Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 4 Issue 6 June 2022

Research Article

Polymorphism of Interferon-Gamma (INFG) Gene Promoter in High and Low Immunocompetent Aseel Chicken using PCR-RFLP

Shanti Choudhary[1,2]*, Binita Nautiyal[1,2] and Sanjeev Kumar[1,2]

[1]*Department of Animal Science, M.J.P. Rohilkhand University, Bareilly, (U.P.), India*

[2] *Molecular Genetics Laboratory, Central Avian Research Institute, Izatnagar, India*

***Corresponding Author:** Shanti Choudhary, Department of Animal Science, College of Animal Biotechnology, GADVASU, Ludhiana, Punjab, India.

DOI: 10.31080/ASVS.2022.04.0406

Received: May 2, 2022

Published: May 19, 2022

Abstract

The interferon-gamma (INFG) is a significant modulator of macrophage function, one of the candidate genes implicated in the immune response to various avian diseases. The objective of this study was to explore the polymorphism of INFG gene promoter of Aseel, the Indian native chicken using polymerase chain reaction -Restriction Fragment Length Polymorphism (PCR-RFLP). Based on immunological traits, 48 Aseel birds (24 males with high and low titer and 24 females with high and low titer values) were selected from 301 birds. DNA was isolated from blood, and PCR amplification of the INFG promoter region was conducted using two sets of primer, set I for the full-length promoter and set II for a partial-length promoter. Primer set I and set II to produce a product length of 670 bp and 495 bp, respectively. Agarose gel electrophoresis of PCR amplified product and DNA sequencing showed amplification of the INFG gene promoter region. The RFLP analysis of full-length promoter with enzymes, *EcoR1* and *Taq 1* and partial length promoter with enzymes *Alu 1, Hinf1, Dde 1,* and *Taq 1* was monomorphic. However, the full-length promoter with enzyme *Tsp5091* was polymorphic and produced 168, 123, 104, 99, 88, 64, and 54 bp size fragments. Allele A (168 bp, 123 bp, 99 bp, 88 bp, 64 bp, and 54 bp fragments) and allele B (123 bp, 104 bp, 99 bp, 88 bp, 64 bp, and 54 bp fragments) showed all possible genotypes A.A., AB, and B.B. with genotypic frequencies of 0.17, 0.30 and 0.53, respectively. Heterozygotes demonstrated more immunocompetence traits (H.A. titer to SRBC, serum lysozyme activity, and IgG level). The INFG gene promoter was mainly monomorphic, and polymorphism was associated with high immunocompetence in Aseel. We conclude that DNA analysis of the IFNG promoter gene may serve as a genetic marker for higher immune responsiveness.

Keywords: Aseel; INFG; polymorphism; PCR-RFLP; Immunocompetence

Introduction

Immunocompetence is the ability of the body to produce an immune response following exposure to antigens, in particular to the pathogen causing various diseases. Low immunocompetent birds, in comparison to high immunocompetence, have greater chances of picking up the infection, and in chickens, mortality has always been a significant concern. The emergence and re-emergence of poultry diseases cause considerable losses to the poultry industry. Artificial selection combining immunocompetence traits appears to be a possible predisposition [1] and can bring noticeable improvement in the poultry population after some generations [2].

Molecular breeding of chicken exploiting candidate genes of immunocompetence to enhance disease resistance in the poultry industry. Genetic variation is a vital feature of the poultry population where underlying diversity is utilized under the selection methods. The genetic variation in non-coding regions (regulatory polymorphism) discerned the preponderance over exonic, coding regions polymorphism [3]. Therefore, polymorphism in the regulatory area, like the promoter, can be considered the principal cause of underlying genetic variation in a population.

Along with other immune response genes, interferon-gamma (INFG), a cytokine, is a candidate gene. INFG regulates immune response by stimulating or inhibiting activation, proliferation, or differentiation of various cells and plays a critical role in immune system function [4,5]. RNA sequencing data suggest a role of chicken INFG in protection against Newcastle disease virus [6]. DNA polymorphism in immune response genes like INFG has been associated with the nature of antibody response [7]. Studying polymorphisms of immune-related genes related to disease resistance can be utilized in selecting more disease-resistant birds.

This study aimed to understand the polymorphism of the INFG gene in controlling antibody response kinetics in Aseel, the Indian native breed of chicken known for good quality meat [8]. The specific objectives were to identify promoter polymorphisms in IFNG, develop polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) to reveal DNA polymorphisms, and evaluate associations between promoter polymorphisms and antibody response kinetics in Aseel chicken.

Materials and Methods

Ethical statement

The institutional ethical committee approved all experimental procedures and bird care performed in the present study of the institution. A total of 48 birds, both males, and females, having high immunological traits were selected from a population of 301 Aseel birds of the ICAR-Central Avian Research Institute (CARI), Bareilly, India.

Genomic DNA isolation from the experimental population

All 48 birds were grouped according to the Hemagglutination titer, i.e., high and low titer groups. Genomic DNA samples were isolated using the phenol and chloroform extraction method [9]. Briefly, 100 µl of blood was collected from the jugular vein in a heparinized tube and subjected to centrifugation at 3000 rpm for 5 minutes. The supernatant was discarded, and 1 ml of RBC lysis buffer (buffer with 2M tris HCl, 2M NaCl, and 0.4M EDTA, pH 8.0) was added and kept at room temperature (R.T.) for 20 minutes. Then proteinase K (final concentration 200 µg/ml) and sodium dodecyl sulfate (final concentration 0.5%) were added and kept overnight in the water bath at 37^0C for incubation. An equal volume of tris-saturated phenol was added and mixed gently for 10 minutes. Two phases were separated by centrifuging at 5000 rpm for 5 minutes, and the upper aqueous phase was transferred to a new microfuge tube, and this step was repeated twice. The aqueous phase was washed with an equal volume of water-saturated ether. Centrifugation was carried out at 5000 rpm for 5 minutes, and then the upper layer was discarded. The viscous phase was kept for perfect evaporation of ether for ~ 2 hours at R.T. DNA was precipitated from the viscous phase by adding chilled absolute ethanol twice the volume and kept at -20^0C for complete precipitation. The purity and quality of DNA were assessed by optical densities (O.D.) ratio at 260 and 280 nm and agarose gel electrophoresis (0.7%). The samples with an O.D. ratio (260nm/280nm) ranging from 1.7 to 1.9 were used in subsequent experiments.

PCR amplification of INFG promoter

PCR was carried out with 100 ng of genomic DNA isolated from all 48 birds. Two sets of primers (set I and set II) were used to amplify the INFG gene promoter. The forward and reverse primers for the set I (5' GT AAG GAA CTT CAG CCA TTG 3'; 5' GAC GAA TGA ACT TCA TCT GCC 3') were synthesized as published earlier Click or tap here to enter text., and the second set II (5' GAA TGA ACT TCA TCT GCC TGT G 3'; 5' CAC GAC AGG AAA GTA CTT TGT G 3') was synthesized using Oligo 4 freeware (https://www.oligo.net/) according to chicken genomic sequence (EMBL accession # Y079221). Primer set I was used to amplifying the full-length promoter region of the IFNG gene and was expected to yield a 670 bp product. Whereas primer set II was expected to yield a product of 459 bp (partial length of INFG promoter region). The PCR reaction conditions were 94°C for 5 minutes; 30 cycles of 94°C for 1 minute; 58°C/53°C for primer set I/set II for 1 minute; 72°C for 1 minute and a final extension at 72°C for 15 minutes. The reaction included 100 ng of template, one × reaction buffer, ten µM of each primer, 0.5 m*M* dNTP, and 1 U *Taq* polymerase in a 25 µl reaction volume. PCR amplified products were visualized in 0.7% agarose gel against a 1 kb DNA marker. Representative samples of purified

products were sequenced to know the specificity of the amplified product.

RE digestion of PCR amplified INFG promoter

Different restriction enzymes (RE) were used for each of the amplified products of the IFNG promoter gene with primer sets I and II to obtain the PCR-RFLP pattern. The RE digestion was carried out in a 20 µl reaction mix per the manufacturer's recommended condition and described elsewhere [10]. Three enzymes, viz., *Tsp 509 I, Eco R1,* and *Taq 1,* were used to digest the amplified product of 670 bp. The reaction mixtures and incubation temperatures are given in table 1.

RE	Reaction components	Amount	Incubation
Tsp509I	*Tsp509*I RE (20U/µl)	0.5µl	65^{0}C for 24 hours
	10X buffer	2.0µl	
	PCR product	8.0µl	
	Autoclaved distilled water	9.5µl	
	Total	20 µ l	
Eco R1	*Eco R1* RE (20U/µl)	0.5µl	37^{o}C for 24 hours
	10X buffer B	2.0µl	
	PCR product	8.0µl	
	Autoclaved distilled water	9.5µl	
	Total	20µl	
Taq1	*Taq1* RE (20U/**µl**)	0.5µl	37^{0}C for 24 hours
	10X buffer B	2.0µl	
	PCR product	8.0µl	
	Autoclaved distilled water	9.5µl	
	Total	20µl	

Table 1: Reaction mixture for RE digestion for full-length IFNG promoter (670 bp).

Restriction enzyme digestion of PCR product of partial promoter (459 bp) was carried out with four different restriction enzymes. The reaction mixtures and temperatures are given in table 2.

RE	Reaction components	Amount	Incubation
Hinf1	*Hinf1* RE (10U/µl)	0.5µl	37^{0}C for 24 hours
	10X buffer	2.0µl	
	PCR product	8.0µl	
	Water	9.5µl	
	Total	20 µl	
Alu1	*Alu1* RE (10U/µl)	0.5µl	37^{o}C for 24 hours
	10X buffer B	2.0µl	
	PCR product	8.0µl	
	Water	9.5µl	
	Total	20µl	
Taq1	*Taq1*(10U/µl)	0.5µl	65^{0}C for 24 hours
	10X bufferB	2.0µl	
	PCR product	8.0µl	
	Water	9.5µl	
	Total	20µl	
Dde1	*Dde1*(10U/**µl**)	0.5µl	37^{0}C for 24 hours
	10X bufferB	2.0µl	
	PCR product	8.0µl	
	Water	9.5µl	
	Total	20µl	

Table 2: Reaction mixture for RE digestion of partial length IFNG promoter (459 bp).

RE digests of PCR amplified products were analyzed on 10% polyacrylamide gel electrophoresis (PAGE) at current 12 A for 5 minutes followed by 10 A for 3-4 hours. Silver staining was done to visualize bands of RE digested product [5].

Results and Discussion

All DNA samples were intact as visualized under U.V. transilluminator on 0.7% agarose gel. The purity of genomic DNA (assessed by OD_{260} to OD_{280}) ranges from 1.7 to 1.9, suggesting the excellent quality of DNA samples. Concentrations of DNA ranged from 116.0 - 703.7 ng/µl.

PCR amplification using primer set I produced 670 bp product (Figure 1A), and primer set II had 459 bp (Figure 1B). DNA sequence analysis of representative amplified products confirmed the INFG promoter region.

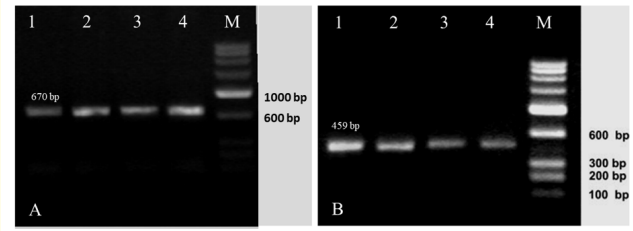

Figure 1: PCR amplification of *IFNG* gene promoter in Aseel chicken. A) the full length of *IFNG* gene promoter from lanes 1-4; B) partial length amplification of *IFNG* gene promoter from lanes 1-4. M = Low range DNA ruler.

PCR-RFLP analysis

The *Tsp 509 I* digestion of full-length promoter generated fragments of 168 bp, 123 bp, 104 bp, 99 bp, 88 bp, 64 bp, and 54 bp (Figure 2 and Table 3). However, fragments of 104 bp, 99 bp, and 104 bp could not be adequately resolved due to slight differences in fragment sizes. Smaller fragments also could not be adequately resolved. Previous reports revealed the presence of *Tsp 509 I* RE sites in the 670 bp of IFNG promoter gene, which generated the fragments of 168 bp, 123 bp, 99 bp, 88 bp, 54 bp (L.L. Genotype) and 123 bp, 104 bp, 99 bp, 88, 64 bp, 54 bp (F.F. Genotype) [4]. Based on the presence and absence of restriction fragments, birds were grouped into three genotypes: A.A., AB, B.B. Present investigation revealed all the three genotypes, i.e., A.A., AB, and B.B., in all the birds analyzed. A.A. genotype had fragments of 168 bp, 123 bp, 99 bp, 88 bp, and 54 bp, B.B. genotype had all the fragments except 168 bp, and A.B. genotype had all fragments of 168 bp, 123 bp, 104 bp, 99 bp, 88 bp, 64 bp, and 54 bp. The genotypic frequencies of A.A., AB, and B.B. were 0.17, 0.30, and 0.53, respectively. Therefore, *Tsp509I* digestion of INFG full-length promoter revealed a polymorphic banding pattern in Aseel native chicken. The RFLP heterozygotes of INFG gene promoter demonstrated a higher magnitude of all immunocompetence traits analyzed in this study, namely, hemagglutination titer to sheep RBC, serum lysozyme activity, and serum IgG level. In comparison to exotic chicken, a high level of IgG in the serum of native breed of chicken has been observed earlier [6,7]. Two genetic groups of backyard chicken with 25% of Aseel inheritance also showed a better immune response to immunocompetence traits [8]. Altogether, Aseel is one of the high immunocompetence trait breeds of native chicken.

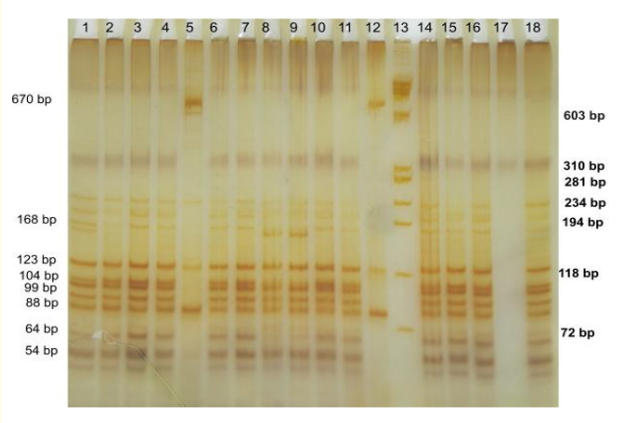

Figure 2: *Tsp5091* PCR-RFLP patterns of IFNG promoter in Aseel chicken showing polymorphic pattern (Lanes from left to right) 1- 11: Males, 12- Uncut, 13 - Marker PhiX 174 DNA / HaeIII digest, 14-18: Aseel Females.

No polymorphism was detected with enzymes *Taq1, EcoR1, Hinf1, Dde1, Alu1, and Taq1* digestion of either full or partial length promoter region of INFG (a representative image figure 3 and table 3). This might suggest INFG is a vital gene for immune response

RE	Product size	Size of digested products	Band pattern
Tsp 5091	670 bp	(i)168 bp, 123 bp, 99 bp, 88 bp, 54 bp (ii) 123 bp, 104 bp, 99 bp, 88 bp, 64 bp, 54 bp	polymorphic
Taq1	670 bp	142 bp, 528 bp	monomorphic
EcoR1	670 bp	143 bp ,527 bp	monomorphic
Hinf1	459 bp	129 bp, 281 bp, 49 bp	monomorphic
Dde1	459 bp	287 bp, 147 bp, 25 bp	monomorphic
Alu1	459 bp	306 bp, 165 bp	monomorphic
Taq1	459 bp	136 bp, 323 bp	monomorphic

Table 3: RE digestion of INFG promoter region and their respective length of DNA fragments.

and is evolutionary conserved. Therefore, no polymorphism has been found on most of the restriction enzymes used in this study. The promoter region is one of the significant regulatory factors of protein expression.

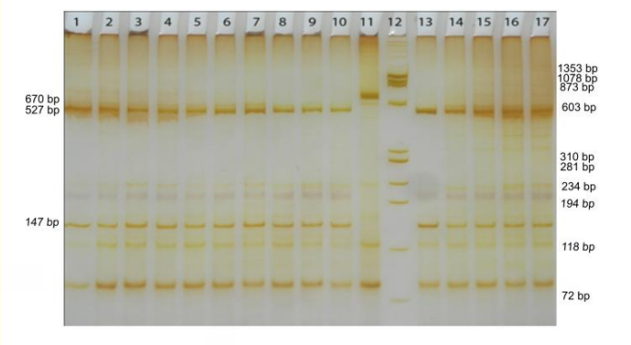

Figure 3: *EcoR1* PCR-RFLP patterns of INFG in Aseel chicken show the monomorphic pattern. Lanes from left to right 1- 10 and 13 Aseel males, 11- Uncut, 12 - Marker PhiX 174 DNA / HaeIII digest, 14-17 Aseel females.

Genetic polymorphisms in immune-related genes influence the susceptibility to disease onset [9]. The current study suggests that IFNG promoter is a conserved gene, and its RFLP polymorphism in Aseel chicken is associated with the high immunocompetence status of selected birds. Polymorphism of IFNG has been associated with disease resistance in other species [11,12]. No polymorphism was identified in the coding region of IFNG either using RFLP or single-stranded conformation polymorphism (SSCP) in chicken [13]. This effect agrees with the important role of INFG in the immune function of avian species.

Conclusions

INFG is a vital gene for immune response and therefore seems to be evolutionarily conserved in species including chickens. Our study in Aseel- a native chicken breed of India, revealed polymorphisms of full-length promoter using the *Tsp509I* enzyme. Most other REs, namely, *Taq1, EcoR1, Hinf1, Dde1, Alu1*, and *Taq1,* showed no polymorphism. Consistent with the literature, the absence of RFLP polymorphisms in the coding region of INFG in Aseel might suggest a strong association with the high immunocompetence status of native chicken. Investigating single nucleotide polymorphisms (SNPs), the relationship with IFNG protein production, and immunocompetence trait association will further illustrate the disease-resistance mechanisms in Aseel chicken.

Bibliography

1. Sivaraman GK., *et al.* "Genetics of immunocompetent traits in a synthetic broiler dam line". *British Poultry Science* 46 (2005): 169-174.

2. Sivaraman GK and Kumar S. "Immunocompetence index selection of broiler chicken lines for disease resistance and their impact on survival rate". *Veterinary World* 6 (2013): 628-631.

3. Mitchison NA. "Polymorphism in regulatory gene sequences". *Genome Biology* 2 (2000): 1-6.

4. Zhou H., *et al.* "Candidate gene promoter polymorphisms and antibody response kinetics in chickens: interferon-gamma, interleukin-2, and immunoglobulin light chain". *Poultry Science* 80 (2001): 1679-1689.

5. Chevallet M., *et al.* "Silver staining of proteins in polyacrylamide gels". *Nature Protocols* 1 (2006): 1852.

6. Yang X., *et al.* "IFN-γ establishes interferon-stimulated gene-mediated antiviral state against Newcastle disease virus in chicken fibroblasts". Acta Biochimica et Biophysica Sinica *(Shanghai)* 52 (2020): 268-280.

7. Kak G., *et al.* "Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases". *BioMolecular Concepts* 9 (2018): 64-79.

8. Rajkumar U., *et al.* "Characterization of indigenous Aseel chicken breed for morphological, growth, production, and meat composition traits from India". *Poultry Science* 96 (2017): 2120-2126.

9. Kawasaki ES. "Sample preparation from blood, cells and other fluids". *PCR Protocol* (1990): 146-52.

10. Kumar R., *et al.* "DNA Polymorphism at IL-2Rγ and IL-15Rα Genes in Aseel Native chicken". *Journal of Applied Animal Research* 32 (2007): 107-110.

11. Furini AADC., *et al.* "Frequency of TNFA, INFG, and IL10 Gene Polymorphisms and Their Association with Malaria Vivax and Genomic Ancestry". *Mediators of Inflammation* (2016): 2016.

12. Khanizadeh S., *et al.* "Association of polymorphism within promoter of the interferon-gamma receptor gene and HBV chronicity among Iranian patients". *International Journal of Infectious Diseases* 16 (2012): e89.

13. Kaiser P., *et al.* "Structure of the chicken interferon-gamma gene, and comparison to mammalian homologues". *Gene* 207 (1989): 25-32.