



Prokaryotic expression and characterisation of recombinant M1 protein of an Indian H5N1 avian influenza virus

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ABSTRACT

The type specific Matrix 1 (M1) protein of an Indian H5N1 avian influenza virus (AIV) was expressed as a histidine-tagged fusion protein in a prokaryotic expression system and characterized. The M1 gene was amplified by reverse transcription PCR using appropriately designed primers and cloned into the expression vector, pET28a(+). The orientation and reading frame of the recombinant expression construct (pET-M1) was confirmed by sequence analysis. The derived amino acid sequence homology between M1 of AIV H5N1 and other reference AIV subtypes was found to be 93.7% to 99.2%. The 33kDA recombinant M1 (rM1) protein was expressed as inclusion body after induction with 1 mM IPTG in *E. coli* BL₂₁ (DE3)pLysS cells. The protein was purified to near homogeneity by affinity chromatography using Ni-NTA agarose column. The yield of the purified rM1 was found to be 2 mg/100 ml of induced culture. The rM1 was found to react specifically with H1-H15 AIV subtype specific sera in Western blot. The results indicated that the purified rM1 protein could be used as antigen for detection of type specific AIV antibodies by immunoassays.

Key words: Avian influenza, Characterization, Expression, Purification, Recombinant M1 protein

The avian influenza viruses (AIVs), belonging to the genus type A influenza virus and family *Orthomyxoviridae*, are enveloped viruses with 8 segmented RNA encoding 11 proteins. Based on the antigenic differences in haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, 17 HA (H1-H17) and 10 NA (N1-N10) subtypes of AIVs have been identified so far (Tong *et al.* 2012). In India, outbreaks of both H9N2 low pathogenic AIV (LPAIV) and H5N1 highly pathogenic AIV (HPAIV) have been reported since 2003 and 2006 respectively (Dubey *et al.* 2009, OIE 2012). Sero-surveillance can be used as a means of assessing the disease status of AIV of a country for export purposes (Suarez *et al.*, 2007). As the cost of imported kits is prohibitive for large scale surveillance of Indian poultry, there is a need to develop type-specific immunoassays as import substitute for sero-surveillance. The production of reliable and universally acceptable diagnostic antigens for

use in serological tests by traditional methods is difficult due to a various technical and biosafety issues. Even though, various expression systems are available, bacterial expression system is commonly used. The nucleoprotein (NP) and matrix 1 (M1) proteins are conserved across the subtypes of AIVs and used for group specific detection of type A influenza viruses. The type specific diagnostic immunoassays reported for AIV were based mostly on recombinant NP (Harley *et al.* 1990, Wu *et al.* 2007). Even though, the M1 protein contains sequences of low variability and high conservation across subtypes of influenza A viruses (Lamb and Krug, 2001), only a very few studies on development of recombinant M1 based diagnostic test for AIV have been reported (Wang *et al.* 2010). In this paper, cloning and expression of M1 protein of an Indian H5N1 virus in *E. coli* and characterization of the recombinant protein by western blot is reported.

MATERIALS AND METHODS

Virus and sera: The H5N1 virus (A/chicken/Navapur/Nandurbar/India/7972/2006 (H5N1)) and sera against H5N1 and H9N2 subtypes were obtained from the AIV repository of HSADL. The reference sera against other subtypes were procured from NVSL, USA

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PCR amplification, cloning and sequencing of M1 gene:

The cDNA was synthesized using the viral RNA extracted from infected amnio-allantoic fluid and Bm-M-1 universal primer (Hoffmann *et al.* 2001) with cDNA synthesis kit. PCR was performed using primers designed with inbuilt *NotI* restriction enzyme site [forward (M1expF - 5' TTGCGGCCGCAAGATGAGTCTTCTAACCGAGGTC 3' and reverse (M1expR - 5' TTGCGGCCGCACACTTGAATC GCTGCATCTG 3')] to amplify the complete reading frame of the M1 gene. Both the amplicon and pET28a (+) prokaryotic expression vectors were digested with *NotI* enzyme, ligated using T4 DNA ligase at 22°C for 1h and transformed into competent *E.coli* JM109 cells. Plasmids extracted from 3 recombinant colonies with QIAprep plasmid extraction kit were sequenced using cycle sequencing kit. The sequences were analyzed using MEGALIGN program of DNASTAR.

Expression, characterization and purification of recombinant M1 (rM1) protein: The *E.coli* BL₂₁ (DE3)pLysS cells transformed with the recombinant plasmid were grown overnight in LB broth supplemented with 34µg/ml chloramphenicol and 50µg/ml kanamycin at 37°C, 200 rpm. 10 ml each of LB lennox, LB miller, Terrific broth and DYT medium were inoculated with fresh overnight culture and incubated at 37°C, 200rpm. The cultures were induced with 1 mM IPTG, collected before and at every hour post induction and pelleted at 5000rpm, for 5 min. rM1 protein was extracted from the total cell pellet (TCP) using protein extraction reagent and was transferred onto a nitrocellulose membrane using a semi dry blotting assembly after SDS-PAGE (Towbin *et al.* 1979). After blocking with 5% milk powder, individual lanes were cut and treated with AIV negative serum, H1-H15 subtype specific and NDV positive sera. The blots were incubated with peroxidase-conjugated anti-chicken IgG for 1 h at room temperature and developed with 3,3'-diaminobenzidine (DAB). One of the strips was treated for 1 h with anti-his (C-term)-HRP antibody and developed as above. Western blot was also carried out using serum treated with *E. coli* cell lysate. The rM1 protein was affinity purified using Ni-NTA resin. The composition of sample application buffer (SAB) was 8M urea, 100 mM NaH₂PO₄, 10 mM Tris Cl, 300 mM NaCl, 1 mM PMSF, (pH7.8.). SAB with 5 mM imidazole pH 7.8, SAB with 20 mM imidazole, pH 6.0 and SAB with 250 mM imidazole, pH 4.0 were used for binding, washing and elution of the protein respectively. Purified protein was dialyzed overnight at 4°C against 0.1X Tris pH8.0 with 1% Triton X-100 and quantitated using protein assay kit.

RESULTS AND DISCUSSION

The M1 gene was amplified using the designed primers (Fig. 1) but the amplicon was larger than the coding sequence of M1 gene (759 bp) as the primers had inbuilt *NotI* restriction enzyme site. Sequencing of the recombinant plasmid (pET-M1) confirmed correct reading frame of the insert with the

vector start codon. The M1 gene sequence was submitted to GenBank (Accession number: EU651887). The derived amino acid sequence gave a per cent identity ranging from 93.7 to 99.2 (Table 1) when aligned with amino acid sequences of reference isolates from H1 to H16 subtypes. This indicated that the M1 protein expressed from this clone could be used for detection of H1 to H16 subtypes of AIV. The newly identified H17 subtype was not taken for analysis as the same has been identified only in bats and not in other species so far.

Terrific broth was found to give a better level of expression as compared to the other media (Fig. 2) indicating that the level of expression of rM1 was influenced by the culture media. The terrific broth is an enriched medium and the recombinant bacteria might have an extended growth phase leading to better expression. After induction with 1 mM IPTG, expression of the recombinant fusion protein of around 33kDa was observed in TCP as inclusion body (Fig. 3) which was more than the reported molecular mass of AIV M1 protein (27kDa) (Lamb and Krug 2001). The M1 protein sequences in the recombinant plasmid was in frame with the upstream and downstream 6 histidine-tag sequence of the pET28a (+) expression vector, which allows the expression of recombinant protein with 6xhistidine-tag at the N-terminal and/or C-terminal end. This increases the molecular mass of expressed protein by around 6kDa. A single band of around 33 kDa was seen in western blot with anti-His-HRP antibody which confirmed the expression of the recombinant protein with histidine tag.

Western blotting showed that the protein reacted specifically with H1 to H15 subtype specific sera and not

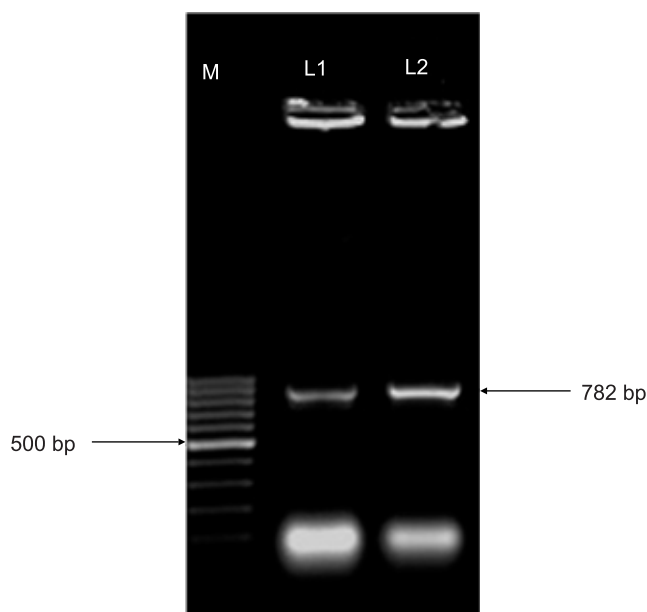


Fig. 1. PCR amplification of M1 gene. M - 100 bp DNA Ladder. L1 and L2 - 782 bp amplicons of M1 gene.

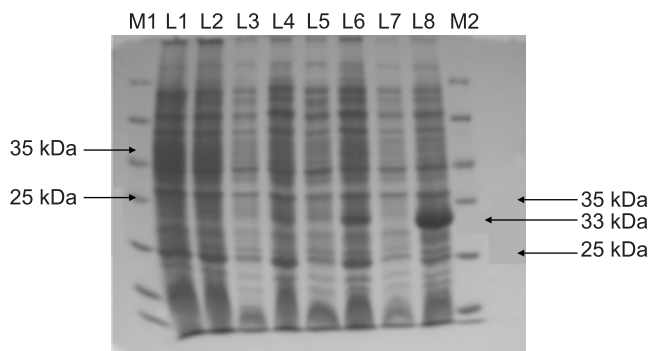


Fig. 2. SDS PAGE analysis of expression of recombinant M1 protein in different media; M1, M2 - Protein molecular weight marker; L1- Uninduced TCP of cells grown in lennox LB broth; L2- Induced TCP of cells grown in lennox LB broth; L3- Uninduced TCP of cells grown in miller LB broth; L4- Induced TCP of cells grown in miller LB broth; L5- Uninduced TCP of cells grown in DYT broth; L6- Induced TCP of cells grown in DYT broth; L7- Uninduced TCP of cells grown in Terrific broth; L8- Induced TCP of cells grown in Terrific broth.

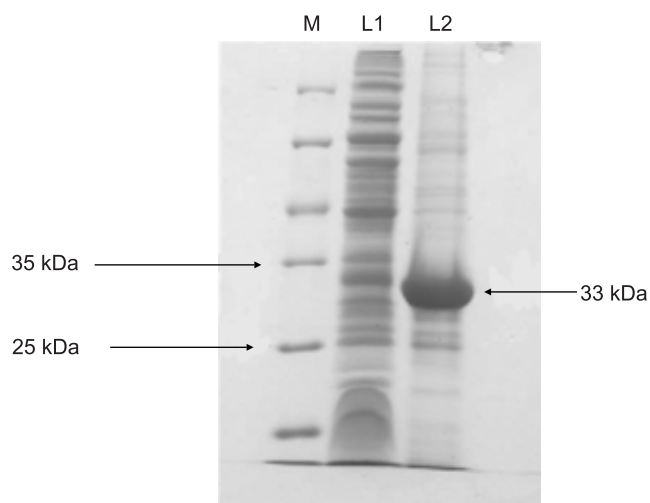


Fig. 3. SDS PAGE analysis of solubility of recombinant M1 protein; M- Protein molecular weight marker; L1- purified supernatant; L2- purified inclusion body.

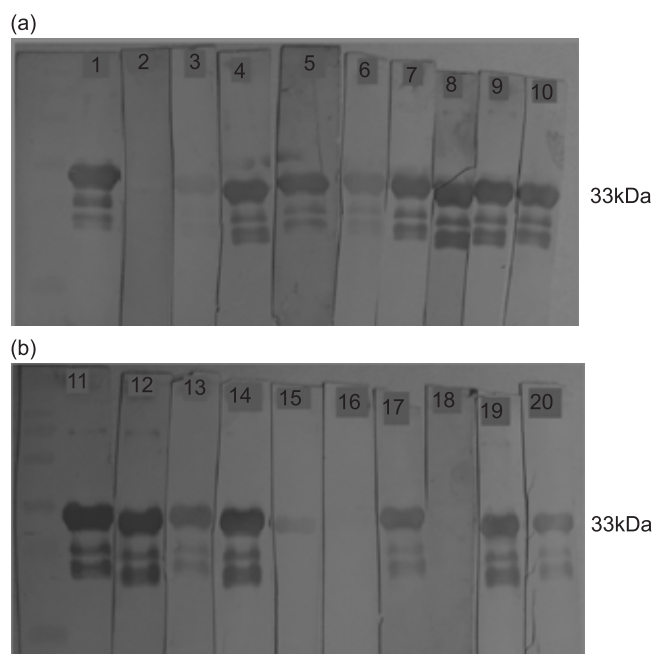


Fig. 4. Immunoblot analysis of recombinant M1 protein with different sera; M-Pre stained Protein Ladder, A/chicken/Navapur/ Nandurbar/India/7972/2006 (H5N1) serum; Negative serum from SPF poultry; A/equine/NJ/8/76- H1N7 serum A/Pintail/Alberta/293/ 77-H2N9 serum; A/DK/Ukraine/1/63-H3N8 serum; A/Mynah/ Mass/71-H4N8 serum; A/TY/Wisconsin/68-H5N9 serum; A/TY/ Ontario/63-H6N8 serum; A/TY/Oregon/71-H7N3 serum; A/TY/ Ontario/6118/67-H8N4 serum; A/Gull/MD/4435/80-H9N8 serum; A/CK/Germany/N/49-H10N7 serum; A/DK/Memphis/546/74- H11N9 serum; A/DK/Alberta/60/70-H12N5 serum; Anti-His (C-term)-HRP Antibody; Peroxidase-conjugated anti-chicken IgG; A/ Gull/MD/704/77-H13N6 serum; NDV hyperimmune serum; A/ Malard/Gurjev/263/82-A/BEL/42-H14N1 serum; A/Shearwater/ W.Australia/2576/79-H15N9 serum.

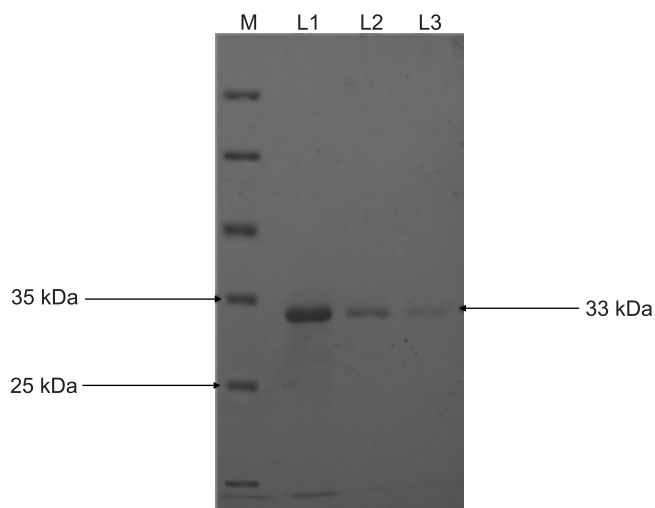


Fig. 5. SDS-PAGE analysis of affinity purified rM1 protein; M- Protein molecular weight marker (Fermentas, USA); L1- Eluted fraction 1; L2- Eluted fraction 2; L3- Eluted fraction 3.

with AIV negative or NDV positive serum (Fig. 4). In addition to the specific 33 kDa band, some other bands were also observed. However, with the sera treated with *E. coli* cell lysate, only the 33 kDa band was observed. This showed that the smaller sized proteins found in the inclusion body preparation were contaminants and not degraded recombinant protein. The protein could not be tested against H16 and H17 subtypes as the referral antisera were not available.

Affinity purification using a polyhistidine-tag usually results in relatively pure protein when the recombinant protein was expressed in prokaryotic host organism. Because its mechanism is only dependent on the primary structure of proteins, polyhistidine-tagging is the option of choice for purifying recombinant proteins in denaturing conditions

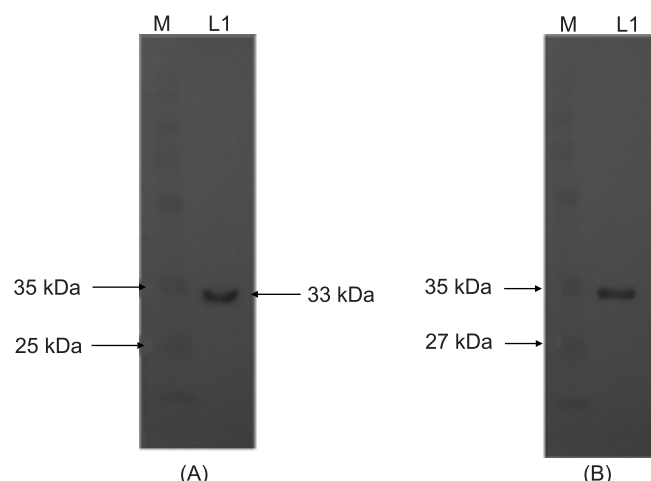


Fig. 6. Immunoblot analysis of affinity purified rM1 protein; A-Blot with A/chicken/Navapur/Nandurbar/India/7972/2006 (H5N1) serum; B- Blot with A/Ck/UP/India/2543/04-H9N2 serum; M- Plus Prestained protein ladder; L1- Affinity purified recombinant M1 protein.

(Spencer *et al.* 2007). In the affinity column purification using Ni-NTA resin under denaturing conditions, the protein was eluted by buffer containing 250 mM imidazole. The final elute showed a band of around 33 kDa rM1 protein without any apparent contaminating proteins as analysed by SDS-PAGE (Fig.5). Two mg of protein was obtained from 100 ml of induced culture. A single specific band was obtained in western blot of purified protein with AIV H5 and H9 subtype specific serum (Fig. 6) indicating the purity and the reactivity of the purified rM1.

The results suggested that the purified rM1 can be used as antigen for development of immuno assays for type specific detection of AIV antibodies as an import-substitute for sero-surveillance of Indian poultry for AIV.

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