



PCR-RFLP and nucleotide sequencing of mx1 gene in chicken

CHINMOY MISHRA¹, DHARMESWAR DAS², PUSHPENDRA KUMAR³ and ARJAVA SHARMA⁴

Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

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ABSTRACT

Mx1 gene is an interferon induced gene which inhibits the proliferation of single stranded RNA viruses including influenza virus. Single nucleotide polymorphism in the Mx1 gene was reported to be associated with resistance to influenza virus. However, the indigenous breeds of India are not screened for Mx1 gene previously. In the present investigation partial intron 13 and partial exon 14 of Mx1 gene was studied using PCR-RFLP technique in 153 Aseel breed of chicken. A 100 bp PCR amplified products were digested with *Rsa* I restriction enzyme which revealed 2 genotypes (RS and SS) and 2 different alleles (R and S). The RR genotype could not be found in this population. The genotype frequency was 0.655 and 0.345 for RS and SS genotypes and 0.33 and 0.67 for R and S alleles, respectively. PCR product of both the alleles were sequenced and submitted to NCBI GenBank data. The analysis of sequence data by DNASTAR programme revealed differences at one (72nd) position in 100 bp fragment. There is an urgent need of wide search for detection of polymorphism in Mx1 gene of chicken and its possible association with the traits of economic importance.

Key words: Aseel, Chicken, Mx1 gene, Polymorphism, PCR-RFLP, Sequencing

Poultry, due to its least demanding nature in terms of infrastructure in the backyard set up, has been widely accepted by the rural poor. 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 (Pinard *et al.* 1992). The resolution of genes controlling disease resistance in chickens is a somewhat daunting, but ultimately rewarding. The genetics of a bird or flock has a profound impact on its ability to resist disease and development of a disease by an animal is the result of the interaction between the genotype of the individual and the environment. Disease therefore occurs when environmental insult meets genetic predisposition.

The avian influenza virus is infectious to both humans and avian species (Subbarao *et al.* 1998). Development of virus-resistant chickens by selective breeding would be beneficial for both the livestock industry and human health. Myxovirus resistance gene i.e. Mx1 gene is an interferon induced gene which inhibits the proliferation of single

stranded negative sense RNA viruses (Lee and Vidal 2002). 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 RFLP in detecting polymorphism is well documented (Gelderman 1975). The present investigation was undertaken to find out the DNA polymorphism in Mx1 gene of chicken using PCR-RFLP and nucleotide sequencing techniques.

MATERIALS AND METHODS

Experimental birds: This study was conducted on 153 birds of Aseel breed of chicken maintained at Poultry Breeding Complex, Anand Agricultural University, Anand, Gujarat, India. About 2 ml of blood was collected randomly in a sterile eppendorf tube containing 0.5 M EDTA (0.1 ml/ 1 ml of blood) as an anti-coagulant for genomic DNA isolation. It was then brought to the laboratory in ice box and stored at 4°C till the isolation of DNA.

Present address: ¹Ph.D. Scholar, ³Principal Scientist, ⁴Head, Animal Genetics Division; ²Joint Director Academic, IVRI, Izatnagar, Bareilly, Uttar Pradesh 243 122 India. (e-mails: ¹drchinmoymishra@gmail.com; ²drdasd@rediffmail.com; ³pushpendra64@gmail.com; ⁴arjava @yahoo.com).

Table 1. Primer sequences used to amplify Mx1 gene

Primer	Sequences	Length of primer	Fragment Size
Forward (M ₁)	5'-CCTTCAGCCTGTTTCTCCTTTTAGGAA-3'	29bp	100 bp
Reverse (M ₂)	5'-CAGAGGAATCTGATTGCTCAGGCGTGTA-3'	28bp	

Isolation of genomic DNA: Genomic DNA was isolated from the blood samples as per Sambrook and Russell (2001) and Padma *et al.* (2004) using proteinase-K and phenol. The purity of genomic DNA was assessed by spectrophotometry. The isolated DNA samples were stored at -20°C for further analysis.

Amplification of Mx1 gene: A 100 bp fragment of Mx1 gene (Li *et al.* 2007) comprising partial intron 13 and partial exon 14 (Fig. 1) was amplified using a set of forward (M₁) and reverse (M₂) primers as given in Table 1. The 25 µl of PCR reaction mixture was prepared using 20 pmoles of each primer, 200 mM of each dNTPs, 2.5 mM MgCl₂, 2.5 ml 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 with the following conditions: initial denaturation of 4 min at 94°C, followed

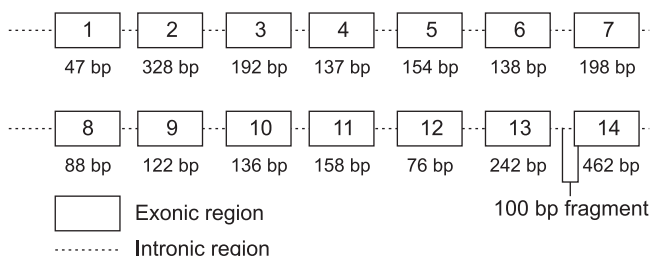


Fig. 1. Structure of Mx 1 Gene

by 35 cycles of denaturation at 94°C, annealing at 58.5°C and extension at 72°C, each of 30 sec and finally the final extension of 10 min at 72°C. The PCR products were checked by agarose gel electrophoresis using 1.5% agarose gel in 0.5 X TBE buffer at 6 volts/cm for one hour. The amplified product was visualized under UV transilluminator.

Restriction enzyme analysis: The amplified PCR products were digested with *Rsa* I restriction enzyme. 10 ml of PCR product was digested with 1 unit of restriction enzyme using suitable 10 X RE buffer at 37°C for 16 h. The RE digested PCR products (about 15 ml) were electrophoresed in 4% agarose gel containing ethidium bromide as staining agent in 0.5 X TBE buffer for 2–3 h at 6 volts/cm. The digested products were visualized and documented under gel documentation system.

Gene and genotype frequency: The genotypes were detected by visualizing RFLP patterns of each sample in the gel. The gene and genotype frequencies were estimated by applying standard procedure (Falconer and Mackey 1996)

$$\text{Genotype frequency} = \frac{\text{Total number of individuals of a particular genotype}}{\text{Total number of individuals of all genotypes}}$$

$$\text{Gene frequency} = \frac{D + \frac{1}{2} H}{N}$$

Where, D, number of homozygotes; H, number of heterozygotes; N, total number of individuals.

DNA sequencing: PCR products were sequenced using the automated dye-terminator cycle sequencing method.

RESULTS AND DISCUSSION

DNA Polymorphism of Mx1 gene: Chicken Mx was first reported in German White Leghorn (Bernasconi *et al.* 1995), but it lacked antiviral activity for influenza virus. However, Ko *et al.* (2002) reported that a specific amino acid substitution between asparagine and serine at position 72nd (present in exon 14 region) determines the differential antiviral activity of chicken Mx protein. For detection of resistant or sensitive allelic gene and subsequent selective breeding of resistant Mx gene, PCR-RFLP technique can be used. Using this method, Mx gene from 153 samples of Aseel breed of chicken were investigated for the first time. The 100 bp fragment of partial intron 13 and partial exon 14 of Mx1 gene was amplified (Fig. 2), followed by RFLP analysis. The RFLP analysis of 100 bp fragment of Mx1 gene revealed 2 genotypes, viz. RS and SS (Fig. 3) in this fragment with 2 alleles, viz. R and S depending on their restriction digestion. In our investigation, RR genotype could not be found, may be due to random mating among the population. The observed



Fig. 2. Amplified 100 bp product of Mx 1 gene

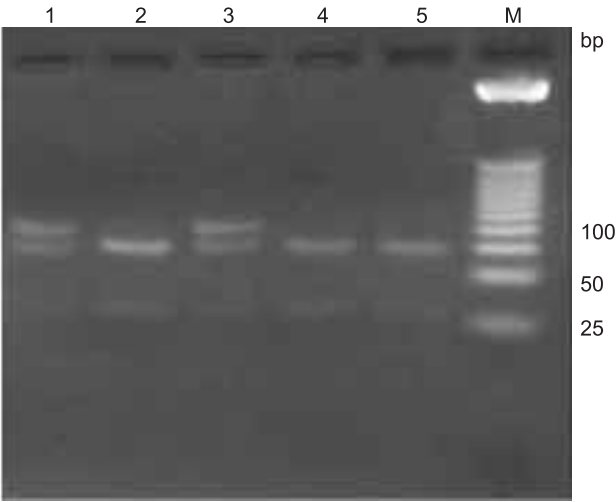


Fig. 3. Polymorphic pattern of Mx1 gene

gene and genotype frequencies are presented in Table 2. The genotype frequency of RS genotype was higher (0.655) than that of SS genotype (0.345). The allelic frequency of R allele was less (0.33), than that of S allele (0.67). In contrast to

Table 2. Allele-wise gene and genotype frequency

Genotype frequency		Allelic frequency	
Genotype	Frequency	Allele	Frequency
RS	0.655	R	0.33
SS	0.345	S	0.67

this, Li *et al.* (2006) reported resistant allelic frequency is more in native breed of chicken than commercial populations. The possible explanations for the distinct differences of the advantageous allele frequencies between native chickens and commercial populations are positive selection on this position in the chicken genome and the negative correlation between important commercial production and disease resistance traits (Zekarias *et al.* 2002). However, Seyama *et al.* (2006) reported that rate of resistant Mx allele was 40.8% and that of sensitive allele was 59.2%. The allelic distribution of Aseel Mx1 gene is nearly similar to that of the wild mouse population (Haller *et al.* 1987).

Nucleotide sequence analysis: Various alleles of 100 bp fragment of Mx1 gene were sequenced by Sanger's dideoxy

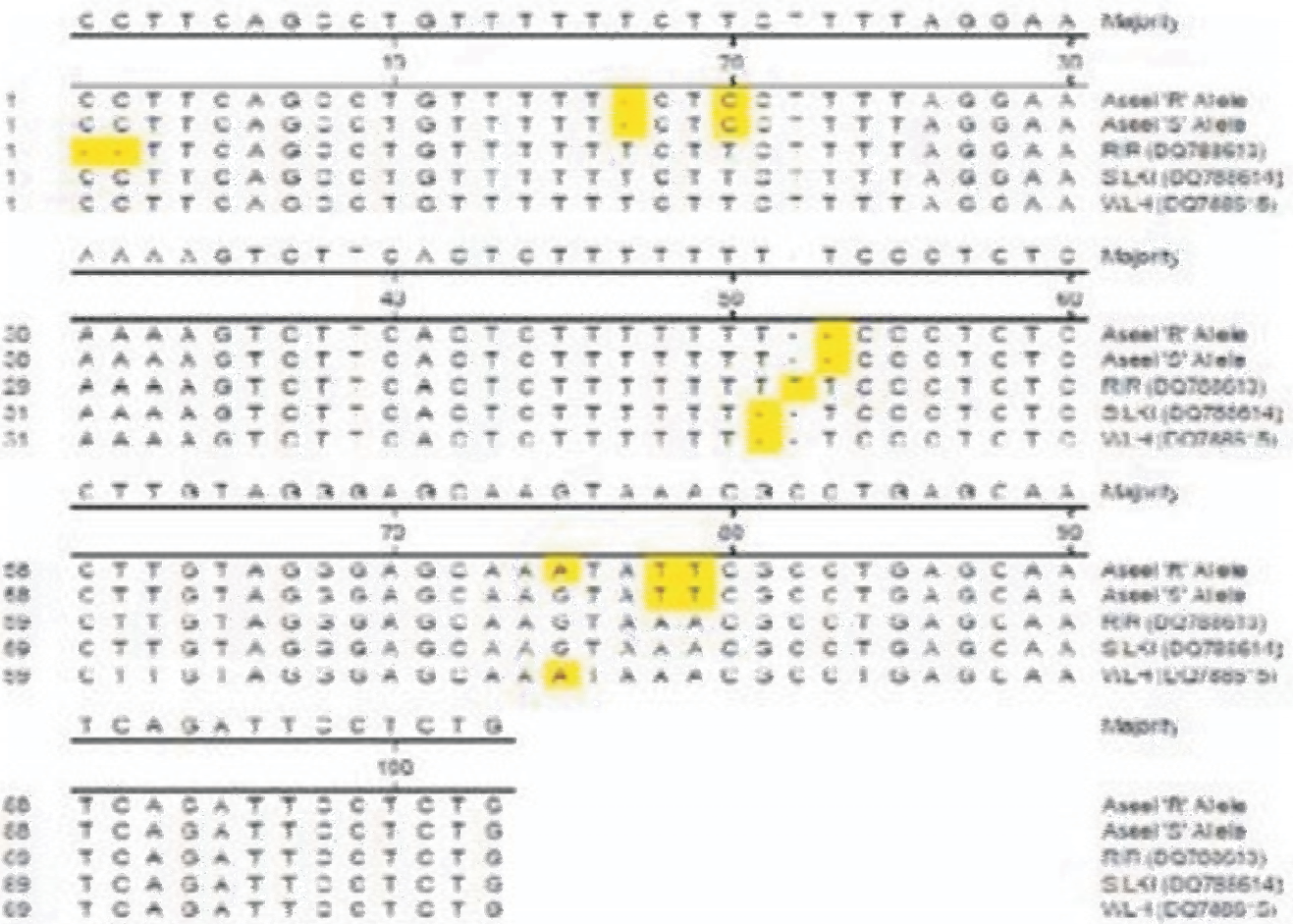


Fig. 4. Nucleotide sequence alignment of 100 bp fragment of Mx1 gene

Table 3. Allele-wise nucleotide differences between Aseel and other breeds of chicken

Breed	Nucleotide position		
	17th	19th	72nd
Aseel 'R' Allele	C	C	A
Aseel 'S' Allele	C	C	G
RIR	T	T	G
SILKI	T	T	G
WLH	T	T	A

chain termination sequencing method in automated sequencer. The sequences obtained were subjected to NCBI BLAST. After comparing with other available sequences of chicken, the amplified fragment was confirmed to comprise partial intron 13 and partial exon 14 of Mx1 gene with a length of 100 bp. These sequences were submitted to NCBI GenBank data with Acc. No. GU129017 and GU129018 for R and S alleles respectively. This was the first report of sequencing of Mx1 gene in Aseel breed.

The sequences of 2 alleles as well as the corresponding sequences of chicken in other breeds (RIR, Silki and WLH) were aligned using MegAlign programme of DNASTAR software (Fig. 4). From the alignment of the allelic variants, it was found that there is variation at single position among these 2 alleles of Aseel breed of chicken (Table 3). Allele R has adenine at 72nd position whereas S allele has guanine at 72nd position. Similarly at 17th and 19th positions, R and S allele have cytosine, whereas RIR, Silki and WLH have thymine at the same positions.

		Similarity					
		1	2	3	4	5	
Divergence	1	■	91.7	83.3	83.3	91.7	1 Allele 'R'
	2	8.9	■	91.7	91.7	83.3	2 Allele 'S'
	3	18.9	8.9	■	100.0	91.7	3 RIR
	4	18.9	8.9	0.0	■	91.7	4 Silki
	5	8.9	18.9	8.9	8.9	■	5 WLH
		1	2	3	4	5	

Fig. 5. Percentage similarity and divergence among alleles

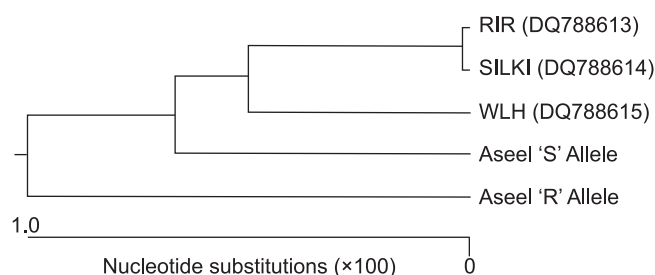


Fig. 6. Phylogenetic tree based on 100 bp fragment of Mx1 gene

G	A	S	K	R	L	S	N	Q	I	P	L	Majority
											10	
G	A	N	I	R	L	S	N	Q	I	P	L	Allele 'R'
G	A	S	I	R	L	S	N	Q	I	P	L	Allele 'S'
G	A	S	K	R	L	S	N	Q	I	P	L	RIR
G	A	S	K	R	L	S	N	Q	I	P	L	Silki
G	A	N	K	R	L	S	N	Q	I	P	L	WLH

Fig. 7. Comparison of amino acid sequence of 100 bp fragment

Percentage similarity between alleles: Percentage similarity study was carried out between nucleotide sequences of 2 alleles of Aseel and the corresponding fragment of Mx1 gene of other chicken breeds (RIR, Silki and WLH) by using DNASTAR Software (Fig. 5) which was found in the range of 83.3% to 100%. This shows that 3 breeds are close to each other with respect to 100 bp fragment of Mx1 gene. Phylogenetic analysis was conducted using MegAlign programme of DNASTAR Software (Fig. 6). Alleles R and S of Aseel were in one cluster while WLH, RIR and Silki breed of chicken in separate cluster. Allele R is more closely related with WLH, whereas allele S is more closely related with Silki breed.

Comparison of amino acid sequences: The deduced amino acid sequences of the 100 bp of Mx1 gene fragment nucleotide sequence was compared with those of the published sequences and the alignment report of the deduced amino acid sequence is presented in Fig. 7. There is asparagine in place of serine in allele R of Aseel and WLH. The isoleucine substitution in place of lysine is due to mismatch primer that is designed for creation of mismatch RFLP site. Allele R of Aseel has more homology with WLH breed than Silki and RIR whereas allele S has more homology with Silki and RIR than WLH. Larger amount of chicken population contains sensitive type serine amino acid in exon 14 region. It was also reported that Mx protein in the wild duck possessed serine at this position and could not inhibit the multiplication of influenza virus (Bazzigher *et al.* 1993). These findings may lead to a conclusion that Mx gene encoding serine at amino acid in exon 14 region is the original ancestor in birds related to chicken species. Nucleotide variations (at 17th and 19th positions) between Aseel and exotic breed of chicken (WLH, RIR and Silki) may not be significant as they are present in the intronic region.

In 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. S allele was predominant, out of the two alleles found in 100 bp fragment of Mx1 gene. Other regions of this gene need to be sequenced for the complete characterization of this gene.

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REFERENCES

- Bazzigher L, Schwarz A and Staeheli P. 1993. No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. *Virology* **195**: 100–12.
- Bernasconi D, Schultz U and Staeheli P. 1995. The interferon-induced Mx protein of chickens lacks antiviral activity. *Journal of Interferon and Cytokine Research* **15**: 47–53.
- Falconer D S and Mackay T F C. 1996. *Introduction to Quantitative Genetics*. 4th edn, pp. 1–12, Addison Wesley Longmann Ltd. Essere, England.
- Gelderman H. 1975. Investigation on inheritance of quantitative characters in animals by gene markers. *Methods of Theoretical and Applied Genetics* **46**: 319–30.
- Haller O, Acklin M and Staeheli P. 1987. Influenza virus resistance of wild mice: Wild-type and mutant Mx alleles occur at comparable frequencies. *Journal of Interferon and Cytokine Research* **7**: 647–56.
- Ko J H, Jin H K, Asano A, Takada A, Ninomiya A, Kida H, Hokiya H, Ohara M, Tsuzuki M, Nishibori M, Mizutani M and Watanabe T. 2002. Polymorphisms and the differential antiviral activity of the chicken Mx gene. *Genome Research* **12**: 595–601.
- Lee S H and Vidal S M. 2002. Functional diversity of Mx proteins: variations on a theme of host resistance to infection. *Genome Research* **12** (4) : 527–30.
- Li X Y, Qu L J, Yao, J F and Yang N. 2006. Skewed allele frequencies of a Mx gene mutation with potential resistance to avian influenza virus in different chicken populations. *Poultry Science* **85**: 1327–29.
- Li X Y, Qu L J, Hou Z C, Yao J F, Xu G Y and Yang N. 2007. Genomic structure and diversity of the chicken Mx gene. *Poultry Science* **86**: 786–89.
- Padma B, Pushpendra Kumar, Choudhary V, Dhara S K, Mishra A, Bhattacharya T K, Bhushan B and Arjava Sharma. 2004. Nucleotide sequencing and PCR-RFLP of insulin-like growth factor binding protein-3 gene in riverine buffalo (*Bubalus bubalis*). *Asian-Australian Journal of Animal Science* **17**: 910–13.
- Pinard M H, Van Arendank J A M, Nieuwland M G B and Vanderzipp A J. 1992. Divergent selection for immune responsiveness in chickens: Estimation of realized heritability with an animal model. *Journal of Animal Science* **70**: 2986–93.
- Sambrook J and Russell D W. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd edn, Vol. 1, Cold Spring Harbor Laboratory Press, New York, USA.
- Seyama T, Ko J H, Ohe M, Sasaoka N, Okada A, Gomi H, Yoneda A, Ueda J, Nishibori M, Okamoto S, Maeda Y and Watanabe T. 2006. Population research of genetic polymorphism at amino acid position 631 in chicken Mx protein with differential antiviral activity. *Biochemical Genetics* **44**: 437–48.
- Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K and Cox N. 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279**: 393–96.
- Zekarias B, Ter Huurne A A, Landman W J, Rebel J M, Pol J M, and Gruys E. 2002. Immunological basis of differences in disease resistance in the chicken. *Veterinary Research* **33**: 109–25.