of individuals with genotype  $ij$ , and  $N$  is the total population size.

Observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) were calculated using the following formulas according to Allendorf *et al.* (2010):

$$H_o = N_h / N$$

$$H_e = 1 - \sum p_i^2$$

where  $H_o$  is the observed heterozygosity,  $N_h$  is the number of heterozygous individuals,  $H_e$  is the expected heterozygosity, and  $p_i$  is the frequency of the  $i$ -th allele.

Hardy–Weinberg equilibrium was evaluated using the Chi-square ( $\chi^2$ ) test according to Hartl and Clark (1997):

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

where  $\chi^2$  represents the Chi-square value,  $O$  is the observed value, and  $E$  is the expected value.

Associations between each identified SNP in the IFN-γ gene and immune-related traits were analyzed independently using the General Linear Model (GLM) in Minitab 19. The statistical model was adapted from Amusan *et al.* (2013), and Tukey's post hoc test was applied for multiple comparisons among genotype means.

$$Y_{ijk} = \mu + B_i + G_j + e_{ijk}$$

where  $Y_{ij}$  is the observed phenotypic value of the measured trait,  $\mu$  is the overall mean,  $B_i$  is the fixed effect of chicken breed ( $i$  = IPB D1 and Merawangarab),  $G_j$  is the fixed effect of genotype, and  $e_{ij}$  is the random residual error assumed to be independent and normally distributed with mean zero and variance  $\sigma_e^2$ .

## RESULTS

### IFN-γ Gene Amplification and SNP Identification

The primer pair successfully amplified the expected 410 bp fragment encompassing exon 4 of the chicken IFN-γ gene. Agarose gel electrophoresis showed a single, distinct PCR product without non-specific amplification (Figure 2), confirming the specificity of the designed primers. Subsequent Sanger sequencing identified two single-nucleotide polymorphisms (SNPs), namely c.304C>G and c.390G>A, within the amplified region of the IFN-γ gene (Figure 3). The c.304C>G polymorphism was identified as a synonymous variant

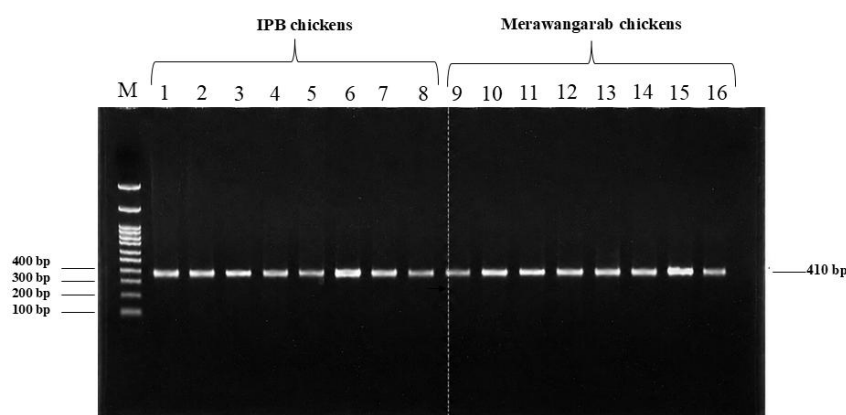


Figure 2. PCR amplification of the IFN- $\gamma$  gene in IPB D1 and Merawangarab chickens showing a 410-bp amplified fragment. Lanes 1–8 represent IPB D1 chickens, lanes 9–16 represent Merawangarab chickens, and M represents the 100-bp DNA ladder.

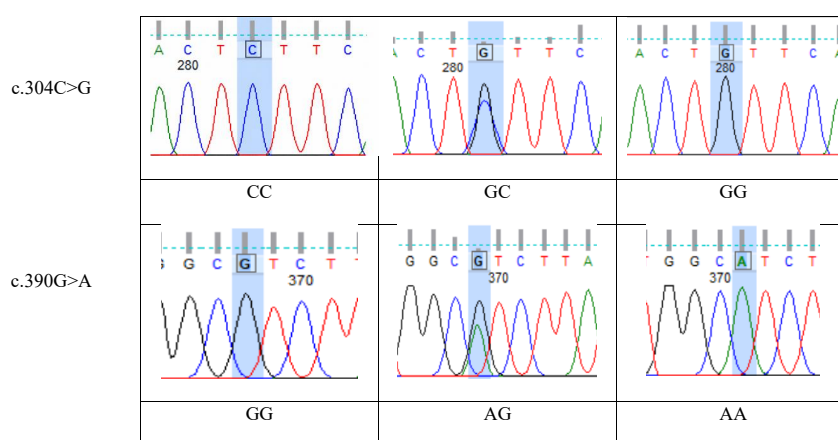


Figure 3. Chromatogram showing mutations (SNPs) in the chicken IFN- $\gamma$  gene

within the coding region, whereas the c.390G>A polymorphism was located in the 3' untranslated region (3'UTR). The sequence chromatograms exhibited clear homozygous and heterozygous nucleotide peaks, confirming the presence of genetic variation at both loci.

#### Descriptive Statistics of Avian Influenza-Related Immune Response Traits in Indonesian Local Chickens

The descriptive statistics of hematological and avian influenza (AI)-related immune response traits in IPB D1 and Merawangarab chickens are presented in Table 1. Mean AI antibody titers were  $1.37 \pm 0.13 \log_2$  HI in IPB D1 and  $1.00 \pm 0.09 \log_2$  HI in Merawangarab chickens, while serum IgY concentrations were  $12.71 \pm 0.52$  and  $12.46 \pm 0.47$  mg/mL, respectively. Erythrocyte counts were  $2.84 \pm 0.07 \times 10^6$  cells/mm<sup>3</sup> in IPB D1 and  $2.84 \pm 0.13 \times 10^6$  cells/mm<sup>3</sup> in Merawangarab chickens, with corresponding hemoglobin concentrations of  $8.79 \pm 0.14$  and  $8.99 \pm 0.30$  g/dL and hematocrit values of  $30.40 \pm 0.65\%$  and  $28.85 \pm 0.98\%$ . The erythrocyte indices, including MCV, MCH, and MCHC, were comparable between the two populations. Total leukocyte counts were  $19.56 \pm 0.37 \times 10^3$  cells/mm<sup>3</sup> in IPB D1 and  $17.36 \pm 0.42 \times 10^3$  cells/mm<sup>3</sup> in Merawangarab chickens. Lymphocytes were the predominant leukocyte

population in IPB D1 and Merawangarab chickens ( $48.13 \pm 0.53\%$  and  $48.85 \pm 0.80\%$ , respectively), followed by heterophils ( $31.21 \pm 0.47\%$  and  $29.52 \pm 0.66\%$ , respectively). Eosinophils, monocytes, and basophils ranged from 10.87–11.99%, 8.87–9.01%, and 0.77–0.78%, respectively. The H/L ratio was  $0.65 \pm 0.02$  in IPB D1 and  $0.61 \pm 0.02$  in Merawangarab chickens.

#### Genetic Diversity of IFN- $\gamma$ Gene Polymorphisms

Two SNPs were identified within exon 4 of the IFN- $\gamma$  gene, namely c.304C>G and c.390G>A. The genotype and allele frequencies of both SNPs are presented in Tables 2 and 4, whereas the observed and expected genotype frequencies under Hardy-Weinberg equilibrium are shown in Tables 3 and 5. For SNP c.304C>G, three genotypes (CC, CG, and GG) were detected in both populations. The CC genotype was the predominant genotype in IPB D1 (0.73) and Merawangarab chickens (0.97), whereas the GG genotype occurred at relatively low frequencies in both populations. Consistently, the C allele was the major allele, accounting for 83% and 97% of alleles in IPB D1 and Merawangarab chickens, respectively (Table 2).

Similarly, SNP c.390G>A exhibited three genotypes (GG, AG, and AA). The GG genotype was the most prevalent genotype in both IPB D1 (0.75) and

Table 1. Descriptive statistics of hematological and immune response traits in IPB D1 and Merawangarab chickens

| Variables                                                  | IPB D1 chickens (n=60) | Merawangarab chickens (n=34) | Reference value                                          |
|------------------------------------------------------------|------------------------|------------------------------|----------------------------------------------------------|
|                                                            | Mean ± SE              | Mean ± SE                    |                                                          |
| Immune response                                            |                        |                              |                                                          |
| Avian influenza antibody titer (log <sub>2</sub> HI)       | 1.37 ± 0.13            | 1.00 ± 0.09                  | Seropositive titer ≥4 log <sub>2</sub> HI <sup>(1)</sup> |
| IgY (mg/mL)                                                | 12.71 ± 0.52           | 12.46 ± 0.47                 | 5-15 <sup>(2)</sup>                                      |
| Erythrocyte profile                                        |                        |                              |                                                          |
| Erythrocytes (×10 <sup>6</sup> cells/mm <sup>3</sup> )     | 2.84 ± 0.07            | 2.84 ± 0.13                  | 2.5-3.9 <sup>(3)</sup>                                   |
| Hemoglobin (g/dL)                                          | 8.79 ± 0.14            | 8.99 ± 0.30                  | 10.2-15.1 <sup>(3)</sup>                                 |
| Hematocrit (%)                                             | 30.40 ± 0.65           | 28.85 ± 0.98                 | 24-43 <sup>(3)</sup>                                     |
| MCV (fL)                                                   | 107.37 ± 3.30          | 105.97 ± 4.31                | 90-140 <sup>(4)</sup>                                    |
| MCH (pg)                                                   | 32.16 ± 1.03           | 31.33 ± 1.49                 | 33-47 <sup>(4)</sup>                                     |
| MCHC (g/dL)                                                | 29.69 ± 0.69           | 31.62 ± 1.00                 | 26-35 <sup>(4)</sup>                                     |
| Leukocyte profile                                          |                        |                              |                                                          |
| Total leukocytes (×10 <sup>3</sup> cells/mm <sup>3</sup> ) | 19.56 ± 0.37           | 17.36 ± 0.42                 | 12-30 <sup>(6)</sup>                                     |
| Differential leukocyte count (%)                           |                        |                              |                                                          |
| Lymphocytes (%)                                            | 48.13 ± 0.53           | 48.85 ± 0.80                 | 45-70 <sup>(4)</sup>                                     |
| Heterophils (%)                                            | 31.21 ± 0.47           | 29.52 ± 0.66                 | 15-40 <sup>(4)</sup>                                     |
| Eosinophils (%)                                            | 10.87 ± 0.25           | 11.99 ± 0.37                 | 0-7 <sup>(3)</sup>                                       |
| Monocytes (%)                                              | 9.01 ± 0.31            | 8.87 ± 0.34                  | 1-8 <sup>(7)</sup>                                       |
| Basophils (%)                                              | 0.78 ± 0.01            | 0.77 ± 0.01                  | 0-1 <sup>(3)</sup>                                       |
| Heterophil/Lymphocyte ratio                                | 0.65 ± 0.02            | 0.61 ± 0.02                  | 0.27-1.54 <sup>5</sup>                                   |

Note:  $\bar{x}$  = mean; SE = standard error;  $n$  = sample size; Min = minimum value; Max = maximum value. Reference values: <sup>(1)</sup>WOAH (2021), <sup>(2)</sup>Gaetani *et al.* (2017), <sup>(3)</sup>Samour (2015), <sup>(4)</sup>Jain (1993), <sup>(5)</sup>Talebi *et al.* (2005), <sup>(6)</sup>Weiss and Wardrop (2006), <sup>(7)</sup>Baudouin *et al.* (2021).

Table 2. Genotype and allele frequencies of the IFN- $\gamma$  gene SNP c.304C>G in IPB D1 and Merawangarab chickens

| Breed                 | N  | Genotype count |    |    | Genotype frequency IFN- $\gamma$ |      |      | Allele frequency IFN- $\gamma$ |      |
|-----------------------|----|----------------|----|----|----------------------------------|------|------|--------------------------------|------|
|                       |    | CC             | CG | GG | CC                               | CG   | GG   | C                              | G    |
| IPB D1 Chicken and    | 60 | 44             | 11 | 5  | 0.73                             | 0.18 | 0.08 | 0.83                           | 0.18 |
| Merawangarab chickens | 34 | 33             | 0  | 1  | 0.97                             | 0.00 | 0.03 | 0.97                           | 0.03 |
| Total                 |    | 77             | 11 | 6  | 0.82                             | 0.12 | 0.06 | 0.88                           | 0.12 |

Note: N = number of chickens; CC, CG, and GG represent genotypes; C and G represent allele frequencies.

Table 3. Observed (O) and expected (E) genotype frequencies of the IFN- $\gamma$  gene SNP c.304C>G in IPB D1 and Merawangarab chickens

| Breed                 | N  | Genotype IFN- $\gamma$ (c.304C>G ) |       |    |       |    |      | $\chi^2$ | P-value |
|-----------------------|----|------------------------------------|-------|----|-------|----|------|----------|---------|
|                       |    | CC                                 |       | CG |       | GG |      |          |         |
|                       |    | O                                  | E     | O  | E     | O  | E    |          |         |
| IPB D1 Chickens       | 60 | 44                                 | 40.83 | 11 | 17.32 | 5  | 1.83 | 8.713    | 0.0128* |
| Merawangarab chickens | 34 | 33                                 | 32.02 | 0  | 1.94  | 1  | 0.02 |          |         |
|                       |    | 77                                 |       | 11 |       | 6  |      |          |         |

Note: O = observed genotype count; E = expected genotype count under Hardy-Weinberg equilibrium,  $\chi^2$  = chi-square test statistic; P = probability value.  $p < 0.05$  indicates a statistically significant deviation from the Hardy-Weinberg equilibrium.

Merawangarab chickens (0.74). The G allele was the predominant allele, with frequencies of 0.84 and 0.78 in IPB D1 and Merawangarab chickens, respectively (Table 4). The observed and expected genotype frequencies under the Hardy-Weinberg equilibrium for both SNPs are presented in Tables 3 and 5. Comparison of genotype distributions between IPB D1 and Merawangarab chickens revealed a significant difference for SNP c.304C>G ( $\chi^2 = 8.713$ ,  $p = 0.013$ ), indicating population-specific variation at this locus. In contrast, no significant difference was detected for SNP c.390G>A ( $\chi^2 = 3.784$ ,  $p = 0.151$ ), suggesting comparable genotype distributions between the two populations.

#### Association of IFN- $\gamma$ Gene SNP c.304C>G with Avian Influenza-Related Immune Response Traits

The associations between the IFN- $\gamma$  gene SNP c.304C>G and avian influenza-related immune response traits are presented in Table 6. Significant genotype effects were observed for avian influenza (AI) antibody titer ( $p < 0.001$ ), total leukocyte count ( $p < 0.001$ ), and eosinophil percentage ( $p = 0.021$ ). In contrast, no significant associations were observed for serum IgY concentration, hematocrit, MCV, MCH, MCHC, lymphocyte percentage, heterophil percentage, monocyte percentage, basophil percentage, or the heterophil-to-lymphocyte ratio ( $p > 0.05$ ).

Table 4. Genotype and allele frequencies of the IFN- $\gamma$  gene polymorphism (c.390G>A) in IPB D1 and Merawangarab chickens

| Breed                 | N  | Genotype count |    |    | Genotype frequency IFN- $\gamma$ |      |      | Allele frequency IFN- $\gamma$ |      |
|-----------------------|----|----------------|----|----|----------------------------------|------|------|--------------------------------|------|
|                       |    | GG             | AG | AA | GG                               | AG   | AA   | G                              | A    |
| IPB D1 Chickens       | 60 | 45             | 11 | 4  | 0.75                             | 0.18 | 0.07 | 0.84                           | 0.16 |
| Merawangarab chickens | 34 | 25             | 3  | 6  | 0.74                             | 0.09 | 0.18 | 0.78                           | 0.22 |
| Total                 |    | 70             | 14 | 10 | 0.74                             | 0.15 | 0.11 | 0.82                           | 0.18 |

Note: N = number of chickens; GG, AG, and AA represent genotypes; G and A represent allele frequencies.

Table 5. Observed (O) and expected (E) genotype frequencies of the IFN- $\gamma$  gene SNP c.390G>A in IPB D1 and Merawangarab chickens

| Breed                 | N  | Genotype IFN- $\gamma$ (c.390G>A) |       |    |       |    |      | $\chi^2$ | P-value |
|-----------------------|----|-----------------------------------|-------|----|-------|----|------|----------|---------|
|                       |    | GG                                |       | AG |       | AA |      |          |         |
|                       |    | O                                 | E     | O  | E     | O  | E    |          |         |
| IPB D1 Chickens       | 60 | 45                                | 42.50 | 11 | 15.99 | 4  | 1.50 | 3.784    | 0.151   |
| Merawangarab chickens | 34 | 25                                | 20.65 | 3  | 11.69 | 6  | 1.65 |          |         |
|                       |    | 70                                |       | 14 |       | 10 |      |          |         |

Note: O = observed genotype count; E = expected genotype count under Hardy-Weinberg equilibrium;  $\chi^2$  = chi-square test statistic; P = P-value.  $p \geq 0.05$  indicates no statistically significant deviation from the Hardy-Weinberg equilibrium.

Table 6. Association of the IFN- $\gamma$  Gene SNP c.304C>G with Avian Influenza-Related Immune Responses in IPB D1 and Merawangarab chickens

| Variables                                                  | IPB D1 chickens ( $\bar{x} \pm SE$ ) |                            |                            | Merawangarab chickens ( $\bar{x} \pm SE$ ) |                           |        | P-value |
|------------------------------------------------------------|--------------------------------------|----------------------------|----------------------------|--------------------------------------------|---------------------------|--------|---------|
|                                                            | CC (44)                              | GC (11)                    | GG (5)                     | CC (33)                                    | GC (1)                    | GG (0) |         |
| Immune response                                            |                                      |                            |                            |                                            |                           |        |         |
| Avian influenza antibody titer (log <sub>2</sub> HI)       | 1.14±0.19 <sup>a</sup>               | 1.82 ± 0.42 <sup>a</sup>   | 2.40 ± 0.68 <sup>a</sup>   | 0.27 ± 0.09 <sup>b</sup>                   | 1 ± 0.00                  | -      | 0.000*  |
| IgY (mg/mL)                                                | 12.19 ± 0.61                         | 13.35 ± 1.00               | 15.88 ± 2.02               | 12.41 ± 0.49                               | 13.93 ± 0.00              | -      | 0.260   |
| Erythrocyte Profile                                        |                                      |                            |                            |                                            |                           |        |         |
| Erythrocytes (×10 <sup>6</sup> cells/mm <sup>3</sup> )     | 2.74 ± 0.08                          | 3.11 ± 0.15                | 3.03 ± 0.17                | 2.87 ± 0.13                                | 1.98 ± 0.00               | -      | 0.227   |
| Hemoglobin (g/dL)                                          | 8.74 ± 0.18                          | 8.73 ± 0.25                | 9.36 ± 0.31                | 8.87 ± 0.29                                | 13.00 ± 0.00              | -      | 0.786   |
| Hematocrit (%)                                             | 30.73 ± 0.89                         | 30.09 ± 0.85               | 28.20 ± 1.07               | 28.85 ± 0.99                               | 29.00 ± 0.00              | -      | 0.606   |
| MCV (fL)                                                   | 110.79 ± 4.01                        | 99.61 ± 6.68               | 94.31 ± 7.28               | 104.74 ± 4.19                              | 146.46 ± 0.00             | -      | 0.202   |
| MCH (pg)                                                   | 33.18 ± 1.32                         | 28.46 ± 1.11               | 31.30 ± 2.30               | 31.16 ± 0.98                               | 65.66 ± 0.00              | -      | 0.238   |
| MCHC (g/dL)                                                | 29.39 ± 0.97                         | 29.27 ± 1.29               | 33.23 ± 0.45               | 31.22 ± 0.94                               | 44.83 ± 0.00              | -      | 0.315   |
| Leukocyte profile                                          |                                      |                            |                            |                                            |                           |        |         |
| Total leukocytes (×10 <sup>3</sup> cells/mm <sup>3</sup> ) | 18.98 ± 0.47 <sup>a</sup>            | 21.29 ± 0.61 <sup>a</sup>  | 20.85 ± 1.46 <sup>a</sup>  | 17.17 ± 0.38 <sup>b</sup>                  | 23.50 ± 0.00 <sup>b</sup> | -      | 0.000*  |
| Differential leukocyte count (%)                           |                                      |                            |                            |                                            |                           |        |         |
| Lymphocytes (%)                                            | 48.53 ± 0.60                         | 48.03 ± 1.41               | 44.83 ± 1.55               | 48.74 ± 0.82                               | 52.48 ± 0.00              | -      | 0.334   |
| Heterophils (%)                                            | 31.00 ± 0.56                         | 31.18 ± 1.29               | 33.07 ± 0.94               | 29.73 ± 0.64                               | 22.70 ± 0.00              | -      | 0.059   |
| Eosinophils (%)                                            | 10.54 ± 0.27 <sup>b</sup>            | 11.62 ± 0.51 <sup>ab</sup> | 12.11 ± 0.98 <sup>ab</sup> | 11.94 ± 0.38 <sup>a</sup>                  | 13.48 ± 0.00              | -      | 0.021*  |
| Monocytes (%)                                              | 9.14 ± 0.35                          | 8.39 ± 0.90                | 9.25 ± 1.29                | 8.81 ± 0.35                                | 10.64 ± 0.00              | -      | 0.799   |
| Basophils (%)                                              | 0.79 ± 0.01                          | 0.78 ± 0.02                | 0.74 ± 0.02                | 0.78 ± 0.01                                | 0.70 ± 0.00               | -      | 0.374   |
| Heterophil/Lymphocyte ratio                                | 0.65 ± 0.02                          | 0.66 ± 0.05                | 0.74 ± 0.05                | 0.62 ± 0.02                                | 0.43 ± 0.00               | -      | 0.148   |

Note:  $\bar{x}$  = mean; SE = standard error. Means within the same row bearing different superscript letters (a, b) differ significantly ( $P < 0.05$ ). Numbers in parentheses indicate the sample size for each genotype. CC, CG, and GG represent genotypes of the IFN- $\gamma$  gene SNP c.304C>G. (\*) Significant at  $p < 0.05$ .

#### Association of IFN- $\gamma$ Gene SNP c.390G>A with Avian Influenza-Related Immune Response Traits

The associations between IFN- $\gamma$  gene SNP c.390G>A and avian influenza-related immune response traits are presented in Table 7. Significant genotype effects were observed for AI antibody titer ( $p = 0.001$ ), total leukocyte count ( $p = 0.009$ ), heterophil percentage ( $p = 0.001$ ), and the heterophil-to-lymphocyte (H/L) ratio ( $p = 0.014$ ). Chickens carrying the GG genotype exhibited the highest AI antibody titer, whereas those carrying the AA genotype exhibited the lowest. In contrast, no significant associations were detected for serum IgY concentration, erythrocyte count, hemoglobin concentration, hematocrit, MCV, MCH,

MCHC, lymphocyte percentage, eosinophil percentage, monocyte percentage, or basophil percentage ( $p > 0.05$ ).

#### DISCUSSION

Descriptive statistics indicated that IPB D1 and Merawangarab chickens exhibited relatively favorable physiological conditions, with erythrocyte counts of  $2.84 \times 10^6$  cells/mm<sup>3</sup>, total leukocyte counts ranging from 17.36 to  $19.56 \times 10^3$  cells/mm<sup>3</sup>, and heterophil-to-lymphocyte (H/L) ratios of 0.61–0.65 (Table 1). Hematological parameters, particularly erythrocyte and leukocyte profiles, are useful indicators of physiological condition and immunological status in chickens (Kuswandi *et al.*, 2025). Regarding humoral immune

Table 7. Association between the IFN- $\gamma$  Gene SNP c.390G>A and Avian Influenza-Related Immune Responses in IPB D1 and Merawangarab chickens

| Variables                                                  | IPB D1 chicken ( $\bar{x} \pm SE$ ) |                             |                            | Merawangarab chickens ( $\bar{x} \pm SE$ ) |                            |                           | P-value |
|------------------------------------------------------------|-------------------------------------|-----------------------------|----------------------------|--------------------------------------------|----------------------------|---------------------------|---------|
|                                                            | GG (45)                             | AG (11)                     | AA (4)                     | GG (25)                                    | AG (3)                     | AA (6)                    |         |
| Immune response                                            |                                     |                             |                            |                                            |                            |                           |         |
| Avian influenza antibody titer (log <sub>2</sub> HI)       | 1.53 ± 0.21 <sup>a</sup>            | 0.91 ± 0.34 <sup>ab</sup>   | 0.75 ± 0.75 <sup>ab</sup>  | 0.40 ± 0.11 <sup>b</sup>                   | 0.00 ± 0.00 <sup>b</sup>   | 0.00 ± 0.00 <sup>b</sup>  | 0.001*  |
| IgY (mg/mL)                                                | 12.37 ± 0.52                        | 13.70 ± 1.10                | 13.83 ± 1.37               | 12.42 ± 0.61                               | 12.90 ± 1.65               | 12.40 ± 0.48              | 0.891   |
| Erythrocyte profile                                        |                                     |                             |                            |                                            |                            |                           |         |
| Erythrocytes (×10 <sup>6</sup> cells/mm <sup>3</sup> )     | 2.85 ± 0.07                         | 2.67 ± 0.22                 | 3.16 ± 0.22                | 2.97 ± 0.17                                | 2.23 ± 0.11                | 2.61 ± 0.19               | 0.248   |
| Hemoglobin (g/dL)                                          | 8.71 ± 0.15                         | 8.85 ± 0.30                 | 9.55 ± 1.01                | 9.38 ± 0.38                                | 7.13 ± 0.13                | 8.33 ± 0.26               | 0.058   |
| Hematocrit (%)                                             | 29.80 ± 0.74                        | 32.00 ± 2.08                | 32.75 ± 0.75               | 29.80 ± 1.19                               | 28.00 ± 2.00               | 25.33 ± 1.52              | 0.195   |
| MCV (fL)                                                   | 104.69 ± 3.15                       | 119.10 ± 12.10              | 105.15 ± 8.12              | 105.68 ± 5.55                              | 125.30 ± 2.66              | 97.51 ± 2.17              | 0.365   |
| MCH (pg)                                                   | 31.37 ± 0.95                        | 36.17 ± 3.97                | 29.99 ± 1.16               | 33.21 ± 1.98                               | 32.08 ± 0.94               | 32.41 ± 1.46              | 0.449   |
| MCHC (g/dL)                                                | 29.97 ± 0.85                        | 28.69 ± 1.79                | 29.31 ± 3.47               | 31.93 ± 1.23                               | 25.66 ± 1.27               | 33.28 ± 1.58              | 0.276   |
| Leukocyte profile                                          |                                     |                             |                            |                                            |                            |                           |         |
| Total leukocytes (×10 <sup>3</sup> cells/mm <sup>3</sup> ) | 19.57 ± 0.45 <sup>ab</sup>          | 18.88 ± 0.87 <sup>abc</sup> | 21.22 ± 1.03 <sup>a</sup>  | 17.73 ± 0.49 <sup>c</sup>                  | 16.21 ± 1.17 <sup>bc</sup> | 16.38 ± 0.99 <sup>c</sup> | 0.009*  |
| Differential leukocyte (%)                                 |                                     |                             |                            |                                            |                            |                           |         |
| Lymphocytes (%)                                            | 48.03 ± 0.62                        | 47.22 ± 1.27                | 51.73 ± 0.80               | 48.40 ± 0.90                               | 46.79 ± 3.04               | 51.76 ± 1.90              | 0.182   |
| Heterophils (%)                                            | 31.17 ± 0.55 <sup>a</sup>           | 32.36 ± 0.95 <sup>a</sup>   | 28.49 ± 1.97 <sup>ab</sup> | 29.78 ± 0.66 <sup>ab</sup>                 | 34.96 ± 1.76 <sup>a</sup>  | 25.75 ± 0.89 <sup>b</sup> | 0.001*  |
| Eosinophils (%)                                            | 10.87 ± 0.27                        | 11.40 ± 0.64                | 9.42 ± 1.04                | 12.23 ± 0.40                               | 10.95 ± 1.55               | 11.48 ± 1.08              | 0.060   |
| Monocytes (%)                                              | 9.16 ± 0.37                         | 8.22 ± 0.74                 | 9.60 ± 1.01                | 8.81 ± 0.42                                | 6.55 ± 0.30                | 10.26 ± 0.22              | 0.216   |
| Basophils (%)                                              | 0.78 ± 0.01                         | 0.79 ± 0.03                 | 0.77 ± 0.01                | 0.78 ± 0.01                                | 0.76 ± 0.02                | 0.75 ± 0.00               | 0.796   |
| Heterophil/Lymphocyte ratio                                | 0.66 ± 0.02 <sup>a</sup>            | 0.69 ± 0.04 <sup>ab</sup>   | 0.55 ± 0.04 <sup>b</sup>   | 0.63 ± 0.02 <sup>a</sup>                   | 0.76 ± 0.08 <sup>a</sup>   | 0.50 ± 0.04 <sup>b</sup>  | 0.014*  |

Note:  $\bar{x}$  = mean; SE = standard error. Means within the same row bearing different superscript letters (a, b, c) differ significantly ( $p < 0.05$ ). Numbers in parentheses indicate the sample size for each genotype. GG, AG, and AA represent genotypes of the IFN- $\gamma$  gene SNP c.390G>A. (\*) Significant at  $p < 0.05$ .

status, serum IgY concentrations were comparable between IPB D1 (12.71 mg/mL) and Merawangarab (12.46 mg/mL) chickens, consistent with the 12.60 mg/mL reported for IPB D1 chickens by Lestari *et al.* (2025). These concentrations were higher than those reported in Sentul hens (9.55  $\pm$  1.25 mg/mL) (Setyawati *et al.*, 2019) and remained within the normal range of serum IgY concentrations in chickens (5–15 mg/mL) (Gaetani *et al.*, 2017). In contrast, mean AI antibody titers were relatively low in both populations, ranging from 1.00 to 1.37  $\log_2$  HI. According to WOA (2021), an HI titer of  $\geq 4 \log_2$  (1:16) is considered positive for AI antibodies when 4 HAU of antigen is used, whereas titers  $< 4 \log_2$  are below the positive threshold. The titers observed in the present study were also below the protective levels reported in previous studies (Mirzaie *et al.*, 2020; Ikhsan *et al.*, 2021). As the chickens were neither vaccinated against AI nor experimentally challenged with the virus, the detected antibodies likely reflected naturally acquired immune responses following environmental exposure. This interpretation is consistent with Rouygari *et al.* (2025), who reported higher and more sustained HI antibody responses in vaccinated than in non-immunized chickens. Collectively, these findings indicate that despite relatively favorable physiological conditions and adequate baseline humoral immunity, AI-specific antibody responses remained low in both populations, suggesting that variation in AI-related immune responses may also be influenced by intrinsic host genetic factors.

The comparable physiological and immunological conditions of the two populations suggest that variation in avian influenza (AI)-related immune responses may be associated with host genetic factors. In this study, two polymorphisms were identified in exon 4 of the

IFN- $\gamma$  gene: c.304C>G, a synonymous coding variant, and c.390G>A, located in the 3' untranslated region (3'UTR). Variations within these regions may influence gene regulation or expression without directly altering protein structure. The biological relevance of these findings is supported by the established role of IFN- $\gamma$  in antiviral immunity, particularly through the induction of interferon-stimulated genes (ISGs) and activation of antiviral signaling pathways that suppress AI virus replication (Freier *et al.*, 2025; Guo *et al.*, 2025). Thus, the identification of exon 4 polymorphisms extends current knowledge of IFN- $\gamma$  genetic variation potentially associated with AI-related immune responses in Indonesian chickens.

Genotype distributions at c.304C>G differed between IPB D1 and Merawangarab chickens, whereas no significant difference was observed at c.390G>A. The predominance of the CC genotype and C allele in Merawangarab chickens indicates greater genetic homogeneity at this locus than in IPB D1 chickens. This pattern may reflect differences in the genetic backgrounds and breeding histories of the two populations, with IPB D1 developed through a structured breeding program and Merawangarab derived from crosses between Merawang and Arabian chickens. Consistent with this interpretation, Wang *et al.* (2025) reported that demographic history, genetic introgression, and population differentiation can shape genetic structure and allele frequency variation in chicken populations. Therefore, the observed differences in IFN- $\gamma$  genotype distribution may reflect genetic differentiation between IPB D1 and Merawangarab chickens and potentially contribute to variation in AI-related immune responses.

The significant association between SNP c.304C>G and avian influenza (AI) antibody titer suggests that this locus may contribute to variation in antiviral humoral immune responses. Chickens carrying the GC and GG genotypes exhibited higher AI antibody titers than those with the CC genotype, suggesting a potential association of the G allele with enhanced antibody responses. Antibody production is a key component of adaptive immunity (Lam *et al.*, 2024), and IFN- $\gamma$  may modulate humoral responses by enhancing antigen presentation, activating macrophages and other antigen-presenting cells, and regulating B-cell activation and differentiation (Burke & Young, 2019). Consistent with this mechanism, increased IFN- $\gamma$  expression has been associated with enhanced antibody production and antiviral responses during avian influenza virus infection (Karpala *et al.*, 2011; Alqazlan *et al.*, 2022; Pan *et al.*, 2023). Although c.304C>G is a synonymous variant that does not alter the IFN- $\gamma$  amino acid sequence, synonymous variants may affect gene expression through changes in mRNA secondary structure, codon usage, translational efficiency, mRNA stability, or linkage disequilibrium with nearby functional variants (Oelschlaeger, 2024; Lin *et al.*, 2023; Jiang *et al.*, 2023). These findings support c.304C>G as a potential candidate molecular marker for AI-related immune responsiveness, although further functional validation is required before its application in marker-assisted selection.

Beyond the AI antibody titer, SNP c.304C>G was significantly associated with total leukocyte count and eosinophil percentage, further suggesting its involvement in both innate and adaptive immune responses. Genotype-dependent variation in leukocyte counts may reflect differences in immune responsiveness (Wang *et al.*, 2023), while eosinophils contribute to antiviral defense through cytokine production and interactions with other immune cells (Arnold & Munitz, 2024).

SNP c.390G>A showed broader associations with immune-related traits, including AI antibody titer, total leukocyte count, heterophil percentage, and the heterophil-to-lymphocyte (H/L) ratio. Chickens carrying the GG genotype exhibited higher AI antibody titers, suggesting a potential association with enhanced humoral immune responsiveness. This finding is consistent with previous studies showing that polymorphisms in immune-related genes involved in cytokine signaling and antigen presentation contribute to variation in antibody responses in chickens (Luo *et al.*, 2013; Zhang *et al.*, 2015; Wang *et al.*, 2022; Lestari *et al.*, 2023). The associations with leukocyte count, heterophil percentage, and H/L ratio further support the immunoregulatory role of IFN- $\gamma$  in antiviral defense (He *et al.*, 2024). Previous studies have similarly linked immune-related genetic variation, including IFNG variants, to H/L ratio (Wang *et al.*, 2020), while variation in this ratio has been associated with immune responsiveness and inflammatory status (Krzysica *et al.*, 2025). IFN- $\gamma$ -related pathways may also regulate heterophil-lymphocyte balance through modulation of Th1/Th2 cytokine responses (Pu *et al.*, 2023). Because

c.390G>A is located within the 3' untranslated region (3'UTR), these associations may involve regulatory effects on gene expression or linkage disequilibrium with nearby functional variants rather than direct alterations in protein structure. Thus, c.390G>A may contribute to variation in antiviral immune responsiveness, although its underlying functional mechanisms require further validation.

The immune-related associations observed in this study may also reflect differences in the genetic backgrounds of the two populations. IPB D1 was developed through a structured breeding program involving indigenous and commercial genetic resources and has previously been reported to exhibit relatively high IgY concentrations (Lestari *et al.*, 2025). In the present study, IPB D1 also tended to exhibit higher AI antibody titers and total leukocyte counts than Merawangarab chickens, potentially reflecting differences in their genetic backgrounds and breeding histories. Nevertheless, the associations between IFN- $\gamma$  polymorphisms and multiple immune-related traits support their potential as candidate markers for marker-assisted selection (MAS). However, because AI-related immune responsiveness is a complex trait influenced by both genetic and environmental factors, these SNPs should complement phenotypic selection and be validated in larger, genetically diverse populations before their application in breeding programs.

Several limitations should be considered when interpreting these findings. The relatively small sample size ( $n = 94$ ) may limit statistical power and generalizability. Moreover, the chickens were neither vaccinated against AI nor experimentally challenged with the virus, and the measured antibody titers therefore reflected responses under natural exposure conditions rather than controlled immune responses. Accordingly, c.304C>G and c.390G>A should be regarded as candidate markers of immune responsiveness rather than direct markers of AI resistance or susceptibility. Furthermore, c.304C>G is a synonymous coding variant, whereas c.390G>A is located within the 3' untranslated region (3'UTR), and the functional mechanisms underlying their associations with immune-related traits remain unclear. Further studies involving larger and genetically diverse populations, combined with gene expression and transcriptomic analyses, controlled vaccination or challenge experiments, and functional molecular assays, are needed to validate these associations and evaluate the potential application of these SNPs in marker-assisted selection.

## CONCLUSION

Two SNPs (c.304C>G and c.390G>A) were identified in the IFN- $\gamma$  gene of Indonesian local chickens and were significantly associated with several AI-related immune traits. The c.304C>G polymorphism was associated with avian influenza antibody titer, total leukocyte count, and eosinophil percentage, whereas c.390G>A was associated with avian influenza antibody titer, total leukocyte count, heterophil percentage, and

the heterophil-to-lymphocyte ratio. These findings indicate that genetic variation within the IFN- $\gamma$  gene is associated with variation in immune responses related to avian influenza in Indonesian local chickens. Therefore, IFN- $\gamma$  may be a promising candidate gene for identifying molecular markers associated with AI-related immune responsiveness. Nevertheless, further validation in larger and independent populations, together with functional characterization and controlled challenge or vaccination studies, is required before these polymorphisms can be applied in marker-assisted breeding programs.

### CONFLICT OF INTEREST

C. Sumantri is a member of the editorial board of the *Tropical Animal Science Journal* and was not involved in the evaluation or publication decision of this manuscript. The authors declare that there are no competing financial, personal, or institutional interests associated with this study.

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### DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

The authors used Paperpal and Grammarly to assist with language editing, including grammar correction, vocabulary refinement, and improvement of sentence structure and overall manuscript clarity. All revisions were carefully reviewed by the authors, who take full responsibility for the accuracy, integrity, and scientific content of this publication.

### REFERENCES

- Allendorf, F. W., Hohenlohe, P. A., & Luikart, G. (2010). Genomics and the future of conservation genetics. *Nature Reviews Genetics*, 11(10), 697–709. <https://doi.org/10.1038/nrg2844>
- Alqazlan, N., Astill, J., Raja, S., & Sharif, S. (2022). Strategies for enhancing immunity against avian influenza virus in chickens: A review. *Avian Pathology*, 51(3), 211–235. <https://doi.org/10.1080/03079457.2022.2054309>
- Amusan, S. A., Ikeobi, C. O. N., Adebambo, A. O., Agaviezor, B. O., Wheto, M., Durosaro, S. O., Adenaike, A. S., Ilori, B. M., Adedeji, T. A., & Adebambo, O. (2013). Effect of chicken genotype on growth performance and feed consumption in the development of broiler lines. *Nigerian Journal of Animal Production*, 40(2), 1–6. <https://doi.org/10.51791/njap.v40i2.1131>
- Arnold, I. C., & Munitz, A. (2024). Spatial adaptation of eosinophils and their emerging roles in homeostasis, infection and disease. *Nature Reviews Immunology*, 24, 858–877. <https://doi.org/10.1038/s41577-024-01048-y>
- Ayuti, S. R., Khairullah, A. R., Lamid, M., Al-Arif, M. A., Warsito, S. H., Silaen, O. S. M., Moses, I. B., Hermawan, I. P., Yanestria, S. M., Delima, M., Ferasyi, T. R., & Aryaloka, S. (2024). Avian influenza in birds: Insights from a comprehensive review. *Veterinary World*, 17(11), 2544–2555. <https://doi.org/10.14202/vetworld.2024.2544-2555>
- Baudouin, K. A., Kamagate, S., Bleyere, N. M., & Yapo, A. P. (2021). Hematological profile of broilers and local chickens in Korhogo, Côte d'Ivoire. *International Journal of Agriculture, Environment and Bioresearch*, 6(2), 14–23. <https://doi.org/10.35410/IJAEB.2021.5618>
- Burke, J. D., & Young, H. A. (2019). IFN- $\gamma$ : A cytokine at the right time, is in the right place. *Seminars in Immunology*, 43, 101280. <https://doi.org/10.1016/j.smim.2019.05.002>
- Chen, J., Feng, X., Desierto, M. J., Keyvanfar, K., & Young, N. S. (2015). IFN- $\gamma$ -mediated hematopoietic cell destruction in murine models of immune-mediated bone marrow failure. *Blood*, 126(24), 2621–2631. <https://doi.org/10.1182/blood-2015-06-652453>
- Depison, D., Gushairiyanto, G., Harahap, R. S., Muthalib, R. A., Azis, A., Alwi, Y., & Wibowo, S. E. (2025). Integrating quantitative traits and growth hormone gene polymorphism in Indonesian crossbred chickens for genetic improvement and marker-assisted selection. *Veterinary World*, 18(8), 2169–2180. <https://doi.org/10.14202/vetworld.2025.2169-2180>
- Frank, K., & Paust, S. (2020). Dynamic natural killer cell and T cell responses to influenza infection. *Frontiers in Cellular and Infection Microbiology*, 10, 425. <https://doi.org/10.3389/fcimb.2020.00425>
- Freier, L., Stuff, J., Götzke, N., Preisinger, R., Grund, C., Tiemann, I., Weigend, S., & Blohm, U. (2025). Baseline immune profiles of local chicken breeds: Linking biodiversity, animal health, and vaccination response. *Poultry Science*, 104(10), 105565. <https://doi.org/10.1016/j.psj.2025.105565>
- Gaetani, C., Ambrosi, E., Ugo, P., & Moretto, L. M. (2017). Electrochemical immunosensor for detection of IgY in food and food supplements. *Chemosensors*, 5(1), 10. <https://doi.org/10.3390/chemosensors5010010>
- Guo, Y., Zhou, Z., Gao, H., Zhang, S., Chen, Z., Chang, H., Cao, H., Gao, L., Wang, Y., & Zheng, S. J. (2025). Identification of the T-box transcription factor T-bet in chickens and its role in immune response. *Poultry Science*, 104(10), 105589. <https://doi.org/10.1016/j.psj.2025.105589>
- Hartl, D. L., & Clark, G. C. (1997). *Principles of Population Genetics*. Sinauer Associates, Sunderland.
- He, X., Zhang, S., Zou, Z., Gao, P., Yang, L., & Xiang, B. (2024). Antiviral effects of avian interferon-stimulated genes. *Animals*, 14(21), 3062. <https://doi.org/10.3390/ani14213062>
- Ikhsan, H. M. (2021). Avian influenza antibody titer of laying hens in production phase at District Penebel Tabanan Regency. *Journal of Applied Veterinary Science and Technology*, 2(2), 26–30. <https://doi.org/10.20473/javest.V2.I2.2021.26-30>
- Indriyani, N., Murtini, S., Darwati, S., & Sumantri, C. (2024). Production performance and humoral immunity in IPB D1-D3 chickens. *Advances in Animal and Veterinary Sciences*, 12(4), 782–790. <https://doi.org/10.17582/journal.aavs/2024/12.4.782.790>
- Irmaya, D., Suyadi, S., Depison, D., & Ardiantoro, A. (2024). The quantitative characteristics of cross-bred native chicken (Sentul  $\times$  Arab chicken and Merawang  $\times$  Arab chicken). *Buletin Peternakan*, 48(3), 161–165. <https://doi.org/10.21059/buletinpeternak.v48i3.89400>
- Jain, N. C. (1993). *Essentials of veterinary hematology*. Lea & Febiger.
- Ji, B., Sun, T. T., Ma, Z. L., Lu, Q. F., Hu, W. L., Jian, Z. H., Jiang,

- Y., & Chen, P. F. (2015). Possible association of IFN- $\gamma$  gene -316A/G SNP with humoral immune response to killed H5N1 HPAI vaccine in a Red Junglefowl population. *Journal of Interferon & Cytokine Research*, 35(11), 844–849. <https://doi.org/10.1089/jir.2015.0018>
- Jiang, Y., Neti, S. S., Sitarik, I., Pradhan, P., To, P., Xia, Y., Fried, S. D., Booker, S. J., & O'Brien, E. P. (2023). How synonymous mutations alter enzyme structure and function over long timescales. *Nature Chemistry*, 15, 308–318. <https://doi.org/10.1038/s41557-022-01091-z>
- Kaiser, P., Wain, H. M., Rothwell, L. (1998). Structure of the chicken interferon-gamma gene, and comparison to mammalian homologues. *Gene*, 207(1):25–32. [https://doi.org/10.1016/S0378-1119\(97\)00600-8](https://doi.org/10.1016/S0378-1119(97)00600-8)
- Karpala, A. J., Bingham, J., Schat, K. A., Chen, L. M., Donis, R. O., Lowenthal, J. W., & Bean, A. G. D. (2011). Highly pathogenic (H5N1) avian influenza induces an inflammatory T helper type 1 cytokine response in the chicken. *Journal of Interferon & Cytokine Research*, 31(4), 393–400. <https://doi.org/10.1089/jir.2010.0069>
- Kim, H. R., Lee, K. N., Lee, Y. J., & Her, M. (2025). Comparison of gross lesions in poultry naturally infected with high pathogenicity avian influenza H5N6 and H5N1 viruses in South Korea, 2023–2025. *Transboundary and Emerging Diseases*, 2025, 1736453. <https://doi.org/10.1155/tbed/1736453>
- Krzysica, P., Hollemans, M., Lammers, A., Smits, C., Savelkoul, H. F. J., de Vries, S., & Tijhaar, E. (2025). Sanitary conditions modulate proinflammatory cytokine response of broilers. *Poultry Science*, 104(12), 105984. <https://doi.org/10.1016/j.psj.2025.105984>
- Kuswandi, W., Budiman, C., Khaerunnisa, I., & Sumantri, C. (2025). Rearing system and immune status influence the small intestinal microbiota of IPB-D3 chickens: A full-length 16S rRNA metagenomic approach. *Veterinary World*, 18(8), 2206–2221. <https://doi.org/10.14202/vetworld.2025.2206-2221>
- Lam, N., Lee, Y., & Farber, D. L. (2024). A guide to adaptive immune memory. *Nature Reviews Immunology*, 24, 810–829. <https://doi.org/10.1038/s41577-024-01040-6>
- Lestari, D., Murtini, S., Ulupi, N., Gunawan, A., & Sumantri, C. (2023). Novel MHC BLB2 gene polymorphism and its association with IgY concentration and Newcastle disease antibody titer in IPB D1-D2 chickens. *Archives Animal Breeding*, 66(3), 275–283. <https://doi.org/10.5194/aab-66-275-2023>
- Lestari, D., Murtini, S., Ulupi, N., Gunawan, A., Khaerunnisa, I., Anggraeni, A., & Sumantri, C. (2025). Disease resistance traits of IPB-D2 chickens: Characterization of IgY concentrations, Newcastle disease antibody titers, and leukocyte profiles. *Veterinary World*, 18(1), 172–177. <https://doi.org/10.14202/vetworld.2025.172-177>
- Lin, B. C., Katneni, U., Jankowska, K. I., Meyer, D., & Kimchi-Sarfaty, C. (2023). In silico methods for predicting functional synonymous variants. *Genome Biology*, 24, 126. <https://doi.org/10.1186/s13059-023-02966-1>
- Luo, C., Qu, H., Ma, J., Wang, J., Li, C., Yang, C., Hu, X., Li, N., & Shu, D. (2013). Genome-wide association study of antibody response to Newcastle disease virus in chicken. *BMC Genetics*, 14(1), 42. <https://doi.org/10.1186/1471-2156-14-42>
- Maheshwari, H., Sasmita, A. N., Farajallah, A., Achmadi, P., & Santoso, K. (2017). Effect of temperature on leukocyte differential and malondialdehyde (MDA) levels in quail (*Coturnix coturnix japonica*). *Bioma*, 13(2), 22–30. [https://doi.org/10.21009/Bioma13\(2\).4](https://doi.org/10.21009/Bioma13(2).4)
- Ministry of Agriculture of the Republic of Indonesia. (2026). Report of highly pathogenic avian influenza (H5N1) outbreaks in chickens in Riau, South Sulawesi, West Java, Banten, and Lampung Provinces (WAHIS Event ID: 7230).
- Directorate General of Livestock and Animal Health Services.
- Mirzaie, K., Shoushtari, A., Bokaie, S., Fallah Mehrabadi, M. H., & Peighambari, S. M. (2020). Trend of changes in the titer of antibody against avian influenza virus H9N2 during raising period in vaccinated and unvaccinated broiler farms in Qazvin Province, Iran: A cohort study. *Archives of Razi Institute*, 75(1), 9–16. <https://doi.org/10.22092/ari.2018.120089.1183>
- Nei, M., & Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press.
- Novitasari, D., & Anwar, C. (2020). Seroprevalence of avian influenza virus subtype H5 among poultry workers of central traditional markets in Indonesia. *Journal of World's Poultry Research*, 10(4), 643–648. <https://doi.org/10.36380/jwpr.2020.73>
- Oelschlaeger, P. (2024). Molecular mechanisms and the significance of synonymous mutations. *Biomolecules*, 14(1), 132. <https://doi.org/10.3390/biom14010132>
- Pan, X., Liu, Q., de Jong, M. C. M., Forlenza, M., Niu, S., Yan, D., Teng, Q., Li, X., Beerens, N., & Li, Z. (2023). Immunoadjuvant efficacy of CpG plasmids for H9N2 avian influenza inactivated vaccine in chickens with maternal antibodies. *Veterinary Immunology and Immunopathology*, 259, 110590. <https://doi.org/10.1016/j.vetimm.2023.110590>
- Pawestri, H. A., Eggink, D., Isfandari, S., Thanh, T. T., van Doorn, H. R., Setiawaty, V., & de Jong, M. D. (2020). Viral factors associated with the high mortality related to human infections with clade 2.1 influenza A/H5N1 virus in Indonesia. *Clinical Infectious Diseases*, 70(6), 1139–1146. <https://doi.org/10.1093/cid/ciz328>
- Prawira, A. Y., Yuneldi, R. F., Khaerunnisa, I., Furqon, A., Lestari, D., Phadmacanty, N. L. P. R., Budiman, C., & Sumantri, C. (2026). Growth performance and muscle histological characteristics of IPB-D3 chickens reared under intensive and free-range systems. *Veterinary World*, 19(1), 282–294. <https://doi.org/10.14202/vetworld.2026.282-294>
- Pu, X., Pan, Y., Xiang, Q., Lu, M., Xu, L., Yan, R., Li, X., & Song, X. (2023). Inhibitory effect of *Eimeria maxima* IFN- $\gamma$  inhibitory molecules on the immune function of T cell subsets in chickens. *Poultry Science*, 102(12), 103098. <https://doi.org/10.1016/j.psj.2023.103098>
- Pujiastuti, I. N. E., Lestari, S. R., & Gofur, A. (2017). Gambaran hematologi mencit (*Mus musculus*) model toksisitas subkronis. *Scripta Biologica*, 4(2), 75. <https://doi.org/10.20884/1.sb.2017.4.2.371>
- Putri, N. T., Murtini, S., Ulupi, N., Khaerunnisa, I., & Sumantri, C. (2022). Polymorphisms of the *Thy-1* gene in IPB-D2 chicken line: Association with IgY and ND antibody. *Indonesian Journal of Animal and Veterinary Sciences*, 27(4), 186–194. <https://doi.org/10.14334/jitv.v27i4.3029>
- Rouygar, M. R., Mayahi, M., Vasfi Marandy, M., & Boroomand, Z. (2025). Serological baseline, antibody stability and efficacy of different types of avian influenza (H5) vaccines. *Journal of Veterinary Science*, 26(1), e4. <https://doi.org/10.4142/jvs.24045>
- Sajjadi, N. C., Abolnik, C., Baldinelli, F., Brown, I., Cameron, A., de Wit, S., Dhingra, M., Espeisse, O., Guerrin, J. L., Harder, T., Ho, J., Chua, T. H., Hussein, K., Lyons, N., Monne, I., Okamuro, Y., Pacheco, D. T., Pavade, G., Poncon, N., Prajitno, T. Y., Rojas, J. G., Swayne, D. E., & Stegeman, A. (2025). Vaccination and surveillance for high pathogenicity avian influenza in poultry: Current situation and perspectives. *Biologicals*, 91, 101840. <https://doi.org/10.1016/j.biologicals.2025.101840>
- Samour, J. (2015). *Diagnostic value of hematology in clinical avian medicine*. Spix Publishing.
- Sarma, P. R. (1990). *Clinical methods: The history, physical, and*

- laboratory examinations (3<sup>rd</sup> ed.). Butterworths.
- Setyawati, M. P., Ulupi, N., Murtini, S., & Sumantri, C. (2019). Production performance, reproduction and immunity of Sentul hens at different IgY concentrations. *Buletin Peternakan*, 43(1), 17–21. <https://doi.org/10.21059/buletinpeternak.v43i1.35180>
- Simancas-Racines, A., Reytor-González, C., Toral, M., & Simancas-Racines, D. (2025). H5N1 avian influenza: A narrative review of scientific advances and global policy challenges. *Viruses*, 17(7), 927. <https://doi.org/10.3390/v17070927>
- Song, H., Liu, X., Gao, X., Li, J., Shang, Y., Gao, W., Li, Y., & Zhang, Z. (2022). Transcriptome analysis of pre-immune state induced by interferon gamma inhibiting the replication of H9N2 avian influenza viruses in chicken embryo fibroblasts. *Infection, Genetics and Evolution*, 103, 105332. <https://doi.org/10.1016/j.meegid.2022.105332>
- Talebi, A., Asri-Rezaei, S., Rozeh-Chai, R., & Sahraei, R. (2005). Comparative studies on haematological values of broiler strains (Ross, Cobb, Arbor-Acres and Arian). *International Journal of Poultry Science*, 4(8), 573–579. <https://doi.org/10.3923/ijps.2005.573.579>
- Thiam, M., Barreto Sánchez, A. L., Zhang, J., Zheng, M., Wen, J., Zhao, G., & Wang, Q. (2021). Association of heterophil/lymphocyte ratio with intestinal barrier function and immune response to *Salmonella enteritidis* infection in chicken. *Animals*, 11(12), 3498. <https://doi.org/10.3390/ani11123498>
- Wang, H., Liu, T., Wang, G., Zhao, X., Wang, C., Li, F., Muhatai, G., & Qu, L. (2025). Research on the historical dynamics of Baicheng oil chicken populations. *Animals*, 15(13), 1952. <https://doi.org/10.3390/ani15131952>
- Wang, J., Zhang, J., Wang, Q., Zhang, Q., Thiam, M., Zhu, B., Ying, F., Elsharkawy, M. S., Zheng, M., Wen, J., Li, Q., & Zhao, G. (2023). A heterophil/lymphocyte-selected population reveals genetic regulation of immune defense in chickens. *Communications Biology*, 6, 196. <https://doi.org/10.1038/s42003-023-04559-x>
- Wang, J., Zhu, B., Wen, J., Li, Q., & Zhao, G. (2020). Genome-wide association study and pathway analysis for heterophil/lymphocyte (H/L) ratio in chicken. *Genes*, 11(9), 1005. <https://doi.org/10.3390/genes11091005>
- Wang, W., & Zhang, L. (2022). Genome-wide association study on two immune-related traits in Jinghai Yellow chicken. *Brazilian Journal of Poultry Science*, 24(3), eRBCA-2021-1565. <https://doi.org/10.1590/1806-9061-2021-1587>
- Weiss, D. J., & Wardrop, K. J. (2006). *Schalm's veterinary hematology* (6th ed.). Wiley-Blackwell.
- World Organisation for Animal Health (WOAH). (2021). Avian influenza (including infection with high pathogenicity avian influenza viruses). In *manual of diagnostic tests and vaccines for terrestrial animals* (Chapter 3.3.4). World Organization for Animal Health. [https://www.woah.org/fileadmin/Home/fr/Health\\_standards/tahm/3.03.04\\_AI.pdf](https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.03.04_AI.pdf)
- Yuk, S. S., Lee, D. H., Park, J. K., Tseren-Ochir, E. O., Kwon, J. H., Noh, J. Y., Lee, J. B., Park, S. Y., Choi, I. S., & Song, C. S. (2016). Pre-immune state induced by chicken interferon gamma inhibits the replication of H1N1 human and H9N2 avian influenza viruses in chicken embryo fibroblasts. *Virology Journal*, 13(1), 71. <https://doi.org/10.1186/s12985-016-0527-1>
- Zhang, L., Li, P., Liu, R., Zheng, M., Sun, Y., Wu, D., Hu, Y., Wen, J., & Zhao, G. (2015). The identification of loci for immune traits in chickens using a genome-wide association study. *PLoS ONE*, 10(3), e0117269. <https://doi.org/10.1371/journal.pone.0117269>