RESEARCH ARTICLE

Genetic variations in *Toll-like receptor-4* and *Myeloid differentiation protein-2* Genes among Giriraja and Indigenous Chicken

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Abstract

The present study aimed to identify genetic polymorphism in chicken Toll-like receptor-4 (*TLR4*) and Myeloid differentiation protein-2 (*MD-2*) genes in Indigenous and Giriraja birds. Blood samples were collected from 50 each of indigenous and Giriraja birds and genomic DNA was isolated. Published primers were used to amplify chicken *TLR4* (exon 3) and *MD-2* (exon 1) genes. The polymorphism was determined in *TLR4* and *MD-2* genes using RFLP analysis employing *Taq*I and *Ase*I restriction enzymes, respectively. RFLP analysis of *TLR4* gene revealed a monomorphic pattern both in Indigenous and Giriraja birds. Whereas RFLP analysis of *MD-2* gene in both Indigenous and Giriraja birds showed polymorphic patterns. In indigenous birds, genotypic frequencies were 0.02, 0.14, and 0.84 for AA, AB, and BB, respectively, and gene frequencies were 0.09 and 0.91 for alleles A and B. In Giriraja birds, genotypic frequencies were 0.02, 0.46, and 0.52 for AA, AB, and BB, respectively, and gene frequencies were 0.25 and 0.75 for alleles A and B. Sequence Alignment of A and B alleles of *MD-2* gene showed a SNP (G → A) at nucleotide position 73 bp in B allele.

Keywords: Disease resistance, Genetic polymorphism, *MD-2*, PCR-RFLP, *TLR4.*

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Introduction

Giriraja is an improved colored bird developed by the Department of Poultry Science, Veterinary College, Bengaluru by crossing Red Cornish, White Cornish, White Rock, and New Hampshire, which is suitable for both backyard rearing and intensive system of management. The birds exhibit better growth than local varieties and are suited for mixed and backyard farming (Somu, 2015). The significance of indigenous birds and improved backyard varieties like Giriraja to the rural economy is enormous. Indigenous birds and Giriraja are well recognized for their tropical adaptability and disease resistance (Somu, 2015; Padhi, 2016).

The candidate genes (CGs) may be structural genes or genes involved in regulating a metabolic pathway. The working hypothesis assumes that a molecular polymorphism within the CGs is related to phenotypic variation (Li *et al*., 2013a). Many CGs have been involved in disease resistance, but only a few genes have been identified to have an actual role. Various candidate genes responsible for disease resistance are *TLR4, MD-2, CD28, LITAF,* and *MIF* (Malek *et al*., 2004).

Toll-like receptor 4 (*TLR4*) gene is a phagocyte cell surface receptor, which plays an important role in the recognition of lipopolysaccharide (LPS) of gram-negative bacteria, including *Salmonella enteritidis* (Akira and Takeda, 2004). *TLR4* has been identified as the most important marker having an association with a resistance of chicken to salmonella, which is the main cause of mortality in birds. Chicken *TLR4* encodes an 843-amino-acid protein that contains a leucine-rich repeat extracellular domain, a short transmembrane domain typical of type I transmembrane proteins, and a Toll–interleukin-1R signaling domain characteristic of all TLR proteins and is found on chromosome 17. *TLR4* gene spans about 11698 bp (AY064697.1), and the structure of this gene starts with the promoter region, 3 exons and 2 introns (Ulupi *et al*., 2013).

Myeloid differentiation protein-2 (*MD-2*) is necessary for correct intracellular distribution, cell surface expression, and *TLR4* LPS recognition on the cell surface of bacteria(Nagai

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et al., 2002), which interacts with CD14 on the surface of the macrophages, thereby signaling the *TLR4/ MD-2* complex (Akashi *et al*., 2001). This pathway leads to the transcription of immune response genes against bacterial infections. Hence for the expression, recognition, and intercellular distribution of the *TLR4, MD-2* is necessary. The *MD-2* gene in chicken is located on chromosome 2 and spans about 6625 bp. The structure of this gene includes five exons and three introns (AY064697.1).

Chicken *TLR4* and *MD-2* polymorphisms in the proteins have been associated with variable susceptibility to Salmonella infection (Leveque *et al*., 2003). So, these two genes have been easily identified and detected by using PCR-RFLP techniques (Malek *et al*., 2004; Khatab *et al*., 2017). The present study aimed to determine the polymorphism of *TLR4* and *MD-2* genes to find effective alleles influencing disease resistance traits in indigenous and Giriraja chicken.

Materials and Methods

Blood Sample Collection and Isolation of DNA

About 100 random blood samples were collected from 50 indigenous (Desi/ local chicken of Karnataka) birds and 50 Girirajabirds maintained under All India Coordinated Research Project (AICRP) on Poultry Breeding and Department of Poultry Science, Veterinary College, Bengaluru, respectively. The conventional Phenol: Chloroform: Isoamyl alcohol (P: C: I) method, as recommended by Khosravinia *et al*. (2007), was used to isolate genomic DNA from venous blood. The purity and yield of genomic DNA were ascertained by spectrophotometer and 0.8% agarose gel electrophoresis.

Polymerase Chain Reaction

Amplification of part of exon 3 of *TLR4* gene was done through employing published primers *viz*., Forward: 5' GAAACGTTGTCAGAGGTTCCTATG 3' and Reverse: 5' ACTTTGGTCCACCCATAC TAATTT 3' (Leveque *et al*., 2003: Khatab *et al*., 2017). The amplification was performed in a total volume of 25 µL consisting of 12.5 µL of Red PCR master mix, 1 µL (10 pmol/ µL) each of forward and reverse primer, 9.5 µL of PCR grade water, and 1 µL of template DNA. The PCR reaction was carried out with an initial denaturation temperature of 95 °C (5 min), 35 cycles of 95°C (30 sec), 58.4°C (45 sec) and 72°C (45 sec), followed by a final extension at 72 °C (10 min).

Amplification of part of exon 1 of *MD-2* gene was done by employing published primers *viz*., Forward: 5' GTAACAACAAAGGCAGAA 3' and Reverse: 5' AGAAAAATCCACTGACTCC 3' (Malek *et al*., 2004: Shavakand, 2011: Tohidi *et al*., 2012: Nduna, 2015: Karaffova *et al*., 2017). The amplification was carried out in a total volume of 25 µL consisting of 12.5 µL of Red PCR master mix, 0.5 µL (5 pmol/µL) each of forward and reverse primer, 10.5 µL of PCR grade water, and 1 µL of template DNA. The PCR reaction was carried out with an initial denaturation temperature of 95°C (5 min), 35 cycles of 95°C (60 sec), 52°C (30 sec), and 72°C (60 sec), followed by a final extension at 72°C (10 min).

The PCR amplified products were confirmed by resolving on 1.5% agarose in parallel with 100 bp DNA ladder at a constant voltage of 100 V for 60 minutes in 1X TAE buffer.

Restriction Fragment Length Polymorphism Analysis

The PCR products of the *TLR4* and *MD-2* gene were digested with *Taq*I and *Ase*I restriction enzymes (REs), respectively. The digestion was done in a total volume of 30 µL which consisted of 2 µL of 10X buffer, 10 µL of PCR amplicon, 1 µL of RE, 17 µL of nuclease-free water (NFW) with incubation at 65°C for 3 hours and inactivation at 80°C for 20 minutes and at 37°C for 3 hours and inactivation at 65°C for 20 minutes for *TLR4* and *MD-2* genes, respectively.

The restriction enzyme digested PCR products were resolved on 2% agarose in parallel with 100 bp DNA ladder to visualize bands.

Based on visualization of different band patterns, genotypes were determined. The allelic frequency, genotypic frequency, and observed and expected heterozygosity were calculated.

Sequence Analysis

The PCR products corresponding to different patterns were custom sequenced using primers used for amplification. The sequencing was done at Chromous Biotech private limited, Bangalore, and the resultant sequences were analyzed by MegAlignsoftware. The PCR amplified sequence of *TLR4* and *MD-2* gene was used as query and subjected to nucleotide blast at NCBI for sequence homology searches in public databases.

Results and Discussion

Isolation of Genomic DNA

In the present study, the Phenol: Chloroform: Isoamyl alcohol (25:24:1) method yielded a good amount of DNA from venous blood.

Spectrophotometric readings ratio obtained for genomic DNA at 260 and 280 nm were in the range of 1.7 to 2.0, indicative of good purity of genomic DNA. During this study, about 50 to 150 µg of DNA was obtained per 50 µL of venous blood. However, 132 µg of DNA per 28 µL of whole blood was obtained by Khosravinia *et al*. (2007), and 4.99 ± 0.01 µg of DNA per µL of whole blood was reported by Pirany (2005). Agarose gel (0.8 %) showed clear and distinct bands as observed under UV-transilluminator, which was indicative of good quality DNA.

PCR-RFLP Analysis

The PCR amplification of *TLR4* gene and *MD-2* gene resulted in a single amplified product of 597 and 270 base pairs,

respectively, in all the samples of indigenous and Giriraja birds (Plates 1 and 2).

Chicken *TLR4* gene/*Taq*I analysis yielded only one genotype *viz*., 'AA' (448 and 149 bp fragment) (Plate 3) in both indigenous and Giriraja birds. But Khatab *et al*. (2017) revealed two genotypes in susceptible birds as well as resistant birds against Salmonella infection by *Taq*I restriction enzyme digestion of *TLR4* (exon 3) gene. In the Fayoumi breed, the genotypic frequencies of BB and AB were 0.4 and 0.6, respectively, in susceptible birds and were 0.5 and 0.5 respectively in resistant birds. Regarding Hy-line strain, the genotypic frequencies of BB and AB in susceptible birds were 0.7 and 0.3, respectively, while in resistant birds were 0.5 and 0.5, respectively. Similarly, Malek *et al*. (2004) and Tohidi *et al*. (2012) also have reported two genotypes. While Liu *et al*. (2011), Ulupi *et al*. (2013), and Li *et al*. (2013b) reported three genotypes *viz*., AA, AG, and GG in Kampung with genotypic frequencies of 0.02, 0.36, 0.62, respectively, and the gene frequencies for A and G were 0.49 and 0.51, respectively. Similarly, Nduna (2015) have reported three genotypes, CC, CG, GG, with genotypic frequencies of 0.12, 0.79, and 0.09, respectively. In Howick birds, it was 0.27, 0.67, and 0.07, respectively in Pietermaritzburg birds. It was 0.03, 0.58, and 0.39 in Durban/KwaMashu birds. The allelic frequencies of C and G alleles were 0.52 and 0.48, respectively in Howick birds, it was 0.6 and 0.4, respectively in Pietermaritzburg birds, and it was 0.32 and 0.68, respectively in Durban/KwaMashu birds.

Chicken *MD-2/Ase*I analysis yielded three genotypes in Indigenous birds *viz*., AA, AB, BB (Plate 4) with genotypic frequencies of 0.02, 0.14, and 0.84, respectively, and gene frequencies of 0.09 and 0.91 for alleles A and B, respectively. Similarly, in Giriraja birds, three genotypes *viz*., AA, AB, BB

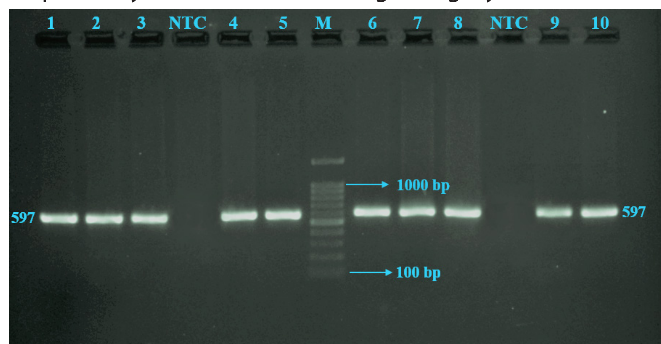

Plate 1: Agarose gel (1.5 %) picture showing PCR amplicons of *TLR4* (exon 3) gene in indigenous (Lanes 1-5) and Giriraja birds (Lanes 6-10); NTC = No Template Control

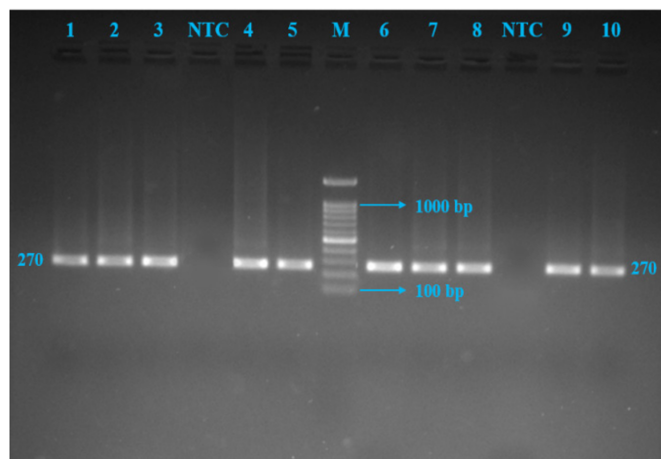

Plate 2: Agarose gel (1.5%) picture showing PCR amplicons of *MD-2* (exon 1) gene in indigenous (Lanes 1-5) and Giriraja birds (Lanes 6-10); NTC = No Template Control

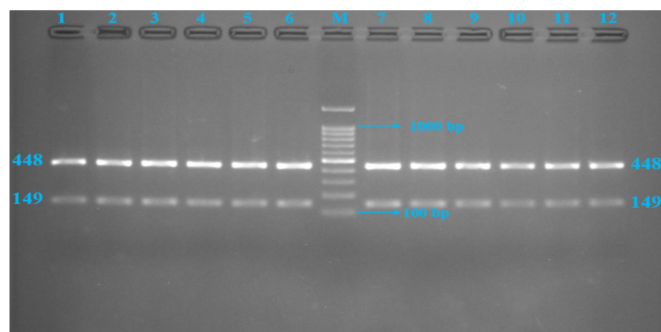

Plate 3: Agarose gel (2%) picture showing RFLP pattern of *TLR4* (exon 3) gene in indigenous (Lanes 1-6) and Giriraja birds (Lanes 7-12)

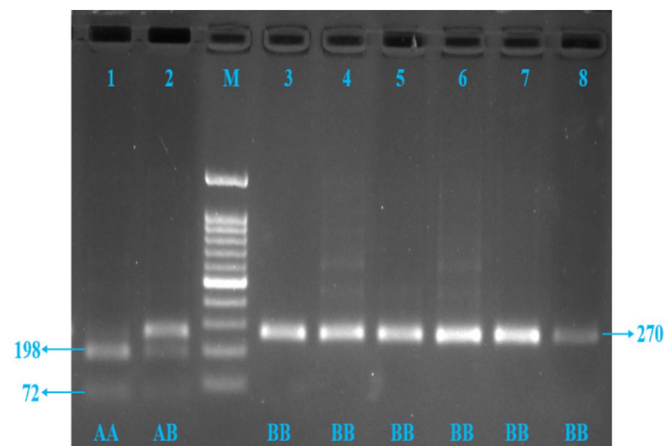

Plate 4: Agarose gel (2%) picture showing RFLP pattern of *MD-2* (exon 1) gene in Giriraja birds

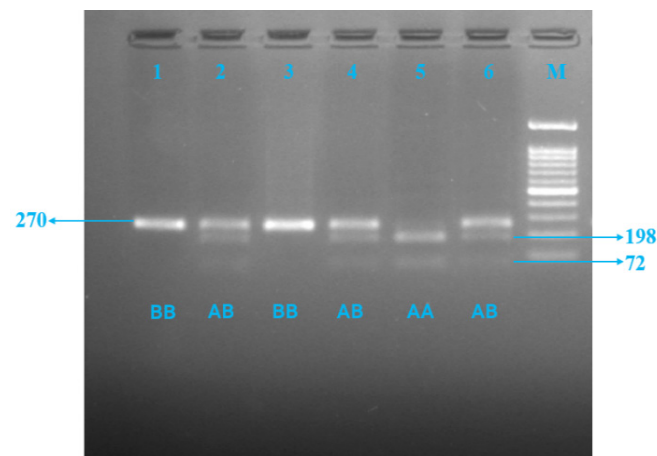

Plate 5: Agarose gel (2%) picture showing RFLP pattern of *MD-2* (exon 1) gene in Indigenous chicken

(Plate 5) were identified with genotypic frequencies of 0.02, 0.46, and 0.52, respectively, and gene frequencies of 0.25 and 0.75 for alleles A and B, respectively. This study agrees with the Nduna (2015), who reported three genotypes *viz*., AA, AB, BB with genotypic frequencies of 0.24, 0.21, and 0.55, respectively in Howick 0.09, 0.36, and 0.55, respectively in Durban/KwaMashu birds. The respective allelic frequencies for A and B were 0.35 and 0.65 in Howick birds and 0.27 and 0.73 in Durban/KwaMashu birds. Contrary to the present study, Malek *et al*. (2004) in Fayoumi and Leghorn breeds reported two genotypes AG and GG, Nduna (2015) reported two genotypes with genotypic frequencies of 0.2 for AG and 0.8 for GG in Pietermaritzburg birds with allelic frequency of 0.10 for A allele and 0.90 for G allele. Tohidi *et al*. (2012) also reported two genotypes with genotypic frequencies for AG and GG as 0.23 and 0.77, respectively in indigenous birds and for red jungle fowl it was 0.08 and 0.92, respectively. The contradictory report in the present study compared to the earlier reports may be attributed to the genetic makeup, the breeding program followed, and the sample size of birds involved.

The Chi-square test indicated that the studied indigenous and Giriraja populations were in Hardy-Weinberg equilibrium (Table 1).

Sequence Analysis

Sequence analysis of A allele of *TLR4* gene in both indigenous and Giriraja birds has confirmed the sequence identity and expected size of 597 bp. Multiple sequence alignment of A allele of *TLR4* of indigenous and Giriraja birds revealed G > A transition in exon 3 at 56th position when compared to *Gallus gallus* reference sequence (Plate 6). However, Malek *et al*. (2004), Tohidi *et al*. (2012), and Nduna (2015) reported a G > C transversion at position 3954 bp in the intronic region, and Li *et al*. (2013b) reported the same at position 1894 bp of TLR4 gene in Qingyuan Partridge hens and Baier hens. Likewise, Ahmed (2010) reported T > G transversion at base 477 position and T > A transversion at 609 positions in Leghorn and both Fayoumi lines.

Sequence analysis of A and B alleles of *the MD*-2 gene in both indigenous and Giriraja birds confirmed the sequence identity and expected size of 270 bp. Multiple Sequence

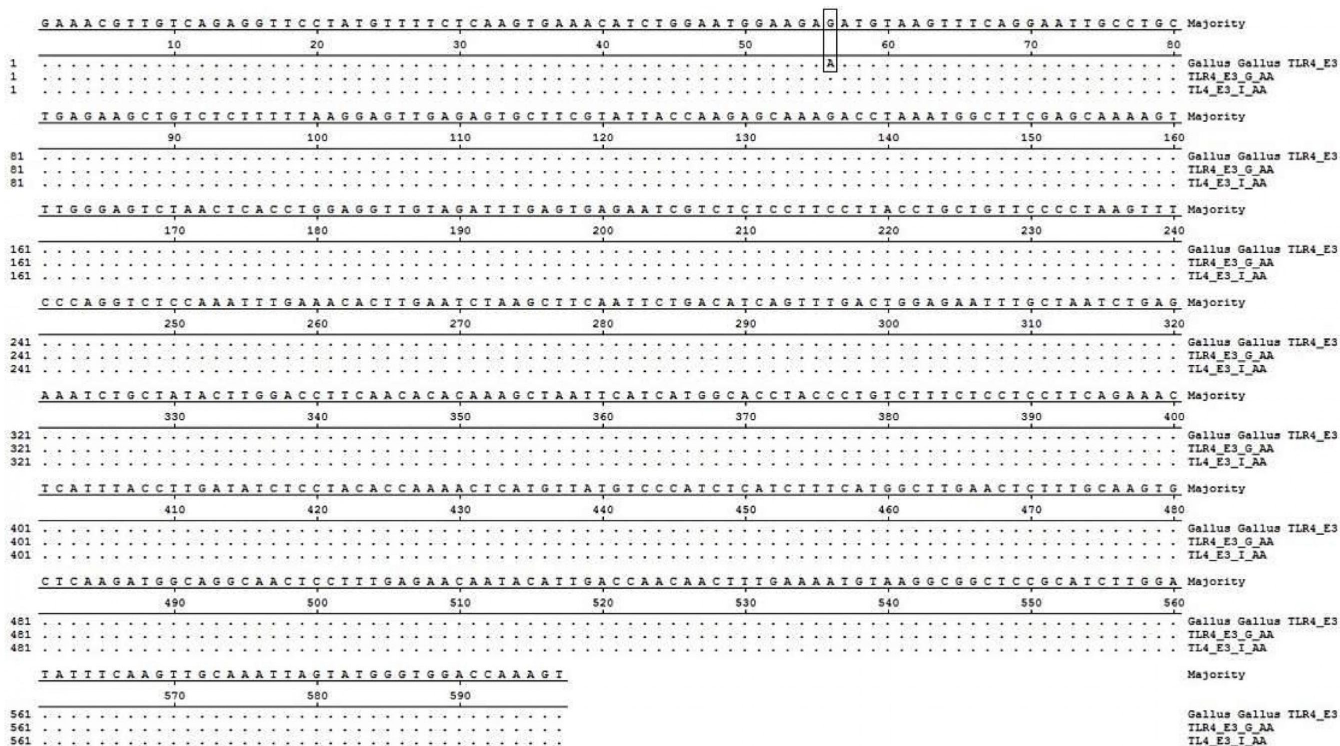

Plate 6: Multiple sequence alignment of *TLR4*gene 'A' allele of Indigenous, Giriraja birds, and *Gallus gallus* reference sequence showing G>A transition at 56th position

Table 1: Allelic and genotypic frequencies of *MD-2/Ase*I polymorphism, observed and expected heterozygosity and χ^2 value in Indigenous and Giriraja birds.

Breed	*Allele frequency*		*Genotypic frequency*			*Observed Hetero-zygosity (Ho)*	*Expected Hetero-zygosity (He)*	*Chi-square value*
	A	*B*	*AA*	*AB*	*BB*			
Indigenous	0.09	0.91	0.02	0.14	0.84	0.14	0.164	1.056^{NS}
Giriraja	0.25	0.75	0.02	0.46	0.52	0.46	0.375	2.569^{NS}

Note: NS – Non significant

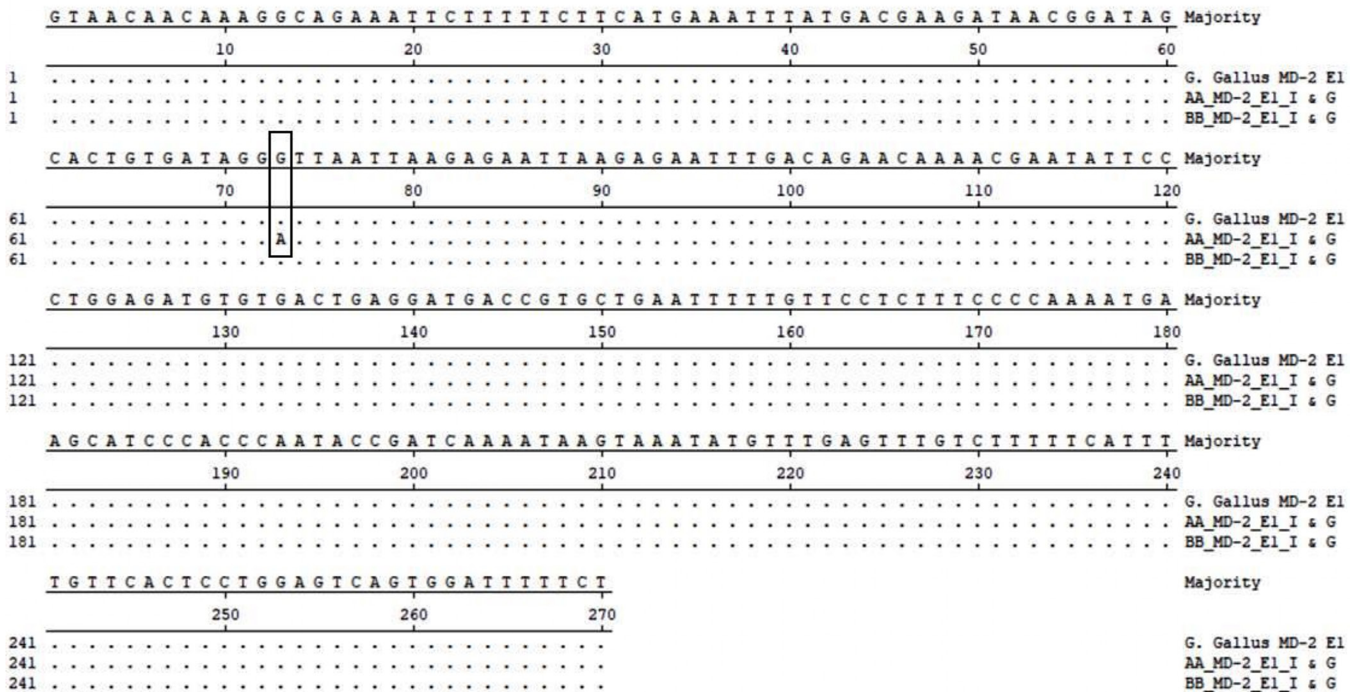

Plate 7: Multiple sequence alignment of *MD-2*gene A allele, B allele of indigenous (I) and Giriraja (G) birds, and *Gallus gallus* reference sequence showing G>A transition at 73rd position.

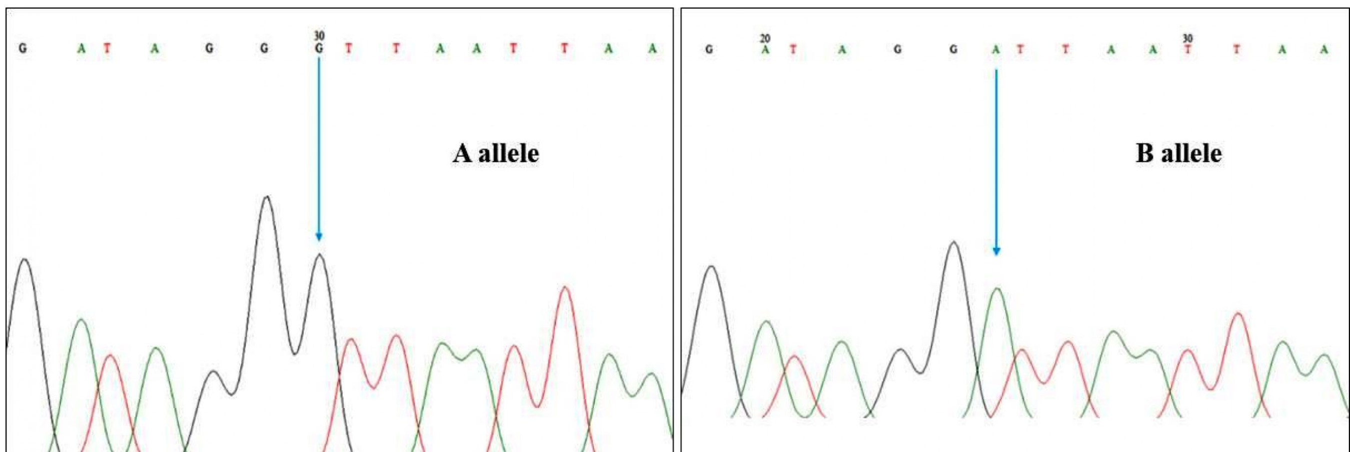

Plate 8: Chromatograms showing G → A transition in A allele of *MD-2* gene in both indigenous and Giriraja birds

alignment of A alleles revealed G > A transition at 73rd position when compared to *Gallus gallus* reference sequence (Plates 7 and 8), which is in agreement with Malek *et al*. (2004) and Nduna (2015). The sequence of B allele of MD-2 was found to be similar to the Gallus gallus reference sequence.

The PCR amplified sequence of *TLR4* and *MD-2* was used as query and subjected to nucleotide BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). Chicken TLR4 gene sequence revealed 100% identity with Aseel breed (KU235270.1), Malay breed (KU235264.1), and Brahma (KU235256.1) and 99.83% identity with Pekin Bantam (KU235327.1) and Japanese Bantam (KU235313.1). Chicken MD-2 gene sequence revealed 100, 99.26, 94.8, 90 and 87.36 per cent identity with *G. gallusdomesticus* (XM_004939913.3), *G. gallusdomesticus* finished cDNA (BX932589.2), *Meleagrisgallopavo* (XM_010709049.2), *Coturnix japonica* (XM_015855803.1) and *Numidameleagris* (XM_021388468.1), respectively.

Conclusion

The present study demonstrated that the *TLR4* locus (exon 3) was monomorphic in both Indigenous and Giriraja birds. However, the limited sample size used in this study warrants further studies involving larger sample sizes from wider

populations. The present study successfully demonstrated the genetic variability in chicken *MD-2* locus (exon 1) in Indigenous and Giriraja birds using PCR-RFLP analysis. *MD-2* gene may be considered as the potential gene marker for within breed selection for disease resistance in chicken provided that association is established between genotype and disease resistance traits. Thus, there is a need to conduct an extensive study within the breed for association analysis between *MD-2* gene variants and disease resistance traits.

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