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Association of Inducible Nitric Oxide Synthase (iNOS) Gene Diversity with Immunity Characteristics in Kampung Chicken

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Abstract: The immune system of Kampung chickens is vital for maintaining health and productivity, especially in combating bacterial infections. Among the genetic components involved, the inducible nitric oxide synthase (iNOS) gene is crucial for producing nitric oxide (NO), a molecule with strong antimicrobial properties. This study investigated the association between iNOS (AluI | g.15056T>C) gene polymorphism and immune traits in Kampung chickens to enhance disease resistance. Blood samples from 100 Kampung chickens were genotyped using PCR-RFLP, and immune parameters such as leukocyte count, macrophage activity, and bacterial resistance were evaluated. Statistical analysis revealed significant differences among TT, TC, and CC genotypes. The CC genotype exhibited superior performance, including the highest macrophage activity (91.74±1.92%), activated macrophage capacity (2279.49), and bacterial death rate (60.81±3.54%). These findings suggest that the CC genotype enhances NO production, strengthening the immune response to bacterial pathogens like *Staphylococcus aureus* and *Salmonella Pullorum*. Additionally, the polymorphism contributes to improved genetic diversity and immune efficiency in Kampung chickens. The study highlights the potential of incorporating the iNOS CC genotype in selective breeding programs to produce chickens with enhanced resilience. Future research should focus on interacting environmental factors with iNOS expression to optimize its application in chicken production.

Keywords: Disease Resistance; Kampung Chickens; Nitric Oxide; Selective Breeding

Introduction

The chicken immune system is vital for health and productivity, especially in combating diseases. Nitric oxide (NO), produced by the enzyme inducible nitric oxide synthase (iNOS), plays a key role with its strong antimicrobial and anti-inflammatory properties (Andrés et al., 2022). Genetic diversity in the iNOS gene has been shown to contribute to variations in immune responses in chickens (Song et al., 2022). Polymorphisms in the iNOS gene, such as single nucleotide polymorphisms (SNPs), can affect NO production, which in turn impacts the ability of chickens to combat bacterial, viral, or parasitic infections (Alkie et al., 2019; Martins et al., 2022). Recent studies indicate that chickens with specific genetic variations in the iNOS gene tend to exhibit more excellent disease resistance, making it a crucial target in breeding programs to enhance poultry resilience (Mazuryk et al., 2024).

Understanding the genetic basis for disease resistance in chickens can lead to more efficient breeding strategies that produce healthier and more robust poultry. Farmers can reduce their reliance on antibiotics and other

medications by identifying and selecting for these specific genetic variations, promoting overall animal welfare and sustainability in the poultry industry. With continued research and advancements in genetic technology, the potential for improving poultry health and productivity through targeted breeding programs is promising. This knowledge can be used to predict the health performance of poultry, especially in intensive production environments that often induce stress and weaken immunity (Alotiby, 2024). For example, a study by Song et al. (2022) found that higher expression of the iNOS gene was associated with improved resistance to respiratory diseases in broiler chickens.

Moreover, genomic technologies and genetic analyses have enabled further identification of the role of the iNOS gene in the chicken immune system. Genomic research by Verwoolde et al. (2020) revealed that variations in the iNOS gene not only influence immunity but also impact the metabolic efficiency of chickens, making it broadly significant for sustainable poultry production. This finding is supported by Asfor et al. (2021), who highlighted the importance of the iNOS gene along with the MHC gene as key genetic markers for selecting chickens with high disease resistance.

Despite extensive research, significant knowledge gaps persist in understanding how diversity in the iNOS gene specifically influences immune traits in chickens. Prior studies, such as Huang et al. (2024), highlight the strong impact of environmental factors on iNOS gene expression, underscoring the need for further investigation into these interactions. Furthermore, while the potential of iNOS gene diversity to enhance disease resistance is recognized, its integration into commercial chicken breeding programs remains underexplored and underutilized (Souillard et al., 2024).

This study aims to explore the relationship between iNOS gene diversity and immune traits in chickens while providing new insights that can be applied to breeding chickens with enhanced disease resistance. This approach is expected to contribute to achieving sustainable improvements in chicken production efficiency.

Material and Method

Experimental Animals

This research was conducted in July 2023. Blood samples from 100 Kampung chickens were obtained from the Animal Breeding and Genetics Laboratory at the Faculty of Animal Science, University of Mataram, Indonesia. All chickens utilized in the present investigation were cared for according to animal welfare principles. They were kept and nourished under uniform circumstances to reduce environmental impact.

Polymorphism Study

DNA extraction was performed following the procedure of the Genomic DNA Mini Kit Geneid (ISO 9001:2008 QMS). The DNA was then quantified using a NanoDrop spectrophotometer to ensure enough DNA for downstream applications. Once the DNA was quantified, it was stored at -20°C to maintain its integrity until further analysis was performed (Bhoyar et al., 2024). The extracted DNA will be used for genotyping studies to investigate potential genetic markers associated with the disease phenotype. For Polymerase chain reactions (PCR) amplification, the forward (5-CCAAGGACTTACAGGTGTGG-3) and reverse (5-CCAGGATGTTTGGGCTGTTG-3) primers were developed based on the intron 24 of the chicken iNOS genomic sequence (GenBank accession No. ENSGALG00000038096). The PCR was conducted in a 25 µL reaction volume containing 10 µL of DNA template, 12.5 µL of PCR master mix, 1 µL of forward primer, 1 µL of reverse primer, and 0.5 µL of Taq DNA polymerase. The PCR reaction was conducted under these conditions: predenaturation at 95 °C for 5 minutes, followed by 35 cycles consisting of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 30 seconds; the final extension was performed at 72 °C for 5 minutes. Genotyping was conducted by restriction fragment length polymorphism (RFLP) analysis. The PCR product of intron 24 of iNOS, measuring 449 bp, was subjected to digestion with 3 U of AluI restriction enzyme (Thermo Fisher Scientific, Waltham, MA). The

PCR product (5 µL) was subjected to digestion overnight at 37 °C. The detection of RFLPs in 100 Kampung chickens was conducted using electrophoresis on a 2% agarose gel in 0.5X TBE, supplemented with 2.5 µL of ethidium bromide, and the RFLP patterns were observed using a UV transilluminator (AlphaImager®EP). The restriction patterns consisted of a single uncut fragment of 449 bp (designated as the T allele) and two fragments of 310 bp and 139 bp (designated as the C allele).

Using Popgene software version 1.31, the frequency of genotypes and alleles, heterozygosity (Ho and He), Hardy-Weinberg equilibrium, polymorphism information content (PIC), and fixation index (Fst) were analyzed.

Number of Leukocytes and Their Differentiation

The number of leukocytes and their differentiation were determined using the counting chamber and Rapid methods (Yamanishi et al., 2007). 20 µL of chicken blood was dissolved in 380 µL of Turk solution (1 mL of 1% gentian violet in water, 1 mL of glacial acetic acid, and 100 mL of distilled water). The total number of leukocytes was determined by counting viable cells in four different areas under a light microscope (100X magnification), then multiplied by 50 to get the concentration per mm^3.

Phagocytosis Test

A phagocytosis test was conducted to assess macrophage activity and capacity. This involved preparing macrophage cells and bacteria (*Staphylococcus aureus*) and conducting a phagocytosis assay (Putra et al., 2021). Macrophages were extracted from the peritoneal fluid by injecting 5 mL of NaCl into the cavity. Macrophage activity and capacity, which indicate the effectiveness and capability of the macrophages, were calculated using the following formula by Cordeiro et al. (2023).

Clearance Test

The clearance test detected Specific immune responses in the blood samples (Gajic et al., 2022). The clearance test method compared the growth of normal bacterial (S. Pullorum) populations with those receiving specific treatment. The effect of the treatment on bacterial growth was assessed after 24-48 hours of incubation at 35±1°C. To prepare the bacterial culture, the culture is first revitalized in a nutrient medium at 36±1°C for 18-24 hours, followed by a sub-culture in Brain Heart Broth medium at the same temperature for another 18-24 hours.

Statistical Analysis

The study analyzed the iNOS genetic variation and immune traits using the SAS 9.1.3 software from the SAS Institute in Cary, NC, USA. The model utilized in the analysis, as described by Maharani et al. (2019), is as follows:

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

The equation represents the observation of immune traits, where Y_{ij} is the trait observation, μ is the average value, G_i is the genetic effect, and e_{ij} is the random residual effect.

Results and Discussion

Polymorphism of The iNOS Gene

After digesting iNOS PCR products with AluI, you observed three fragments: 449, 310, and 139 bp for the TC genotype, 310 and 139 bp for the CC genotype, and 449 bp for the TT genotype (Figure 1). The observed PCR-RFLP patterns indicate the presence of a polymorphism at the iNOS g.15056T>C locus in Kampung chickens. This polymorphism is crucial for understanding genetic diversity within the Kampung chicken population, which may be associated with specific phenotypic characteristics, such as disease resistance, production performance, or environmental adaptation. Previous studies have shown that the iNOS gene plays a significant role in regulating immune responses, particularly in producing nitric oxide (NO), a key molecule in combating bacterial and viral infections (Bath et al., 2021; Evseev & Magor, 2019). The genotype distribution, allele frequency, and genetic parameters at the g.15056T>C locus in Kampung chickens are displayed in Table 1.

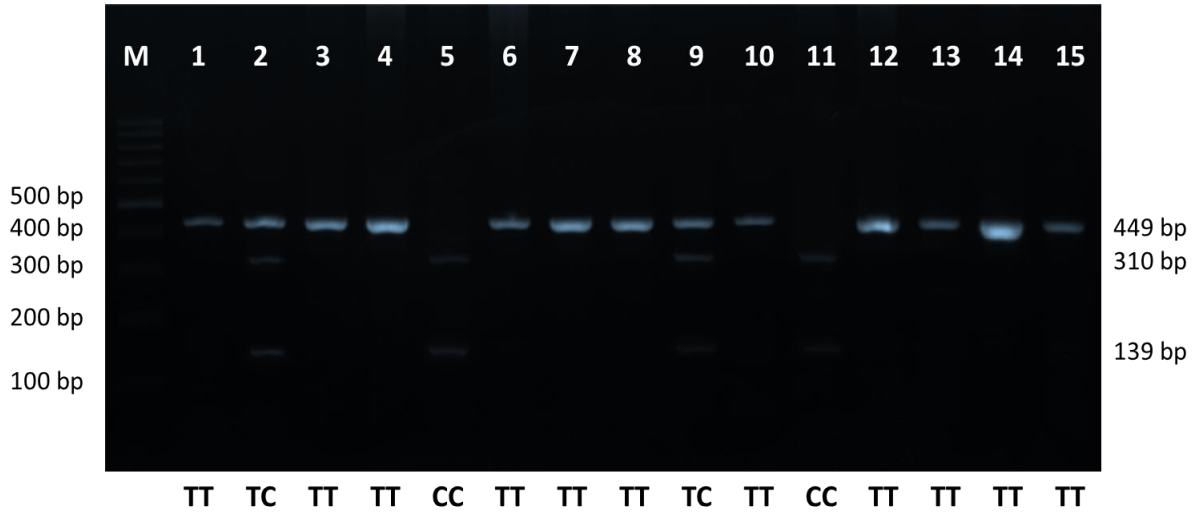

Figure 1. PCR-RFLP Patterns of the iNOS g.15056T>C Locus in Kampung Chickens Using *Alu*I Restriction Enzyme on a 2% Agarose Gel

Table 1. Genotype Distribution, Allele Frequency, and Genetic Parameters at the iNOS g.15056T>C Locus in Kampung Chickens

Locus	Restriction enzyme	Parameter		Kampung Chicken
g.15056T>C	*Alu*I	Genotype	TT	0.640
			TC	0.150
			CC	0.210
		Allele	T	0.715
			C	0.285
		Ho		0.150
		He		0.407
		PIC		0.407
		p-value		$p < 0.001$
		Chi^2		39.850

Ho= observed heterozygosity. He= expected heterozygosity. PIC= polymorphism information content. Chi^2 = Chi-square.

Table 1 shows the genotype distribution of TT (0.640), TC (0.150), and CC (0.210). The frequency of the T allele (0.715) is higher than that of the C allele (0.285), indicating the dominance of the T allele in the Kampung chicken population studied. The genotype distribution pattern aligns with Hardy-Weinberg equilibrium, as verified by the Chi-square value ($\chi^2 = 39.850$; $p < 0.001$), which indicates Hardy-Weinberg disequilibrium. This disequilibrium may be caused by natural selection, migration, or specific genetic pressures within the population (Ehrlich et al., 2020).

The Ho value of 0.150 indicates that this population's frequency of heterozygous individuals is relatively low. The He value of 0.407 reflects a moderate level of genetic variation at this locus. The PIC value of 0.407 suggests that this locus has a moderate level of polymorphism, making it useful for population genetic analysis and trait association studies.

Polymorphism in the iNOS gene can influence its function, which is associated with nitric oxide (NO) production. NO is a key molecule in innate immune responses,

including combating bacterial and viral infections (Herbert & Panagiotou, 2022). Therefore, this polymorphism may contribute to differences in disease resistance among individuals in the Kampung chicken population. Previous studies have also shown that genetic polymorphisms in immune-related genes can affect production performance and adaptation to environmental stress (Goel et al., 2021).

The PCR-RFLP results and genetic analysis of the iNOS g.15056T>C locus reveal significant polymorphism in the Kampung chicken population. The allele frequency and genotype distribution provide insights into this population's genetic diversity. The Hardy-Weinberg disequilibrium indicates the potential presence of selective factors within this population. This polymorphism holds great potential for molecular breeding programs, particularly to enhance disease resistance and production efficiency in Kampung chickens.

Association of iNOS Gene Polymorphism with İmmune Traits

Each genotype was assessed based on total leukocyte count (measured in units of $10^3/mm^3$) and the percentages of three types of white blood cells: heterophils, lymphocytes, and monocytes. The mean values and standard deviations for each genotype are presented in Table 2.

Table 2. The Correlation Between iNOS Gene Genotype and Leukocyte Count and Differentiation in Kampung Chickens

Genotype	N	İmmune Traits			
		Leukocyte (10^3 mm^{-3})	Heterophils (%)	Lymphocyte (%)	Monocyte (%)
TT	10	20.28±3.75	51.75±6.48	47.51±6.61	1.95±0.64
TC	10	18.05±3.40	41.68±6.18	58.12±5.91	2.14±0.61
CC	10	18.16±3.01	45.03±5.84	52.53±5.67	2.97±0.53

N = Number of samples

The TT genotype exhibited the highest leukocyte count (20.28±3.75), followed by the CC (18.16±3.01) and TC (18.05±3.40) genotypes. Although there is a slight variation among the genotypes, the total leukocyte count indicates that the TT genotype has a higher immune response regarding leukocyte production than the TC and CC genotypes. Previous studies have shown that total leukocyte counts are often influenced by genetic factors associated with the body's ability to respond to infections (Wlaźlak et al., 2023). Heterophils are white blood cells that play an important role in responding to bacterial infections. The TT genotype has the highest percentage of heterophils (51.75±6.48), indicating a potentially stronger immune response to bacterial infections. Conversely, the TC genotype has the lowest percentage of heterophils (41.68±6.18), which may suggest differences in immune responses to specific pathogens between genotypes. This is supported by studies that found genetic factors can influence the number and activity of heterophils in response to infections (Redmond et al., 2011).

Lymphocytes are involved in adaptive immune responses, including fighting viruses and other pathogens. The TC genotype has the highest percentage of lymphocytes (58.12±5.91), indicating a greater tendency toward immune responses involving lymphocytes. In contrast, the TT genotype has a lower percentage of lymphocytes (47.51±6.61), which may reflect a balance among different types of white blood cells involved in the immune response. Research by Meijerink et al. (2021) showed that lymphocyte distribution could vary based on genetic factors, and their role in adaptive immune responses depends heavily on the type of infection encountered. Monocytes function in clearing dead cells and regulating inflammatory responses. The CC genotype has the highest percentage of monocytes (2.97±0.53), which may indicate a greater involvement in inflammatory processes or responses to chronic infections compared to the TT and TC genotypes. Research by Zmrhal et al. (2023) highlighted the significant role of monocytes in chronic inflammation, which can be influenced by genetic factors related to the body's inflammatory state. These differences illustrate how genetic variation can affect the composition of white

blood cells and the immune response to infections or diseases.

Association of iNOS Gene Polymorphism with Macrophage Activity and Capacity

Table 3 presents the correlation between the three genotypes of the iNOS gene (TT, TC, and CC) with macrophage activity, the capacity of activated macrophages, and the total bacteria ingested by macrophages in Kampung chickens challenged with *Staphylococcus aureus*.

Table 3. The Correlation Between iNOS Genotype and Macrophage Activity and Capacity in Kampung Chickens Challenged with *Staphylococcus aureus*

Genotype	N	Macrophage activity (%)	Capacity macrophages activated^{-1}	Total of bacteria ingested
TT	10	81.31±1.75^{a}	2042.76	82 364.49±92.52^{a}
TC	10	84.16±1.86^{a}	2136.17	89 825.61±84.53^{a}
CC	10	91.74±1.92^{b}	2279.49	103 474.05±69.21^{b}

N = Number of samples; ^{a,b}Means with different superscript in the same column shows significant difference (P<0.05)

Table 3 shows significant differences between the three genotypes regarding macrophage activity, the capacity of activated macrophages, and the total bacteria ingested by macrophages. This difference is most pronounced in the CC genotype, which shows the best results across all three variables tested. The CC genotype exhibits higher macrophage activity (91.74 ± 1.92%) compared to the TT genotype (81.31 ± 1.75%) and TC genotype (84.16 ± 1.86%). The higher macrophage activity in chickens with the CC genotype suggests that this genotype may have genetic variations that support a stronger immune response to *Staphylococcus aureus* infection. Previous studies have also supported the idea that genetic variation in the iNOS gene can affect macrophages ability to respond to infections (Zhang et al., 2023).

The capacity of activated macrophages in the CC genotype (2279.49) is also higher compared to the TT genotype (2042.76) and TC genotype (2136.17). The higher macrophage capacity in the CC genotype can be interpreted as the iNOS gene being more effective in producing NO (nitric oxide), which enhances the macrophages' ability to phagocytize pathogens. This is in line with research showing that increased iNOS expression can improve the phagocytic capacity of macrophages (Sun et al., 2021). The total bacteria ingested by macrophages also shows higher results in the CC genotype (103,474.05 ± 69.21) compared to the TT genotype (82,364.49 ± 92.52) and TC genotype (89,825.61 ± 84.53). These results indicate that macrophages with the CC genotype can better ingest bacteria, reflecting an enhanced adaptive immune response to *Staphylococcus aureus* infection. The increased phagocytic ability is likely influenced by genetic variation in the iNOS gene, which increases NO production and boosts the immune response (Zhang et al., 2023).

Overall, the CC genotype demonstrates the best results in all three parameters tested, indicating that the iNOS gene with this variation may significantly strengthen the immune response of Kampung chickens to *Staphylococcus aureus* infection. This reinforces previous findings that variations in the iNOS gene influence macrophage ability to combat pathogens (Kulkarni et al., 2022).

Association of iNOS Gene Polymorphism with resistance to *Salmonella Pullorum*

Table 4 presents the correlation between the three genotypes of the iNOS gene (TT, TC, and CC) and the resistance of Kampung chickens to *Salmonella Pullorum* infection, measured by the initial and final bacterial concentrations as well as the bacterial death rate.

Table 4. The Correlation Between iNOS Genotype and Resistance to *Salmonella Pullorum* in Kampung Chickens

Genotype	N	Early concentration (10^7cfu mL^{-1})	Final concentration (10^5cfu mL^{-1})	Death rate of bacteria (%)
TT	10	2.40	163±9.42[a]	36.92±4.35[a]
TC	10	2.40	147±8.68[a]	44.94±4.13[a]
CC	10	2.40	95±7.25[b]	60.81±3.54[b]

N = Number of samples; [a,b]Means with different superscript in the same column shows significant difference (P<0.05)

The results show significant differences between the three genotypes regarding resistance to *Salmonella Pullorum*. The CC genotype shows the best results, with a lower final bacterial concentration and a higher bacterial death rate, indicating better resistance to the infection. In all three genotypes, the initial bacterial concentration was the same, 2.40×10^7 cfu/mL, indicating that the bacterial load given to each group was consistent. However, the final bacterial concentration after infection shows significant differences. The CC genotype showed a greater decrease in bacterial concentration ($95 \pm 7.25 \times 10^5$ cfu/mL) compared to TT ($163 \pm 9.42 \times 10^5$ cfu/mL) and TC ($147 \pm 8.68 \times 10^5$ cfu/mL). The more significant decrease in bacterial concentration in the CC genotype indicates that this genotype is more effective in combating the infection. This may be due to genetic variation in the iNOS gene that enhances the immune response to bacteria, as previous studies have shown that iNOS expression plays an important role in controlling bacterial infections (Tachibana et al., 2022).

The CC genotype also showed the highest bacterial death rate (60.81 ± 3.54%), compared to TC (44.94 ± 4.13%) and TT (36.92 ± 4.35%). This result indicates that chickens with the CC genotype can better eliminate *Salmonella Pullorum*. The increased bacterial death rate is likely associated with enhanced nitric oxide (NO) production by iNOS in this genotype, which serves to destroy and eliminate pathogens. Research by Adams et al. (2023) shows that increased iNOS expression in immune cells can strengthen phagocytic ability and bacterial killing. The CC genotype shows better resistance to *Salmonella Pullorum* infection, as evidenced by the lower final bacterial concentration and higher bacterial death rate. This suggests that variations in the iNOS gene affect the chicken's capacity to respond effectively to infections. A study by Adams et al. (2023) demonstrated that variations in the iNOS gene could increase NO production, which is crucial in enhancing resistance to bacterial infections, especially by boosting the activity of macrophages and neutrophils to fight pathogens.

Overall, the CC genotype shows better resistance to *Salmonella Pullorum*, indicating that genetic variation in the iNOS gene is important in enhancing the immune response to infection. Muhsinin et al. (2018) showed that the CC genotypes of the iNOS gene was significantly associated with *Salmonella Pullorum* disease resistance in Sentul chicken compared to the others genotype (TT and TC). This strengthens the finding that higher iNOS expression contributes to improved chicken resistance to bacterial infections (Stefanetti et al., 2023).

Conclusion

This study demonstrates a significant association between the polymorphism of the (iNOS)|AluI g.15056T>C gene and immune characteristics in Kampung chickens. The CC genotype has been proven to have advantages over the TT and TC genotypes in terms of macrophage activity, activated macrophage capacity, and resistance to bacterial infections such as *Staphylococcus aureus* and *Salmonella Pullorum*. These findings also support previous studies highlighting the role of the iNOS gene in enhancing resistance to pathogens in chickens.

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