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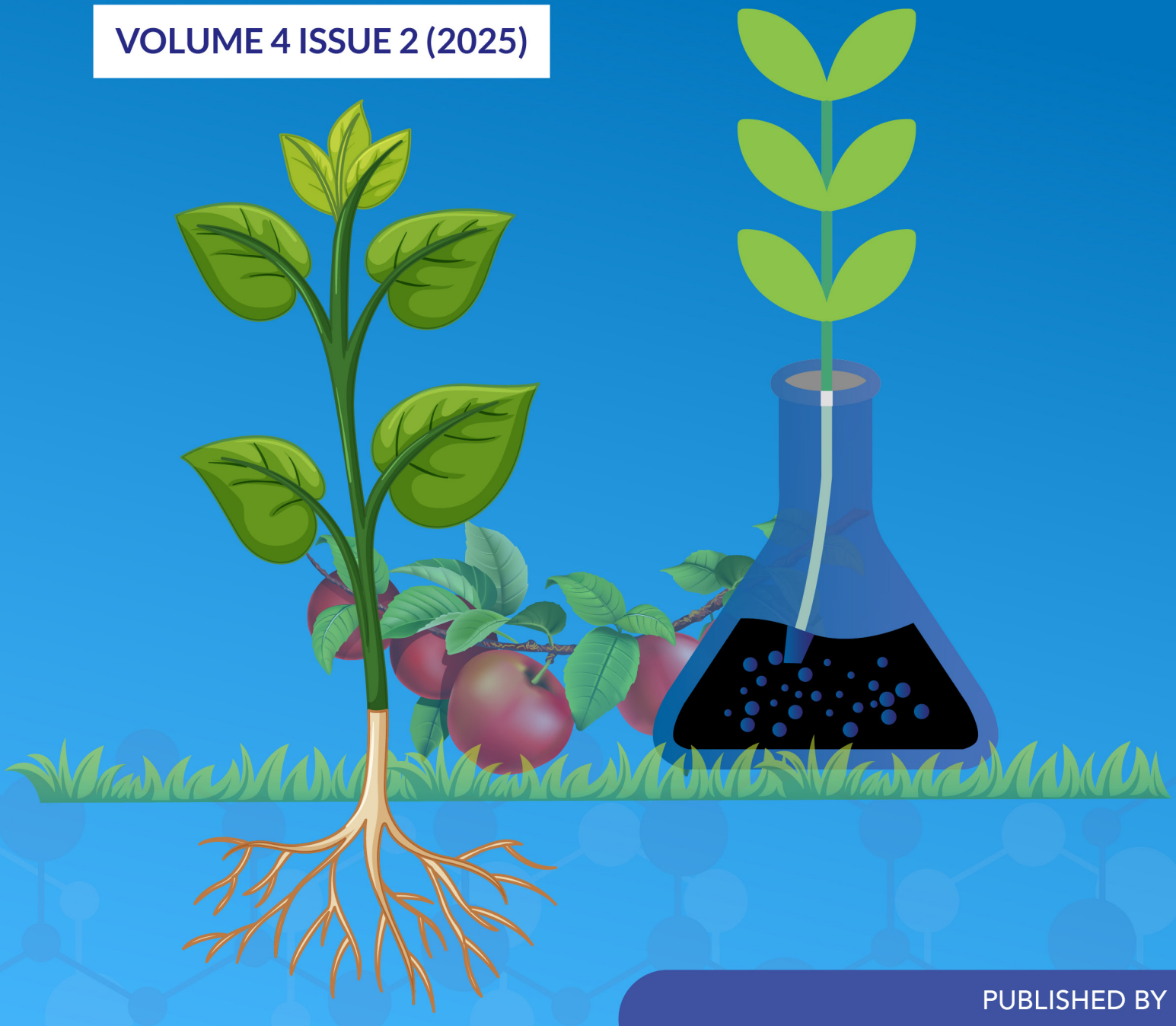

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Variation in Toll-Like Receptor 4 (TLR4) Gene in Chicken Genotypes and Its Association with Resistance to Attenuated Newcastle Virus

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ABSTRACT

Newcastle disease (ND) remains a major constraint to poultry production, and genetic variation in immune-related genes may influence vaccine responsiveness. This study evaluated variation in the Toll-like receptor 4 (TLR4) gene among four chicken genotypes: normal feather (NFC), naked neck (NNC), frizzle feather (FFC), and exotic (EXC), and assessed their antibody responses to attenuated ND vaccination. A total of 100 day-old chicks were reared under uniform intensive management. Birds were vaccinated at two weeks of age, with a booster administered one week later. Blood samples were collected 14 days post-vaccination for determination of haemagglutination inhibition (HI) titre. Genomic DNA was extracted from blood, and the TLR4 gene was PCR-amplified, sequenced, and analyzed for nucleotide and haplotype diversity, mismatch distribution, and phylogenetic relationships. Results showed significant differences in antibody titres among genotypes ($p < 0.05$), with FFC exhibiting the highest response, followed by NNC, NFC, and EXC. Genetic diversity analysis revealed the highest nucleotide diversity in FFC ($\pi = 0.121$) and lowest in EXC ($\pi = 0.031$), with haplotype diversity ranging from 0.822 (NNC) to 1.00 (NFC). Pairwise Fst and Gst values indicated low to moderate differentiation, and phylogenetic analysis showed admixture among genotypes, with two major clades. Mismatch distributions were multimodal and ragged, suggesting complex demographic histories. The findings indicate that indigenous genotypes, particularly FFC and NNC, combine higher genetic diversity with stronger antibody responses to ND vaccination. Polymorphisms in the TLR4 gene may contribute to enhanced immune competence, highlighting the potential of these genotypes as genetic resources for breeding programmes aimed at improving disease resistance and sustainable poultry production.

INTRODUCTION

Poultry production plays a vital role in food security and livelihood in many parts of the world, especially in developing countries, where it provides affordable animal protein and a steady source of household income. Global consumption of poultry meat has increased significantly in recent decades, driven by its relative affordability and nutritional value (Korver, 2023). However, this growth has been accompanied by increasing dependence on a limited number of highly productive commercial breeds, leading to the marginalization of indigenous chickens and a continual erosion of avian genetic resources (Korver, 2023; Senbeta & Keyata, 2024).

In Nigeria, chickens represent the most widely distributed poultry species, with an estimated population exceeding 166 million birds (FAO, 2007; Chikezie, 2021). Indigenous chickens, in particular, play a vital socio-economic role in rural communities where they are often reared under traditional scavenging systems. Their ability to survive under harsh environmental conditions, resist endemic diseases, and thrive with minimal inputs makes them invaluable genetic resources for sustainable poultry production (Soglia *et al.,* 2020; Xie *et al.,* 2024; Ekerette *et al.,* 2025a; Ushie *et al.,* 2025). Despite their relatively low productivity compared to exotic breeds, indigenous chickens are recognized as reservoirs of important adaptive genes that can be exploited for genetic improvement (Ajayi, 2010; Kpomasse *et al.,* 2023; Ekerette *et al.,* 2025a, b).

Advances in molecular genetics now provide powerful tools for assessing genetic diversity, population structure, and candidate genes of economic and adaptive importance (Nazari & Pourkazemi, 2023; Wu *et al.,* 2025; Ekerette *et al.,* 2025c). Increasing attention has been given to immune-related genes, as variation in these loci can influence resistance to infectious diseases. Newcastle disease (ND), caused by Newcastle disease virus (NDV), remains one of the most economically devastating poultry diseases worldwide (Hu *et al.,* 2022; Dharmayanti *et al.,* 2023; Efienokwu & Ekerette, 2024; Zereen *et al.,* 2025). Although vaccination is routinely practiced, differences in immune response across chicken genotypes suggest that genetic background significantly influences vaccine efficacy (Chuwatthanakhajorn *et al.,* 2023).

Toll-like receptors (TLRs) form a key part of the innate immune system, recognizing pathogen-

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associated molecular patterns and initiating host defense mechanisms (Wicherska-Pawłowska *et al.,* 2021; Chen *et al.,* 2024). Among these, toll-like receptor 4 (TLR4) has been reported to play a crucial role in immune signaling against viral and bacterial infections (Olejnik *et al.,* 2018; Kim *et al.,* 2023). Polymorphisms in TLR4 may therefore account for differential resistance or susceptibility to infectious diseases, including ND. To better understand the genetic and immunological basis of ND resistance, this study evaluated variation in the TLR4 gene among different chicken genotypes. It assessed their antibody response to Newcastle disease vaccination using the haemagglutination inhibition (HI) titre method. Together, these approaches provide insights into the genetic underpinnings of immune competence in indigenous and exotic chicken populations.

MATERIALS AND METHODS

Location and Management Procedures

A total of 100 day-old chicks, comprising 25 birds each of the normal feather (NFC), naked neck (NNC), frizzle feather (FFC), and exotic (EXC) genotypes, were used for the study. The birds were reared under an intensive management system at the Animal House of the Department of Genetics and Biotechnology, University of Calabar, Nigeria. They were housed in standard cages with natural ventilation. Before arrival, the Animal House was fumigated with an organophosphate insecticide and disinfected with Dettol to minimize the risk of disease outbreaks. The chicks were acclimatized for two weeks and provided with starter feed and water ad libitum during this period. After acclimatization, they were separated into four groups based on genotype and reared in distinct cages under uniform management conditions. The research was approved by the Research Ethics and Linkage Committee of the Faculty of Biological Sciences, University of Calabar (approval number FBS/RELC/2023/001).

Vaccination and Blood Sampling

At two weeks of age, all birds were orally administered 1 ml of live attenuated Newcastle disease vaccine. Fourteen days post-vaccination, blood samples (1 ml per bird) were collected from the wing vein for antibody titre determination. Following a one-week interval, a booster vaccination was administered, and blood samples were again collected 14 days later for the second titre measurement.

Antibody Titre Measurement

Phosphate-buffered saline (PBS) was prepared with sodium chloride (8 g/L), potassium chloride (0.2 g/L), disodium hydrogen phosphate (1.15 g/L), and potassium dihydrogen phosphate (0.2 g/L) in 10 L of distilled water (pH 7.3). Antibody levels were measured using the haemagglutination inhibition (HI) test with a two-fold serial dilution method, ranging from 10^2 to 10^{256}. Serial dilutions were prepared in test tubes, followed by the addition of 1 ml of antigen and 1 ml of serum. Tubes were incubated at room temperature for one hour, and the antibody titre for each sample was recorded as the highest dilution showing visible inhibition of haemagglutination (Efienokwu & Ekerette, 2024).

Extraction of DNA

Genomic DNA was extracted from blood samples of 10 birds per genotype at the Animal Science Molecular Genetics Laboratory, Department of Animal Science, University of Port Harcourt, Nigeria, using the Quick-DNA MiniPrep Kit (Zymo Research, USA) following the protocol of Ekerette *et al.* (2025c). To enhance lysis efficiency, beta-mercaptoethanol was added to the lysis buffer (500 µL per 100 ml). In brief, 200 µL of blood was mixed with 800 µL of lysis buffer in an Eppendorf tube, vortexed for 5 seconds, and incubated at room temperature for 10 minutes. The lysate was transferred to a Zymo-Spin column in a collection tube and centrifuged at 10,000 rpm for 1 min. After discarding the flow-through, the column was washed with 200 µL DNA pre-wash buffer and 500 µL g-DNA wash buffer, with centrifugation steps at 10,000 rpm for 1 min each. DNA was eluted with 50 µL elution buffer after a 5-minute incubation and centrifugation at 15,000 rpm for 30 s. The purified DNA was stored at –20 °C until further use.

PCR Amplification and Sequencing of TLR4 Gene

PCR amplification of the TLR4 gene was performed using primers reported by Wu *et al.* (2014): forward: 5'-AGTCTGAAATTGCTGAGCTCAAAT-3' and reverse: 3'-GCGACGTTAAGCCATGGAAG-5'. Each 25 µL PCR reaction contained: 2 µL genomic DNA, 1 µL of 50 mM MgCl2, 1.5 µL of 2 mM dNTPs, 1.5 µL of 10× PCR buffer, 0.4 µL of each primer, 1 µL of STABVIDA proprietary Taq polymerase, and 17.2 µL double-distilled water. Amplification was performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, USA) under the following cycling conditions: initial denaturation at 95 °C for 5 min; 25 cycles of denaturation at 94 °C for 40 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 7 min. PCR products were purified using the ExoFast protocol. Sequencing of purified PCR products was conducted on an ABI 3730×L sequencer (Applied Biosystems, USA). Each 20 µL sequencing reaction contained ~20 ng of purified PCR product, 8 µL of BigDye Terminator Reaction Mix, 8 µL deionized water, and 2 µL primer. Cycling conditions included 25 cycles of 96 °C for 10 s, 60 °C for 5 s, and 60 °C for 4 min.

Statistical Analysis

Data obtained from antibody titre measurements were subjected to analysis of variance (ANOVA). Mean differences were separated using the least significant difference (LSD) test at 5% probability level. BioEdit software version 7.2.5 was used to view and edit the

sequences. Multiple sequence alignment of all samples was performed using MEGA software (Tamura *et al.,* 2013). Estimation of variations in the aligned regions, including nucleotide diversity (π) and haplotype diversity (Hd), was carried out using DnaSP version 5.1 (Rozas *et al.,* 2017). The mismatch distribution of the TLR4 sequences from the chicken genotypes was also analyzed with DnaSP. A phylogenetic tree was reconstructed using MEGA X, and the visual display of the tree was fine-tuned using iTOL software.

RESULTS AND DISCUSSION

Anitbody Measurement

The antibody titre measurements among the four chicken genotypes following Newcastle vaccination revealed significant differences (Figure 1). At the initial stage, the highest mean loge HI titre was observed in FFC (0.873 ± 0.001), which was significantly higher ($p < 0.05$) than the other genotypes. NNC (0.846 ± 0.001) and NFC (0.841 ± 0.001) did not differ significantly from each other but were lower than FFC. The lowest titre was recorded in EXC (0.816 ± 0.018), which was significantly different from the rest. At the final measurement, a similar pattern was observed. FFC maintained the highest titre (0.853 ± 0.001), followed by NNC (0.848 ± 0.027) and NFC (0.816 ± 0.018). EXC recorded the lowest titre (0.782 ± 0.001d), which was significantly different from all other genotypes.

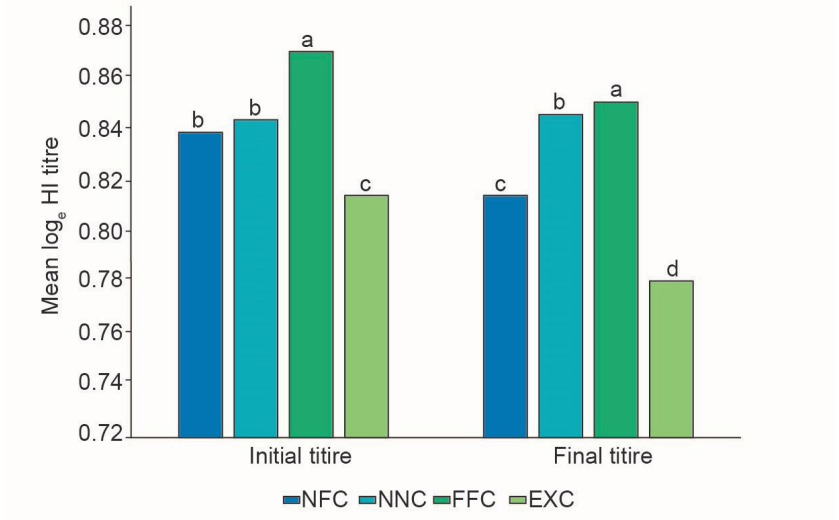

Figure 1: Mean antibody titre of four chicken genotypes following vaccination with attenuated Newcastle vaccine

Genetic Diversity of Four Chicken Genotypes

The genetic diversity indices of the four chicken genotypes revealed varying levels of nucleotide and haplotype diversity (Table 1). The nucleotide diversity (π) ranged from 0.031 in EXC to 0.121 in FFC. The average number of nucleotide differences (K) followed a similar trend, with the highest value recorded in FFC (14.978) and the lowest in EXC (4.689). Haplotype diversity (h) was generally high across the genotypes, with NFC showing the maximum value (1.00), reflecting complete haplotype diversity, while NNC had the lowest (0.822). The number of haplotypes (H) also varied, ranging from 4 in NNC to 10 in NFC, with the overall population exhibiting 21 haplotypes.

Table 1: Genetic diversity indices of four chicken genotypes following vaccination with attenuated Newcastle vaccine

Genotypes	Nucleotide diversity (π)	Haplotype diversity (h)	Haplotype number (H)	Variable sites (S)	Sequence conservation (%)	Average number of nucleotide differences (K)	Number of recombination events (Rm)	Tajima's D
NFC	0.058 ± 0.002	1.00± 0.045	10.0	33.00	44.70	7.422	0	-1.933 ($p < 0.05$)
NNC	0.055 ± 0.0001	0.822 ± 0.005	4.00	16.00	56.30	7.111	0	1.191 ($p > 0.10$)
FFC	0.121 ± 0.001	0.978 ± 0.003	9.00	58.00	42.00	14.978	3	-1.761 ($p > 0.05$)

EXC	0.031 ± 0.000	0.933 ± 0.006	8.00	15.00	54.70	4.689	2	-0.791 ($p > 0.10$)
Overall	0.056 ± 0.0003	0.935 ± 0.001	21.00	59.00	37.60	6.028	3	-2.422 ($p < 0.01$)

The number of variable sites (S) varied widely, from 15 in EXC to 58 in FFC, with the overall dataset containing 59 variable sites. Sequence conservation percentages ranged from 42.0% (FFC) to 56.3% (NNC), with an overall conservation estimate of 37.6%. Recombination events (Rm) were absent in NFC and NNC but detected in FFC (3) and EXC (2), with a total of 3 events recorded. Tajima's D values varied among the genotypes; they were negative in NFC (-1.933, $p > 0.05$), FFC (-1.761, $p > 0.05$), EXC (-0.791, $p > 0.10$), and in the overall population (-2.422, $p > 0.01$), while NNC showed a positive value (1.191, $p > 0.10$).

Pairwise Genetic Differentiation among Four Chicken Genotypes

The pairwise differentiation indices among the four chicken genotypes showed variable levels of genetic differentiation (Table 2). The Fst values ranged from -0.016 (NFC and FFC) to 0.153 (NNC and EXC). Negative Fst values observed in some comparisons (NFC and FFC, NFC and EXC, FFC and EXC) indicate negligible or no measurable differentiation, whereas the highest value between NNC and EXC (0.153) suggests greater differentiation. Similarly, the Gst values varied between -0.015 (FFC and EXC) and 0.091 (NNC and FFC), reflecting differences in genetic diversity partitioning among populations. The average number of nucleotide substitutions per site (Dxy) ranged from 0.033 (NFC and EXC) to 0.081 (NNC and FFC), while the net nucleotide divergence (Da) values were mostly low, ranging between -0.0002 (FFC and EXC) and 0.007 (NNC and EXC, NFC and NNC). The average number of nucleotide differences between populations (Kxy) followed the same trend, ranging from 3.580 (NFC and EXC) to 8.580 (NNC and FFC).

Table 2: Pairwise differentiation among four chicken genotypes following vaccination with attenuated Newcastle vaccine

Population 1	Population 2	Fst	Gst	Dxy	Da	Kxy
NFC	NNC	0.112	0.047	0.061	0.007	6.490
NFC	FFC	-0.016	0.010	0.070	-0.002	7.420
NFC	EXC	-0.006	0.009	0.033	-0.0001	3.580
NNC	FFC	0.06	0.091	0.081	0.006	8.580
NNC	EXC	0.153	0.074	0.044	0.007	4.740
FFC	EXC	-0.005	-0.015	0.054	-0.0002	5.790

Mismatch Distribution

The mismatch distributions for each genotype and for the pooled dataset were multimodal and ragged, each showing multiple peaks rather than a single smooth peak (Figure 2). Specifically, the NFC and NNC exhibited several small peaks across low-to-moderate pairwise differences. The FFC showed pronounced, sharp peaks that extended to larger pairwise differences, while the EXC also presented multiple distinct peaks at low-to-moderate differences. The combined distribution was similarly ragged and multimodal.

Phylogenetic Relationship among Chicken Genotypes

Figure 3 presents the phylogenetic relationships of each chicken genotype. Each genotype exhibited within-group variation, which contributed to the formation of distinct sub-clades among the samples. This observed pattern confirms the presence of genetic heterogeneity within the genotypes, as reflected in the subgrouping of the samples.

The phylogenetic analysis of all the chicken genotypes revealed the presence of two major clades (Figure 4). The first clade contained a single sample from the FFC genotype, suggesting some degree of genetic divergence within this variety. The second major clade comprised the remaining samples across all genotypes, which were intermixed rather than distinctly separated. Within this broader cluster, several sub-clades were observed, each containing a mixture of different chicken genotypes. The clustering pattern indicated a high level of genetic admixture among the genotypes, reflecting a close evolutionary relationship and limited genetic differentiation. Notably, the phylogenetic tree did not segregate the samples strictly according to their genotypes, implying possible gene flow or shared ancestry among the studied populations.

Discussion

The present study examined the variation in the Toll-like receptor 4 (TLR4) gene among four chicken genotypes and its association with antibody response to Newcastle disease (ND) vaccination. The findings provide insights into the interplay between genetic diversity and immune competence in indigenous and exotic chickens.

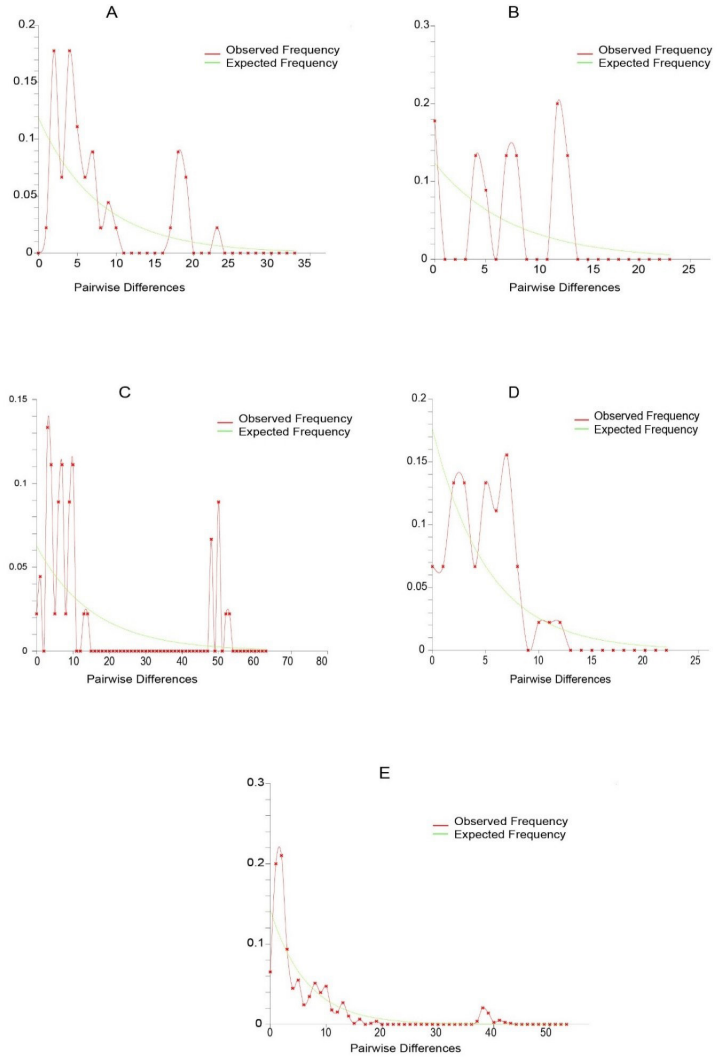

Figure 2: Mismatch distribution of the four chicken genotypes following vaccination with attenuated Newcastle vaccine. A. Normal feather chicken, B. Naked neck chicken, C. Frizzle feather chicken, D. Commercial chicken, and E. All genotypes

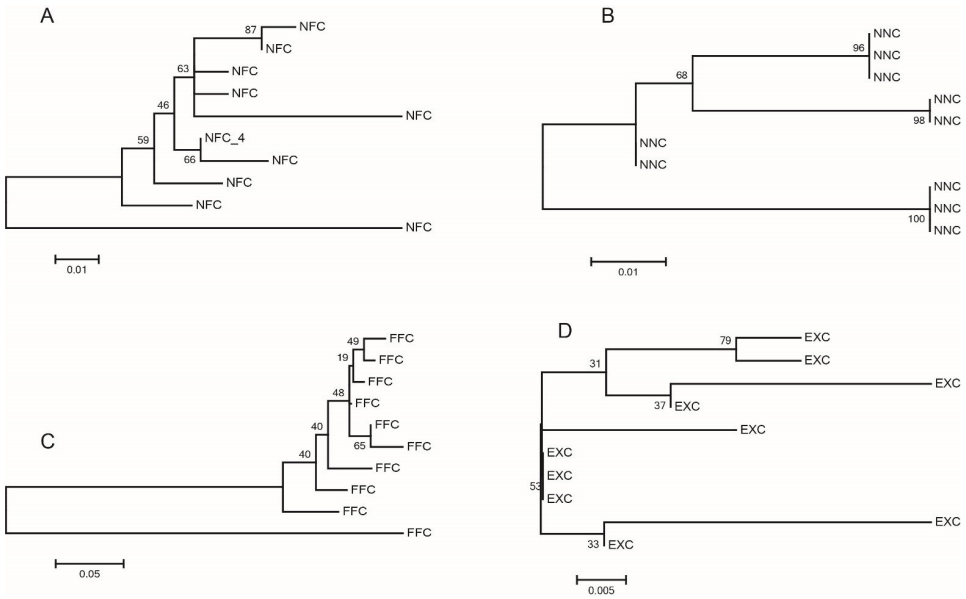

Figure 3: Phylogenetic trees of the four chicken genotypes following vaccination with attenuated Newcastle vaccine. A. Normal feather chicken, B. Naked neck chicken, C. Frizzle feather chicken, and D. Commercial chicken

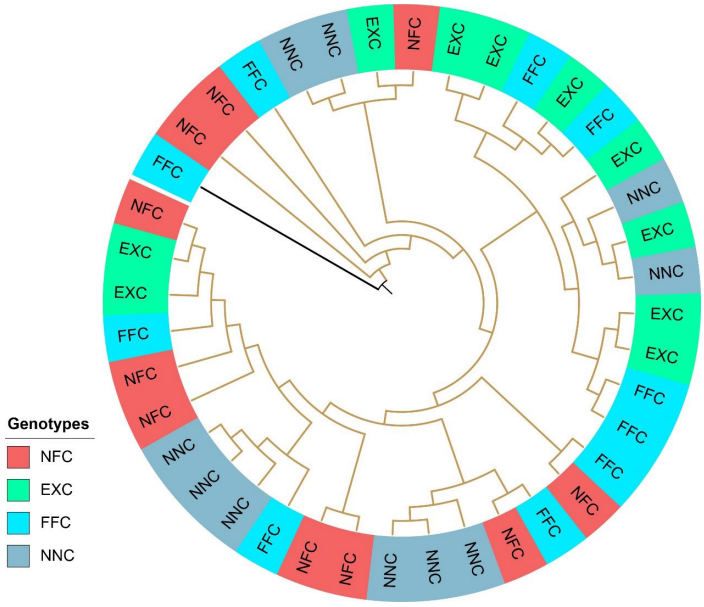

Figure 4: Phylogenetic tree showing the relationship between four chicken genotypes following vaccination with attenuated Newcastle vaccine. Each genotype is indicated by a different colour legend, while the major clades are shown with separate branch colours.

Significant differences in haemagglutination inhibition (HI) titres were observed among the genotypes, with FFC consistently showing the highest titres, followed by NNC, NFC, and EXC. This pattern suggests that FFC possess enhanced humoral immune responsiveness to ND vaccination. Previous studies have demonstrated that genetic background influences vaccine-induced antibody production, likely due to differences in innate immune gene expression and antigen recognition efficiency (Linnik *et al.,* 2016; Clemente-Suárez *et al.,* 2025). The relatively lower titres observed in EXC may reflect reduced adaptive immune responsiveness, possibly due to long-term selective breeding for production traits (Seo *et al.,* 2017; Fu *et al.,* 2023) at the expense of immunocompetence. The higher titres in indigenous genotypes highlight their potential as reservoirs of adaptive genes (Soglia *et al.,* 2020; Xie *et al.,* 2024) that can enhance disease resistance in breeding programmes.

Analysis of genetic diversity indices revealed substantial variation among the genotypes. FFC exhibited the highest nucleotide diversity and average number of nucleotide differences, while EXC showed the lowest diversity. High haplotype diversity in NFC indicates the presence of multiple unique allelic variants, which may enhance population resilience to infectious agents. These findings align with previous reports that indigenous chickens harbor considerable genetic variation due to their adaptation to variable environments and minimal human-directed selection (Soglia *et al.,* 2020; Xie *et al.,* 2024; Ekerette *et al.,* 2025a). Conversely, reduced genetic diversity in EXC is consistent with intensive selective breeding practices aimed at improving growth and productivity, which can inadvertently limit genetic variation related to immunity.

Pairwise Fst and Gst values indicated low to moderate genetic differentiation among genotypes, with negative Fst values in some comparisons suggesting negligible divergence. The highest differentiation was observed between NNC and EXC, reflecting the evolutionary and breeding history of these populations. The phylogenetic analysis further supported this observation, revealing two major clades with extensive admixture among most genotypes, except for one divergent FFC sample. This pattern indicates ongoing gene flow or shared ancestry and underscores the lack of strict genetic isolation among the studied populations. Such admixture is consistent with traditional poultry management systems, where crossbreeding between indigenous and exotic birds is common (Wilkinson *et al.,* 2012; Vargas *et al.,* 20219).

The multimodal mismatch distributions observed across genotypes suggest complex demographic histories, such as selective pressures (Hoelzer *et al.,* 2008). The pronounced peaks in FFC indicate higher genetic variability, consistent with the nucleotide and haplotype diversity data. Conversely, the relatively lower peaks in EXC suggest a more uniform genetic structure, likely resulting from artificial selection and limited effective population size (Fu *et al.,* 2023).

TLR4 plays a critical role in innate immune signaling by recognizing pathogen-associated molecular patterns, including viral components, and initiating inflammatory responses (Olejnik *et al.,* 2018; Kim *et al.,* 2023). Polymorphisms in TLR4 may modulate the strength and efficiency of immune responses, influencing susceptibility or resistance (Noreen *et al.,* 2012), with no exception to NDV. Therefore, the observed variation in TLR4 sequences, combined with differential antibody titres, suggests a potential association between specific TLR4

alleles and enhanced ND vaccine responsiveness. FFC and NNC, which exhibited higher titres, also displayed higher nucleotide diversity and multiple haplotypes, supporting the hypothesis that TLR4 variation contributes to immune competence. The findings emphasize the importance of preserving and utilizing indigenous chicken genetic resources for sustainable poultry production. High genetic diversity and favorable immune responses in FFC and NNC highlight their potential for inclusion in selective breeding programmes aimed at improving ND resistance. In contrast, the low diversity and reduced antibody responses in EXC highlight the potential vulnerability of highly selected commercial lines to infectious diseases. Incorporating TLR4 genotyping into breeding programmes could provide a molecular tool for selecting birds with superior innate and adaptive immune competence, thereby enhancing disease resilience.

CONCLUSION

This study revealed genotype-specific differences in TLR4 diversity and immune response to Newcastle disease vaccination. Frizzle feather (FFC) and naked neck (NNC) chickens exhibited the highest antibody titres and greater genetic diversity, while exotic (EXC) chickens showed the lowest titres and least diversity. Phylogenetic analysis indicated genetic admixture across genotypes, with only one divergent FFC sample. These findings suggest that TLR4 polymorphisms contribute to enhanced disease resistance in indigenous chickens, underscoring their value as genetic resources for breeding programmes aimed at improving Newcastle disease resilience.

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Conflict of interest

The authors declare that there is no existing conflict of interest.

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