Indian J. Anim. Res., 45 (4) : 276 - 282, 2011

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com / indianjournals.com

NUCLEOTIDE SEQUENCING AND PCR-SSCP OF MX1 GENE IN CHICKEN

Chinmoy Mishra*, Dharmeswar Das, Pushpendra Kumar, Kuldeep Khanna[1], Ajit Pratap Singh, Shanker Dayal, Selvaramesh, Tarun Kumar Bhattacharya[2], Bharat Bhushan and Arjava Sharma

Division of Animal Genetics, Indian Veterinary Research Institute, Izatnagar, Bareilly - 243 122, India.

Received : 15-12-2010 Accepted : 08-06-2011

ABSTRACT

Mx1 is an interferon induced gene which inhibits the proliferation of single stranded RNA viruses. The size of Mx1 gene of chicken (*Gallus gallus*) is 21289 bp, having 14 exons and the coding sequence is 2118 bp long. Differential antiviral activity of Mx protein has been proved in various species including chicken. Polymerase Chain Reaction and subsequent Single Stranded Conformation Polymorphism (PCR-SSCP) analysis of 284 bp fragment of this gene was conducted to screen 153 birds of Aseel breed of chicken. Three genotypes *viz.* AB (0.74), BC (0.12) and AC (0.14) with three different alleles *viz.* A (0.44), B (0.43) and C (0.13) were identified. The homozygous genotypes i.e. AA, BB and CC could not be found in this population. Nucleotide sequencing of different alleles was done and submitted to the NCBI GenBank data (Acc. No.-GQ912704, GQ912705 and GQ912706). Nucleotide sequences were aligned with that of the corresponding Mx1 gene sequences of different breeds of chicken using DNASTAR Lasergene Software which revealed variations at five positions (91st, 107th, 113th, 194th and 231st). There is an urgent need of wide search for detection of polymorphism in Mx1 gene of different breeds of chicken and its possible association with the traits of economic importance.

Key words : Mx1 gene, PCR-SSCP, Aseel, Polymormophism, Chicken, Sequencing.

INTRODUCTION

Increasing demands of nutritious food and shrinkage of land has drawn the attention of people towards the livestock production and poultry farming in the present era of globalization. Poultry, due to its least demanding nature in terms of infrastructure in the backyard set up, has been widely accepted by the rural poor. In the world as well as in India, poultry industry has gained its pace because of its industrialization and scientific researches in breeding, feeding, healthcare and management practices. Introgression of disease resistance gene through selective breeding in productive breeds is a powerful method to decrease the input cost in poultry production in terms of health management, treatment and vaccination. That is why scientific communities are searching genes responsible for disease resistance and trying to search a molecular marker which can be used for selection of birds with better resistance to disease.

*Corresponding author's email : drchinmoymishra@gmail.com

[1]Department of Poultry Science, AAU, Anand, Gujarat, India

[2]Project Directorate on Poultry, Rajendra Nagar, Hyderabad, A.P., India

Myxovirus resistance gene i.e. Mx1 gene is an interferon induced gene which inhibits the proliferation of single stranded negative sense RNA viruses. Ko *et al.* (2002) reported the antiviral activity of Mx1 gene in poultry. The chicken (*Gallus gallus*) Mx protein is a predominantly cytoplasmic form and consists of 705 amino acids (Bernasconi *et al.*, 1995). The chicken Mx1 gene has 21289 bp linear DNA present on chromosome number 1. It has 14 exonic regions and coding sequence is 2118 bp long (NCBI Acc. No.-DQ788613). Molecular markers based on the DNA sequences are more reliable and variable. Several DNA based techniques *viz.* PCR-RFLP, RAPD, SSCP, Microsatellite analysis etc. are being adapted to identify quantitative traits for disease resistance. The effectiveness of SSCP in detecting polymorphism is well documented (Orita *et al.*, 1989). The present investigation was undertaken to find out the DNA polymorphism in Mx1 gene of chicken using PCR-SSCP and nucleotide sequencing techniques.

MATERIALS AND METHODS

Isolation of DNA

Genomic DNA was isolated from the collected blood samples of 153 birds of Aseel breed of chicken maintained at Poultry Breeding Complex, Anand Agricultural University, Anand, Gujarat, India (Sambrook and Russell, 2001). The purity of genomic DNA was assessed by spectrophotometry.

Amplification of Mx1 gene

A 284 bp fragment of Mx1 gene (Li *et al.*, 2007) comprising of 5' untranslated region and partial promoter (Figure 1) was amplified using a set of forward and reverse primers designed with the help of Lasergene Software (DNASTAR), as given in Table 1. The 25 pl of PCR reaction mixture was prepared using 20 pmoles of each primer, 200 pM of each dNTPs, 2 mM $MgCl_2$, 2.5 pl of 10X PCR assay buffer, 80-100 ng DNA template and 1 U *Taq* DNA Polymerase. The amplification was carried out using a pre-programmed thermal cycler (PTC-200, M.J. Research) with the following conditions: initial denaturation of 4 min at 94°C, followed by 35 cycles of denaturation at 94°C, annealing at 56°C and extension at 72°C, each of 1 min and finally the final extension of 10 min at 72°C. The PCR products were checked by agarose gel electrophoresis using 1% agarose gel in 0.5 X TBE buffer at 6 volts/cm for one hour (Figure 2).

PCR-SSCP analysis of gene fragment

Single Nucleotide Polymorphisms (SNPs) were screened in this fragment using SSCP technique (Sahoo *et al.*, 2010). The PCR products were resolved on 15% polyacrylamide gel (Table 2). About 8 pl of PCR product was taken in a 0.2 ml PCR tube and 24 pl denaturing formamide dye (Formamide, 95%; Xylene cyanol, 0.025%; Bromophenol blue, 0.025%; 0.5 M EDTA, 4%) was added and mixed properly. The mixture of PCR product and formamide dye were denatured at 95°C for 5 min (by keeping on 95°C hot water) and snap chilled on ice for 15 min. The product was loaded in gel carefully. The electrophoresis was performed at 4°C temperature at 14 mA constant current for 18 h. For visualization of bands, silver staining was carried out as per the method described by Bassam *et al.* (1991). The gel was visualized and documented under gel documentation system. The genotypes were detected by visualizing SSCP patterns of each sample in the gel. The gene and genotype frequencies were estimated by applying standard procedure (Falconer and Mackey, 1996).

Table 1 : Primer sequences used to amplify Mx1 gene.

Primers	Sequences	Length	Fragment Size
Forward	5'-ACCTGTGCCATCTGCCCTCTGA-3'	22	284 bp
Reverse	5'-CACAGCAAGGAGAAACAATTAACTACAT-3'	28	

DNA sequencing

Various genotypes of 284 bp fragment of Mx1 gene were selected for sequencing. The PCR products were run in 1% agarose gel and the product bands were eluted using gel elution kit (GIBCO BRL) for purification. The purified PCR products were sequenced by the Sanger's dideoxy chain termination sequencing method in automated ABI Prism DNA sequencer. The sequences obtained from different birds were subjected to BLAST (www.ncbi.nlm.nih.gov/BLAST) analysis to ascertain that sequences were of Mx1 gene. Nucleotide sequences were then aligned with that of the corresponding Mx1 gene sequences of different breeds using the clustalW method of MegAlign Programme of Lasergene Software (DNASTAR).

RESULTS AND DISCUSSION

DNA Polymorphism of Mx1 gene

The 284 bp fragment of 5' untranslated and partial promoter region of Mx1 gene was amplified (Figure 2), followed by SSCP analysis. The SSCP analysis of 284 bp fragment of Mx1 gene revealed various patterns depending on their single strand conformation. The SSCP analysis revealed 3 genotypes *viz.* AB, BC and AC (Figure 3) in this fragment with three alleles *viz.* A, B and C. The observed gene and genotype frequencies are presented in Table 3.

Table 2 : Composition of polyacrylamide gel mixture.

Reagents	Quantity in 30 ml(15%) approx.
Acry: Bis (50: 1)	9.0 ml
1X TBE	21 ml
10% APS	200 µl
TEMED	40 µl

Table 3 : Allele-wise gene and genotype frequency.

Genotype frequency		Allelic frequency	
Genotype	Frequency	Allele	Frequency
AB	0.74	A	0.44
BC	0.12	B	0.43
AC	0.14	C	0.13

Table 4 : Allele-wise nucleotide differences between Aseel and other breeds of chicken.

Breeds	Nucleotide Positions				
	91st	107th	113th	194th	231st
Aseel 'A' allele	A	C	C	G	C
Aseel 'B' allele	A	C	T	G	C
Aseel 'C' allele	A	C	C	G	T
RIR	A	C	T	G	T
Silki	T	G	C	A	C
WLH	A	C	C	G	T

Note-Nucleotide positions correspond to 1 to 284 positions along the amplified product length from 5' to 3'.

Table 5 : Percentage similarity and divergence among alleles.

	Aseel 'A' allele	Aseel 'B' allele	Aseel 'C' allele	RIR	Silki	WLH
Aseel 'A' allele	-	99.60	99.60	99.30	98.60	99.60
Aseel 'B' allele	0.40	-	99.30	99.60	98.20	99.30
Aseel 'C' allele	0.40	0.70	-	99.60	98.20	100.00
RIR	0.70	0.40	0.40	-	97.90	99.60
Silki	1.10	1.40	1.40	1.80	-	98.20
WLH	0.40	0.70	0.00	0.40	1.4	-

·All values in the table are expressed in percentage.

·Above diagonal values represent per cent similarity and off-diagonal values represent per cent divergences.

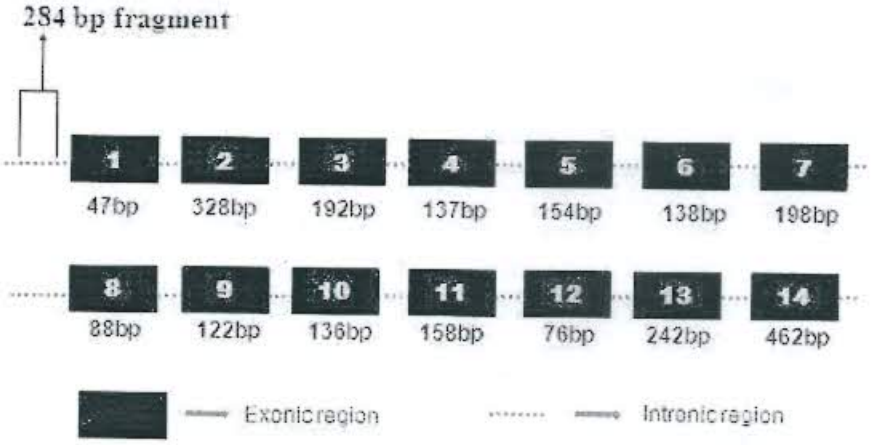

Fig. 1 : Structure of Mx 1 gene.

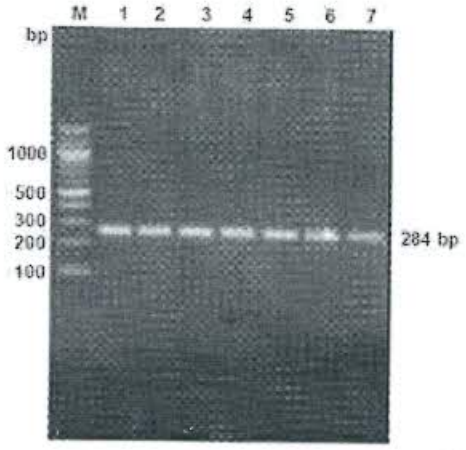

Fig. 2 : PCR product of 284 bp fragment of Mx1 gene Lane M: 100bp DNA ladder Lane 1-7: Amplification of 284 bp fragment.

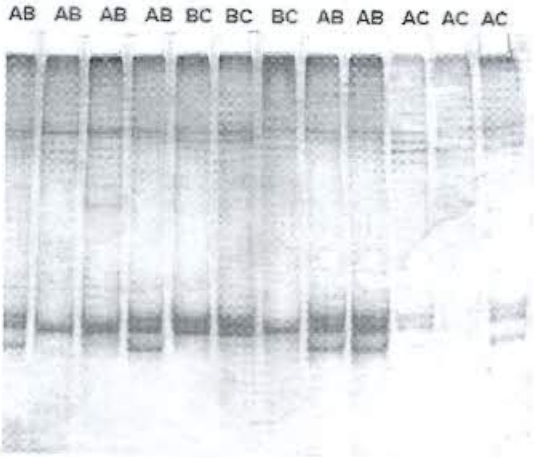

Fig. 3 : PCR-SSCP genotypes of 284 bp fragment of Mx1 gene.

The genotype frequency of AB genotype was highest (0.74), followed by AC (0.14) and BC (0.12). The homozygous genotypes i.e. AA, BB and CC could not be found in this population. The allelic frequency of A allele was highest (0.44), followed by B (0.43) and C (0.13). This indicates that the natural selection might be favouring the AB genotype in general. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment of Mx1 gene in any breed of chicken.

Nucleotide sequence analysis

Various alleles of 284 bp fragment of Mx1 gene were sequenced by Sanger's dideoxy chain termination sequencing method in automated ABI Prism DNA sequencer. The sequences obtained were subjected to NCBI BLAST. After comparing with other available sequences of chicken, the amplified fragment was confirmed to comprise of 5' untranslated region spanning partial promoter of Mx1 gene. These sequences were submitted to NCBI GenBank data with Acc. No.-GQ912704, GQ912705 and GQ912706 for A, B and C alleles respectively.

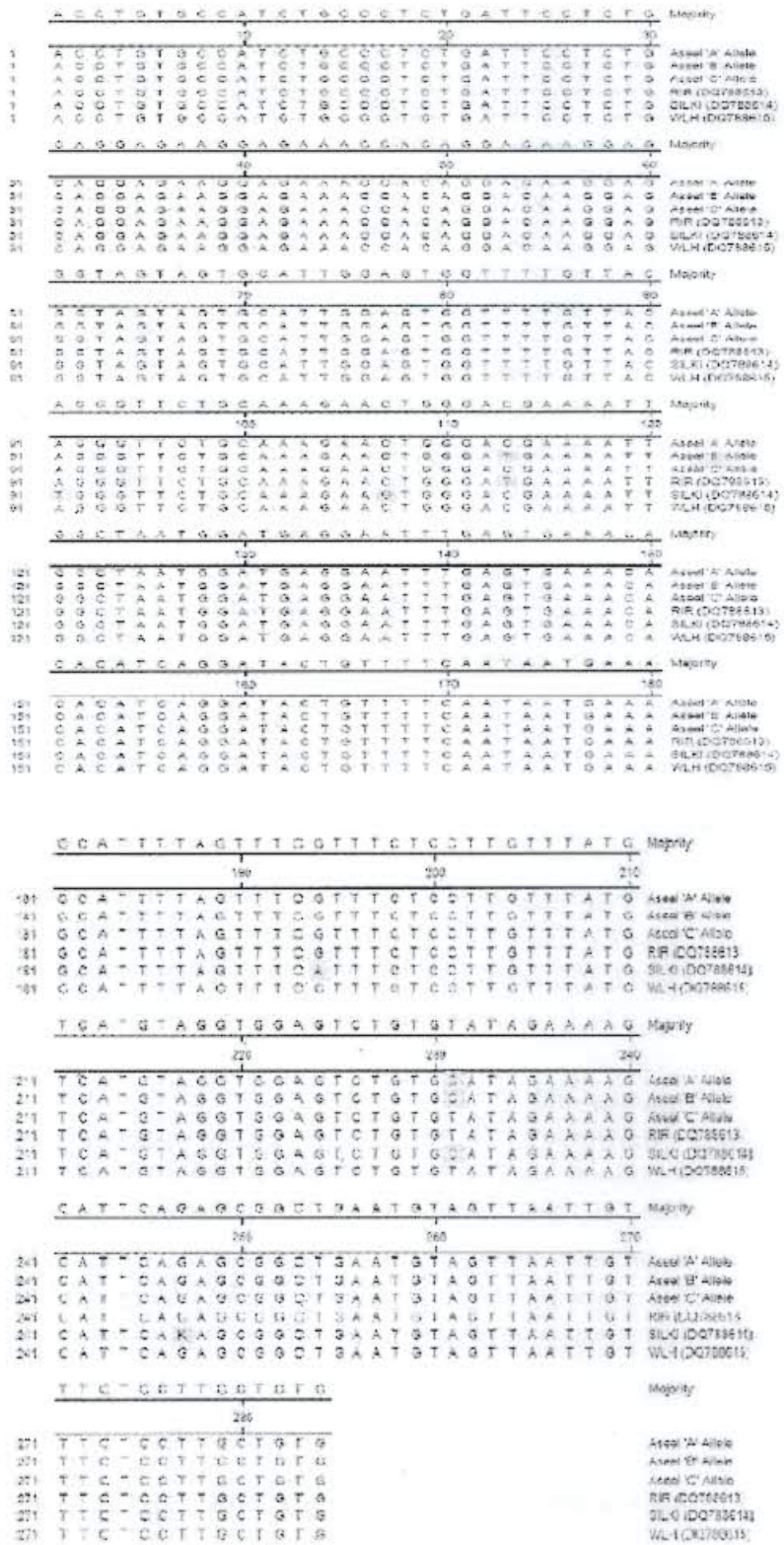

Fig. 4 : Nucleotide sequence alignment of 284 bp fragment of Mx1 gene.

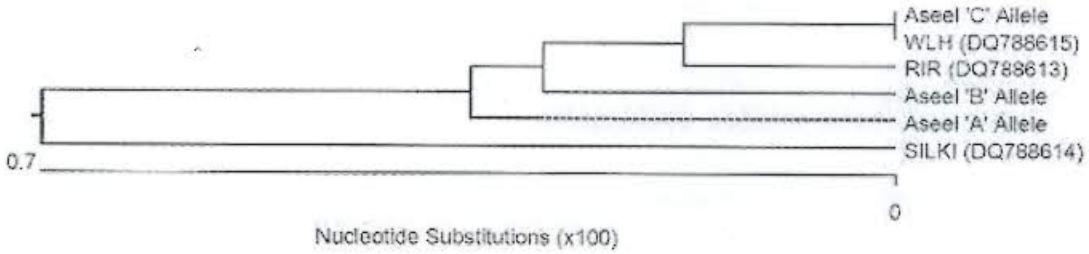

Fig. 5 : Phylogenetic tree based on 284 bp fragment of Mx1 gene.

All the three sequences as well as the corresponding sequences of chicken in other breeds (RIR, Silki and WLH) were aligned using MegAlign programme of DNASTAR software (Figure 4). From the alignment of the allelic variants, it was found that there are differences in 2 positions among these three alleles of Aseel breed of chicken (Table 4). Alleles A and C have cytosine at 113th position whereas B allele has thymine at 113th position. Similarly at 231st position, A and B allele has cytosine, whereas C allele has thymine at the same position.

After analyzing the sequences of allele A, B and C of 284 bp fragment of Mx1 gene of Aseel and RIR, Silki and WLH breeds of chicken, it was found that there were variations at five positions (91st, 107th, 113th, 194th and 231st) as given in Table 4.

Percentage similarity between alleles

Percentage similarity study was carried out between nucleotide sequences of different alleles and the corresponding fragment of Mx1 gene of other chicken breeds (RIR, Silki and WLH) by using DNASTAR Software and presented in Table 5. The per cent similarity between four breeds of chicken was found in the range of 97.9% to 100%. This shows that three breeds are close to each other with respect to 284 bp fragment of Mx1 gene.

Phylogenetic analysis was conducted using MegAlign programme of DNASTAR Software (Figure 5). It was found that alleles A, B and C of Aseel, WLH, RIR are in one cluster and Silki breed of chicken is in separate cluster.

CONCLUSION

Mx1 gene was found to be polymorphic in Aseel breed of chicken. The presence or absence of particular genotype can be used as a marker for breed identification. A allele was predominant, out of the three alleles found in 284 bp fragment of Mx1 gene. Other regions of this gene need to be sequenced for the complete characterization of this gene.

ACKNOWLEDGMENTS

The authors are thankful to the Director, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, India for providing necessary facilities to carry out this work. The first author is thankful to In-charge, Poultry Breeding Complex, Anand Agricultural University, Anand, Gujarat for providing permission for collection of blood samples.

REFERENCES

Bassam, B.J., Caetano-Anolles, G. and Gresshoff, P. M. (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.*, **196**:80-83.

Bernasconi, D., Schwartz, A. and Staeheli, P. (1993). The interferon-induced Mx protein of chickens lacks antiviral activity. *J. Interferon Cytokine Res.*, 15:47-53.