

Research Article

TOLL-LIKE RECEPTOR-4 (TLR4) GENE POLYMORPHISM IN HARINGHATA BLACK CHICKEN BREED

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ABSTRACT: The experiment was conducted on Haringhata Black Chicken breed to investigate the polymorphism of exon-2 of Toll-Like Receptor-4 gene. Genomic DNA extracted from 82 birds was used for Polymerase Chain Reaction (PCR). Amplified polymerase chain reaction (PCR) product (248bp) of exon-2 of TLR4 gene revealed two types of SSCP banding pattern arbitrarily assigned as EF and FF genotypes on 10 percent polyacrylamide gel. The genotype frequencies were found to be 0.634 for EF and 0.366 for FF genotype with respect to exon-2 of TLR4 gene. The frequencies of E and F alleles were estimated as 0.317 and 0.683, respectively. The calculated Chi square value revealed that the population was not in Hardy Weinberg Equilibrium with respect to TLR4 gene in Haringhata Black Chicken.

Key words: Haringhata Black Chicken, TLR4 Gene, Polymorphism, PCR-SSCP.

INTRODUCTION

Toll-Like Receptors popularly known as TLRs are members of cellular receptors belonging to the innate immune system. These receptors recognize molecular patterns known as pathogen associated molecular patterns (PAMPs) which are unique to microbes namely, lipopolysaccharide (LPS), double stranded RNA, flagellin, lipoteichoic acid (LTA) etc. TLRs are present in the organs that are involved in immune responses and in the tissues that are generally exposed to pathogens, including skin, respiratory tract, intestinal and genitourinary tracts, bladder, kidney, spleen and thymus (Slawinska *et al.* 2013).

The chicken genomic Toll-Like Receptor-4 (TLR4) is 5164 bp long, consists 2 introns (813 and 1370 bp) and 3 exons (230, 167 and 2584 bp) (Huang *et al.* 2015). There are 13 members of TLR found in mammals (Beutler 2005) (man and mice). Among various TLRs, TLR4 and TLR5 are observed in high levels in Indian Aseel and Kadaknath breeds when compared to commercial broilers (Dhinakar *et al.* 2011). The higher levels of immunity observed in indigenous chickens may be correlated to such an increased TLR mRNAs expression. Leveque *et al.* (2003) proposed that the polymorphism of chicken

TLR4 was related to susceptibility to salmonella infection. In the present study, polymorphism of exon 2 region of chicken TLR4 gene was carried out on registered breed Haringhata Black Chicken using PCR-SSCP technique.

MATERIALS AND METHODS

Experimental bird

A total of randomly selected eighty-two (82) Haringhata Black chicken at 22nd weeks of age reared at intensive management system of the Haringhata Poultry Farm located at Mohanpur in Nadia District of West Bengal, India were used to investigate the genetic polymorphism of Toll-Like Receptor 4 (TLR4) in this present study.

DNA extraction

Approximately 2 ml of blood sample was collected aseptically from each individual from wing vein in vacutainer containing 200-300 µl of 10 percent EDTA as anticoagulant. The tubes were shaken gently to facilitate thorough mixing of blood with the anticoagulant. The samples were then immediately brought to the laboratory in an icebox containing ice packs

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and stored at 4°C till further use. Genomic DNA was extracted from white blood cells using standard salting out procedure as described by Miller *et al.* (1988) with minor modifications. DNA samples were dissolved in 0.1X TE buffer (pH 8.0). Horizontal submarine agarose gel electrophoresis was performed to check the quality of genomic DNA using 0.8% w/v agarose (low EEO) gel and visualizing the band under gel documentation system. The purity and concentration of DNA samples were estimated by using UV-visible range spectrophotometer. All the DNA samples had 260/280 OD ratios in the range of 1.8 to 2.0, indicating high purity. The extracted DNA samples were stored at -20°C till further use. DNA concentration was adjusted to 30ng/μl before polymerase chain reaction (PCR) amplification.

DNA amplifications (PCR)

The region of exon- 2 of TLR4 gene of Haringhata Black Chicken was amplified by Polymerase Chain Reaction (PCR) technique using the following primers as described by Liu *et al.* (2011). Primers for TLR 4: Forward 5' - ATC TGC CAC AGG TCA TCC-3' Reverse: 5' -AGC CAC GAG ACT CCA AA-3'. PCR was carried out in a final reaction volume of 25 μl. PCR conditions including the cyclic condition and the concentration of different ingredients of PCR reaction mixture was standardized for target gene fragment after carrying out PCR using several combinations. Then standardized PCR reaction mixture and standardized PCR thermocyclic conditions used for amplification of target fragment. Genomic DNA (2 μl) was amplified with 1 μl Taq DNA

polymerase, 3.2 μl MgCl₂, 1.5 μl dNTPs, 2 μl PCR buffer and 1 μl of each primer and Nuclease Free Water (NFW) 13.3 μl in a total volume of 25 μl PCR mixture. The PCR mixture was properly mixed until homogeneous then inserted into the PCR machine for amplification. The amplification for TLR4 gene was carried out using a preprogrammed thermal cycler (Eppendorf Mastercycler) with the following PCR condition: initial denaturation 95°C for 5 min followed by 35 cycles with each cycle denaturation 94°C for 50 sec, annealing 60°C for 30 sec, extension 72°C for 50 sec and final extension at 72°C for 5 min. The amplified amplicons were run at 2% horizontal agarose gel electrophoresis at 70 volts for 120 minutes. Agarose gel (2%) was stained using ethidium bromide to visualize the PCR product.

Single strand conformational polymorphism

The PCR-SSCP technique for genotyping exon-2 region of TLR4 gene revealed a good deal of polymorphism in it. The procedure of SSCP used in this investigation was as per Orita *et al.* (1989) with minor modifications to study the sequence variation. PCR products (4 μl) from each individual sample were thoroughly mixed with 12 μl volume of SSCP loading buffer dye into 200 μl PCR tubes. The tubes were heated in water bath at 94°C for 10 minutes for denaturation. The tubes were immediately snap cooled on ice for 10 minutes to make the single strand DNA for its confirmation. The samples were then loaded immediately onto 10% polyacrylamide gel. The electrodes were connected properly with the power pack and the gel was

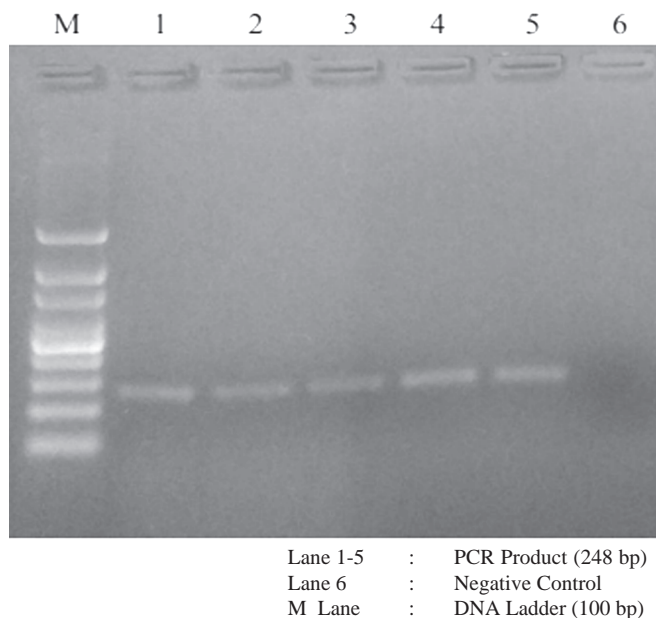


Fig. 1. PCR amplified product of exon-2 region of TLR 4 gene on 2% Agarose gel.

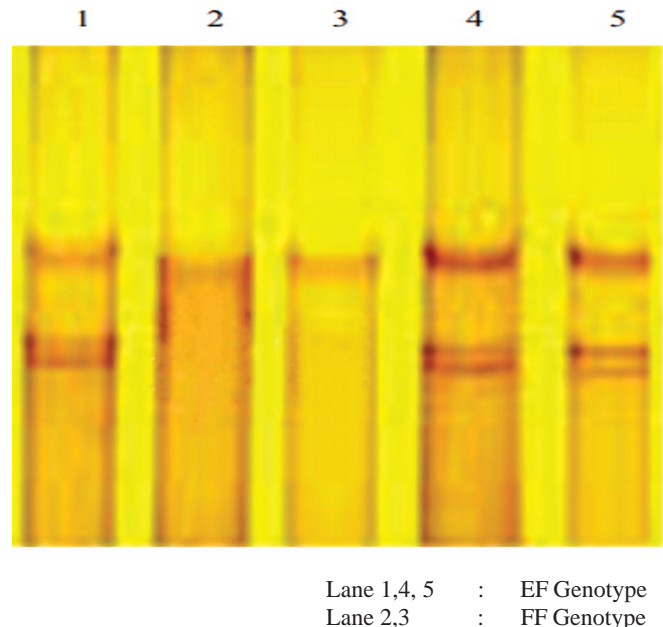


Fig. 2. Different SSCP patterns of 248 bp fragment of exon 2 region of TLR4 gene.

run at the rate of 70 volt for 12 hours at room temperature. Silver staining of polyacrylamide gel was carried out following the method as described by Byun *et al.* (2009) with few modifications. The gel was stained with a solution containing 10% ethanol, 0.5% acetic acid and 0.2% silver nitrate for 20 minutes to identify the DNA sequence variations. The stained gel was then placed on the U-V transilluminator within the gel documentation system (Syngene) and photographs were taken for identification of different SSCP banding patterns. The band patterns were visualized by silver staining and documented by gel documentation system.

Genotypes identification

The procedure for identification of particular genotypes used in this study was adapted from the different banding pattern of single strand conformational polymorphism (SSCP). The genotypes were detected accordingly by observing at every possible combination of the SSCP patterns that could provide identification of alleles. Each pattern was designated as a particular genotype. Genotype frequencies of different SSCP patterns were estimated from the combination of various alleles generated based on the movement of DNA molecules.

Statistical analysis

The following parameters were obtained: the genotype categories and the gene frequency of the alleles that corresponded to the exon-2 of TLR4 genotype. The frequency of genotype and allele were computed following standard procedure (Falconer and Mackay 1996) of direct counting method for codominant loci. The Chi-Square (χ^2) test for goodness of fit was used to find out difference among various genotypes and tested for Hardy-Weinberg Equilibrium. The significance of calculated value was adjudged from the Table values of Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

TLR4 Polymorphism

In the present study, eighty-two (82) DNA samples from Haringhata Black chicken were screened to detect polymorphism in amplified exon-2 fragment of TLR4 gene using PCR-SSCP technique. The yielded PCR product was 248 bp (Fig. 1). The PCR product obtained for exon-2 of TLR4 gene agreed with that reported by Liu *et al.* (2011). However, Ulupi *et al.* (2013) obtained a 220 bp size PCR product for the exon-2 region of TLR4 gene. Good quality PCR amplified products (sufficiently intense and without any spurious bands) as assessed by horizontal submarine agarose gel electrophoresis were

used for SSCP analysis. The electrophoretic picture of different banding pattern and their mobilities during polyacrylamide gel electrophoresis were compiled in a photograph and have been depicted in Fig. 2. The results of polymorphism of this present investigation on Haringhata Black Chicken were carried out by observing at every possible combination of the SSCP patterns that could provide identification of alleles. The banding pattern of present investigation revealed two SSCP variants for exon-2 of TLR4 gene, which was arbitrarily assigned as EF and FF genotype. The EF genotype showed triple band while FF genotype showed single band (Table 1). It was found that out of 82 studied Haringhata Black chicken 52 birds had EF genotype, 30 had FF genotype. The research finding in this present investigation revealed that 63.4 percent of the studied birds had EF genotype, 36.6 percent had FF genotype indicating higher frequency of EF genotype followed by FF genotype for exon-2 region of TLR4 gene in Haringhata Black chicken population under present study. This finding was in contrast with those of Liu *et al.* (2011) and Yan *et al.* (2011) who reported all the three genotypes EE, EF and FF in Chinese chicken breed. The absence of EE genotype in the present study might be due to limited size of the sampled Haringhata Black chicken population. However, Yan *et al.* (2011) observed higher frequency of EF genotype in Chinese chicken bird.

Genotypic frequency

In the analysed flock of Haringhata Black chicken, the genotype and gene frequencies with respect to exon-2 region of TLR4 gene were calculated and have been depicted in Table 2. In the present study, genotype frequency for EF was found to be higher than that of FF genotype in Haringhata Black chicken with respect to exon-2 region of TLR4 gene. The genotype frequencies for EF and FF genotypes were 0.634 and 0.366 respectively for exon-2 region of TLR4 gene. The genotypes observed in present investigation differed from those of Liu *et al.* (2011) and Yan *et al.* (2011) who reported all the three genotypes EE, EF and FF in Chinese chicken breeds. Liu *et al.* (2011) found the genotype frequencies of EE, EF and FF to be 0.251, 0.373 and 0.391 respectively. Yan *et al.* (2011) found the genotype frequencies of EE, EF and FF to be 0.314, 0.400 and 0.286, respectively. However, Yan *et al.* (2011) reported higher frequency of EF genotype (0.400) than that of EE (0.314) and FF (0.286) genotypes. The genotype frequencies observed in the present investigation suggested that Haringhata Black chicken have less diverse genotypes for exon-2 region of TLR4 gene in the sampled population, as compared to the earlier reports.

Table 1. Frequency of SSCP banding patterns of exon-2 region of TLR4 gene in Haringhata Black chicken.

Pattern	Genotype	No. of Bird
Three Band	EF	52
Single Band	FF	30

Table 2. Genotype and allelic frequencies of exon-2 region of TLR4 gene in Haringhata Black chicken.

Genotype	No. of Bird	Genotype Frequency	Allele	Allelic Frequency
EE	0	0.000	E	0.317
EF	52	0.634	F	0.683
FF	30	0.366		

Allelic frequency

In case of exon-2 region of TLR4 gene, the frequencies of E and F alleles were 0.317 and 0.683 respectively (Table 2). The present finding revealed a higher frequency of F allele which was predominant in the studied Haringhata Black chicken population of the farm. The present finding agreed with those of Liu *et al.* (2011) who reported higher frequency of F allele (0.563) than E allele (0.437) in Chinese Chicken. However, Yan *et al.* (2011) reported higher frequency of E allele (0.400) than F allele (0.286) in Chinese Chicken which was in contrary with the present finding. PCR-SSCP analysis of Toll-Like Receptor 4 gene showed polymorphism with two distinct alleles for TLR4 gene and F allele for TLR4 gene was prevalent in the studied Haringhata Black chicken population maintained in the farm.

Hardy-Weinberg equilibrium

Two genotypes EF and FF were observed in the present investigation for exon-2 region of TLR4 gene. The observed number and expected number of birds with EF and FF genotypes have been depicted in Table 3. It is evident that the observed and expected number of EE genotype was 0.00 and 8.24 respectively. The observed and expected number of EF genotype was 52 and 35.51 respectively and that of FF genotype was 30 and 38.25 respectively. Genotypic frequencies were tested for equilibrium using Chi-square test. The calculated Chi square value obtained was 17.676 which is higher than the tabulated value of 3.84 at 5 percent level of significance at 1 degree of freedom (Table 3). Therefore, it can be concluded that the population of Haringhata Black chicken was not in Hardy Weinberg equilibrium with respect to exon-2 region of TLR4 gene. This result

Table 3. Observed and expected number of genotypes with respect to exon-2 region of TLR4 gene in Haringhata Black chicken.

Genotype	Genotype		$\frac{(O-E)^2}{E}$
	Observed Number	Expected Number	
EE	0	8.24	8.240
EF	52	35.51	7.657
FF	30	38.25	1.779
d.f. =1			χ^2 value=17.676

indicated that the population might have under the influence of evolutionary forces like migration, mutation and selection. The present finding was in contrary to the findings of Liu *et al.* (2011).

In conclusion, it is inferred that Toll-Like Receptor-4 gene of Haringhata Black chicken breed was polymorphic that invites further attempt to investigate the association study with important economic traits of this important breed of West Bengal as they performed better under intensive management for generating higher return in well adapted local environment vis-a-vis conservation of this precious germplasm is also essential for posterity use.

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