



Cloning, characterization and expression of GTPase effector domain of chicken Mx1 gene

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The antiviral peptides constitute an important component of the innate immune system and a large number of these molecules have been isolated in vertebrates. Various Mx proteins differ widely with respect to intracellular distribution and biological activities. The Mx1 gene, a member of GTPase gene family, inhibits the multiplication of negative sense RNA viruses. The chicken (*Gallus gallus*) Mx1 protein is predominantly cytoplasmic and consists of 705 amino acids (Bernasconi *et al.* 1995). Its coding sequence has 14 exons consisting of 2118 nucleotides (NCBI Accession Number– DQ788613). Earlier, the chicken Mx1 protein was found to lack antiviral activity (Bernasconi *et al.* 1995); however, later on the existence of antivirally active alleles was reported in some breeds of chicken (Ko *et al.* 2002, Seyama *et al.* 2006, Sasaki *et al.* 2013). The mutation at 631st position (Asn631 is active and Ser631 is inactive) of chicken Mx1 protein is a crucial determinant for inhibition of avian influenza and vesicular stomatitis virus activity (Ko *et al.* 2004). Role of certain chicken Mx1 alleles suppressing influenza virus replication has led to its considerable interest for its introgression into chicken population. The single amino acid change may determine antiviral activity of Mx1 protein. The critical role of genetic variation in the domains of the protein was highlighted which interact with viral proteins for innate defense and co-evolution of host-pathogen relationship.

The Mx1 protein consists of two important domains, viz. dynamin and GTPase effector domain (GED). The GED (amino acid position in mature peptide: 611–702) is an essential participant in clathrin-mediated endocytosis by cells and its efficient GTPase activity depends upon proper protein folding and oligomerization. Most likely, interactions of the C terminus with the N-terminal catalytic domain are necessary for efficient catalytic activity of Mx

proteins (Flohr *et al.* 1999). Interaction with viral components is mediated by the effector domain (Fig. 1) of the Mx1 protein (Johannes *et al.* 1997). So the present study was undertaken to identify the genetic variation in the GED region of Mx1 gene in commercial White Leghorn chicken and its protein expression in prokaryotic system *in vitro*.

The blood sample was collected from WLH chicken with EDTA as anticoagulant. From the blood, polymorphic blood mononuclear cells (PBMC) were isolated by density gradient centrifugation method. In presence of 5% CO₂, the PBMC were cultured in RPMI-1640 medium supplemented with fetal calf serum and antibiotics. After 42 hours of cell culture, they were induced with chicken interferon for Mx1 gene expression. After 6 h of induction, the total RNA was isolated by TRIzol method from the induced cells. The quality and integrity of RNA were checked in formaldehyde agarose gel. The concentration of RNA was estimated in nanodrop (Eppendorf). The RNA if met the criterion (260/280 ratio of 1.8–2.1, 260/230 ratio ≥ 2.0) was used for further analysis.

The first strand of cDNA was synthesized from the mRNA using RT-PCR Kit (MBI Fermentas, USA) as per the manufacturer instructions. The partial cDNA (225 bp) comprising GTPase effector domain of the chicken Mx1 gene was amplified with a set of primers (Forward Primer– 5' TAT CGA GAA TTC ATG AAG GCC TAT TTC ACT G 3', Reverse Primer– 5' TCC GAT AAG CTT CTA GTA TTG GTA GGC TTT G 3'). The concentration of the first strand cDNA was checked in nanodrop (Eppendorf). The thermal profile of PCR programme was standardized to initial denaturation at 94°C (4 min), three stage cyclic steps (34 cycles) of denaturation at 94°C (1 min), annealing 60°C (30 sec), extension at 72°C (30 sec) and a final extension at 72°C (30 sec). The amplified partial cDNA product was visualized in agarose gel electrophoresis and eluted through Mini elute PCR purification kit (Qiagen, USA) as per the supplied protocol. The purified partial cDNA product was cloned in pGEM-T Easy vector (Promega, USA) using T/A cloning strategy. The ligated product was successfully transformed into *E. coli* DH5α strain. The positive clones

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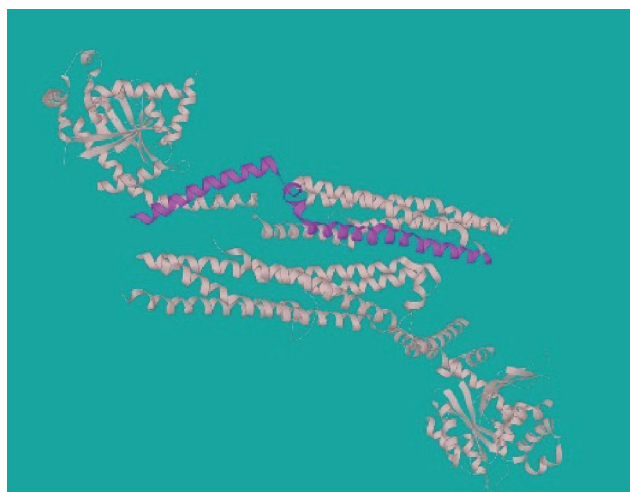


Fig. 1. GTPase effector domain (GED) of Mx1 protein.

with appropriate insert of 225 bp were confirmed by colony PCR. Further confirmation of the insert was done by the isolation of plasmid from the selected culture and subsequent plasmid PCR protocol. The partial cDNA of Mx1 gene was sequenced by Sanger's sequencing method and submitted to GenBank. The partial cDNA chicken Mx1 gene sequence was subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) to pick up similar sequences from public database. The retrieved sequences were translated *in silico* and aligned using MEGA6 software (Tamura *et al.* 2013).

The isolated plasmid from the white colonies of cloning vector (i.e. pGEM-T Easy) carrying partial cDNA chicken Mx1 gene and pPROEX HT α expression vector were double digested with restriction enzymes (i.e. *EcoRI* and *HindIII*) for its directional cloning. The partial cDNA of Mx1 gene and linearized vector were ligated by T4 DNA ligase, transformed into DH5 α cells, plated in presence of ampicillin and incubated at 37°C overnight to get white colonies as positive recombinant plasmids clones. The positive clones were identified by colony PCR using the cDNA specific primers. Further confirmation was done by restriction enzyme digestion of the plasmids extracted from the positive clones using *EcoRI* and *HindIII* enzymes.

When the optical density of the culture reached to optimum, the recombinant colonies were induced with 1 mM concentration of IPTG. The culture was grown for 8 h and the cell samples were collected at 2 h intervals (i.e. 2, 4, 6 and 8 h) simultaneously with the control (uninduced) for analyzing the level of target protein expression. By nickel chelating affinity chromatography, the polyhistidine tagged fusion protein was purified and its purity was checked in SDS-PAGE. The purified protein was dialysed against decreasing concentration of urea for 3 to 4 h and finally against Tris-saline pH 7.4 for 24 h at 4°C for proper refolding. The targeted region of protein production is important due to its functional importance as GED in the matured Mx1 protein that plays a vital role for inhibiting viral multiplication.

Avian influenza is a serious threat to the poultry industry

and, as the potential source of a human pandemic virus, to public health. Different Mx alleles have been reported to confer resistance or susceptibility to influenza virus replication, and so knowledge of their frequencies is important when considering the potential for improvement of modern commercial flocks. Conflicting data exist for the antiviral capability of chicken Mx1 protein. Reports of anti-influenza activity of alleles encoding an Asn631 polymorphism have not been supported by subsequent studies. Here, the WLH bird is analysed along with other poultry species for the relevant Mx codon that confers resistance or susceptibility to influenza virus replication. The partial Mx1 gene cDNA of WLH was cloned (Fig. 2) and its nucleotide sequence was submitted to NCBI GenBank (Accession number- JF932501). When this sequence was subjected to BLAST analysis, similar type of nucleotide sequences (Blackbone, Comtaier, RIR, Quail and Turkey) were retrieved from the public database. These sequences were translated *in silico* to get the precursor polypeptide of 75 amino acids and then aligned by MEGA6 (Table 1). Taking substitution rate into consideration, Jones-Taylor-Thornton model with lowest BIC score (625.88) was found to be the most suitable model for estimation of genetic distance. In the constructed phylogenetic tree with 1000 replicates of bootstrap value, the chicken breeds were found to be in one cluster and other species, viz. turkey and quail were present in different cluster due to their separate evolutionary lineage with multiple amino acid variants (Fig. 3). The comparative amino acid sequence analysis of Mx1 protein revealed 20 variable sites among WLH, RIR, Blackbone (Wuji), Comtaier (native breed of China), quail and turkey species. Out of them, 12 were marked as singleton variable sites and 8 were marked as parsimony informative sites. Only two of these mutations (rs313590198 and rs317224711) were reported earlier in public dbSNP database. So Mx1 protein can be considered to be highly polymorphic between species and less polymorphic within species. The Mx1 protein was reported to lack antiviral activity in WLH breed due to the presence

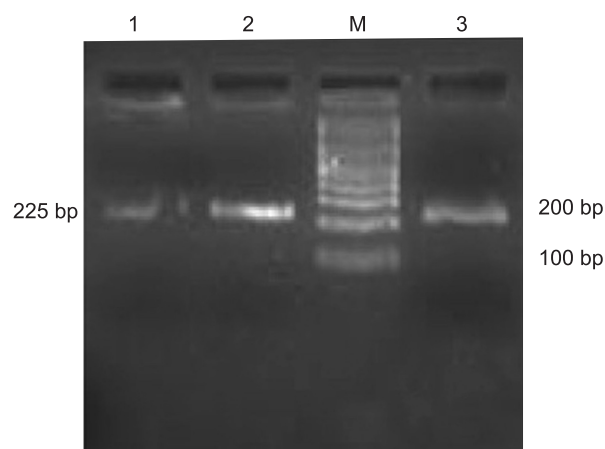


Fig. 2. Plasmid PCR of cloned partial chicken Mx1 gene (225 bp). Lane 1, 2, 3: Cloned Chicken Mx1 gene. Lane M: 100 bp ladder.

Table 1. Amino acid variation sites of partial Mx1 protein in different species

	Amino acid position																			
	8	9	25	27	29	30	33	37	41	45	47	53	57	58	59	62	64	65	68	73
WLH	N	K	H	F	N	Y	T	H	G	I	Y	H	N	Q	Q	L	S	R	H	Y
Wuji	N	K	H	F	N	Y	T	H	G	I	Y	H	N	Q	Q	L	S	R	H	Y
Comtaier	N	K	H	F	N	Y	T	H	G	I	Y	H	N	Q	Q	L	S	R	H	Y
RIR	S	K	H	F	N	Y	T	H	G	I	Y	H	N	Q	Q	L	S	R	H	Y
Quail	S	T	H	Y	D	S	I	N	G	L	H	Q	H	H	R	L	E	Q	H	H
Turkey	–	–	R	F	D	Y	I	H	E	L	H	Q	N	Q	R	V	S	Q	R	Y

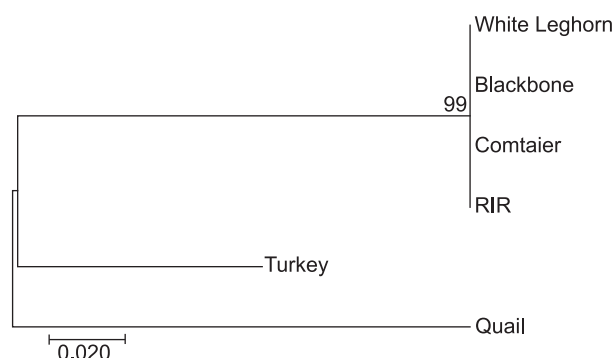


Fig. 3. Constructed phylogenetic tree using partial amino acid sequence of poultry Mx1 gene.

of serine at 8th (631st in matured peptide) amino acid position (Bernasconi *et al.* 1995), but presence of asparagine at 8th position was responsible for antiviral activity in WLH breed (Ko *et al.* 2002). In our present study, we found asparagine at 8th position which additionally confirmed and supported that Mx1 gene of WLH breed have positive antiviral activity. The role of other 19 (17 were novel) polymorphic sites among the species needs further investigation. Our research also confirmed the earlier finding of higher rate of resistant allele in WLH and higher rate of sensitive allele in RIR chicken breeds (Balkissoon *et al.* 2007, Berlin *et al.* 2008).

Amino acid substitution between asparagine and serine determines the differential antiviral activity of chicken Mx1 protein (Ko *et al.* 2007, Li *et al.* 2007, Livant *et al.* 2007, Benfield *et al.* 2010, Ewald *et al.* 2011). This substitution has been found at the GTPase effector domain (GED) region encoded in the beginning of the last exon (Pitossi *et al.* 1993, Haller and Kochs 2002). It has been also reported that Mx protein of duck, Japanese quail, turkey and goose carry sensitive type of serine at this position (Sironi *et al.* 2008, Shastry 2009, Yin *et al.* 2010, Schusser *et al.* 2011, Verhelst *et al.* 2012). These findings suggest that serine at particular location in Mx1 protein was present in the original ancestor of birds related to chicken species. With subsequent time, resistant type of allele was evolved to combat viral infection.

The use of *E. coli* as power house to express various genes has been previously used successfully. With this technique partial chicken Mx1 gene from WLH bird was cloned and expressed. The expression of recombinant protein with a molecular weight of 12.5 KD was verified in SDS-PAGE (Fig. 4). Then the partial Mx1 protein was

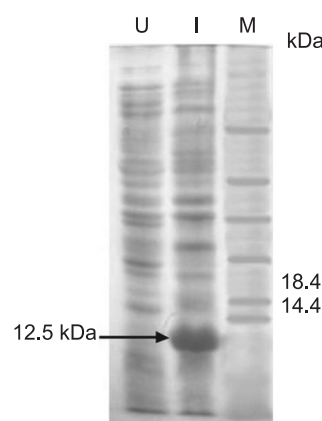


Fig. 4. Prokaryotic expression of partial chicken Mx1 gene. Lane U, Isolated proteins from uninduced prokaryotic cells; Lane I, Isolated proteins from induced prokaryotic cell; Lane M, Protein marker.

expressed and purified due to the 6*Histidine tag using the His-bind Ni-NTA purification method.

SUMMARY

The expression of chicken Mx1 gene in response to chicken interferon was confirmed in our study. However, its antiviral property may be explored if successfully expressed in transgenic chicken with subsequent virus challenge. The present study characterized the GTPase effector domain of Mx1 gene in WLH chicken. The study suggests comparative analysis of this functional domain conferring resistance to infection with myxo-group of virus among resistant and susceptible breeds or strains of chicken for further confounding.

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