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# Development of single-chain Fv against the nucleoprotein of type A influenza virus and its use in ELISA

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Single chain fragment variable (ScFv) antibodies specific to the nucleoprotein (NP) of avian influenza virus (AIV) were developed using a phage display system. The variable heavy ($V_H$) and the variable light ($V_L$) chain gene fragments were derived from spleen cells of Balb/c mouse immunized with a recombinant NP (rNP) antigen (~63 kDa) of H5N1 influenza virus. The $V_H$ and the $V_L$ DNA fragments were assembled through a flexible linker DNA to generate ScFv DNA that was cloned subsequently in a phagemid to express ScFv protein in *Escherichia coli* cells. The specific reactivity of the ScFv with the rNP antigen and viral antigen (H5N1) was confirmed by Western blot and ELISA. A competitive inhibition ELISA (CI-ELISA) was developed using the rNP and the anti-NP ScFv for detection of type-specific antibodies to AIV in chicken sera. The ScFv based CI-ELISA was compared with hemagglutination inhibition (HI) test and agar gel immunodiffusion (AGID) test over 850 sera. Sensitivity of the CI-ELISA was 100% with HI and AGID and specificity was 98.7% with HI and 100% with AGID.



## 1. Introduction

Avian influenza (AI) has emerged as a highly contagious and economically important infection of poultry caused by type A influenza viruses of the family *Orthomyxoviridae* (Lamb and Krug, 1996; Horimoto and Kawaoka, 2001). AI in domestic chickens and turkeys is classified according to the disease severity, with two recognized forms: highly pathogenic avian influenza (HPAI), also known as fowl plague, and low-pathogenic avian influenza (LPAI).

After the first outbreak of HPAI in India in February 2006 (Pattnaik et al., 2006), several outbreaks of HPAI H5N1 virus have occurred in Maharashtra, Gujarat, Madhya Pradesh, West Bengal, Assam, Tripura, Sikkim and Manipur (Tosh et al., 2007; Murugkar et al., 2008; Nagarajan et al., 2009a). All these outbreaks resulted repeatedly in severe economic losses due to high mortality, culling operations, reduced price of chicken and eggs and ban on exports (Dubey et al., 2009). Presence of the LPAI viruses in poultry and other birds has also been reported from India (Nagarajan et al., 2009b; Pawar et al., 2012; Tosh et al., 2008). Presently, the diagnosis of AI is accomplished using various diagnostic assays, such as virus isolation (VI) in embryonated chicken eggs, nucleic acid detection assays (RT-PCR and real-time RT-PCR) and serological assays. Though the nucleic acid detection based tests provide sensitive and specific diagnosis, the serological assays have their own place in surveillance programs and are considered relatively inexpensive and practical way to determine circulation and prevalence of AIV in the avian population (Capua and Cattoli, 2007). The standard serological diagnostics of AIV, recommended by OIE, are hemagglutination inhibition (HI), agar gel immunodiffusion (AGID) tests and enzyme linked immunosorbent assay (ELISA). Although HI and AGID are considered gold standards, these techniques are laborious and ineffective for monitoring and surveillance work involving very large number of samples. The ELISA based serodiagnosis is much more suitable to bulk testing; however, it needs highly purified virus antigens and specific antibodies to achieve the same level of specificity and sensitivity as HI and AGID. Since AI surface glycoproteins have a high rate of antigenic variation, development of assays based on them is problematic. The internal antigenic determinants of the nucleoprotein (NP) are conserved among different strains (Schild et al., 1979; Van Wyke et al., 1980, 1984). This protein can be produced both in prokaryotic and eukaryotic in vitro

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expression systems (Harley et al., 1990; Rota et al., 1990; Voeten et al., 1998; Zhou et al., 1998; Bai et al., 2006; Starick et al., 2006; Wu et al., 2007; Yang et al., 2008; Upadhyay et al., 2009).

Recombinant monoclonal antibodies are important tools for diagnosis as they have specific molecular interactions (Li et al., 2000). The phage display technique is a rapid method of developing recombinant antibodies (RAbs) in the form of single chain fragment variable (ScFv) and is advantageous over the conventional hybridoma technique of developing monoclonal antibodies. Human ScFvs have been produced against the matrix protein (M1) of H5N1 avian influenza virus (Poungpair et al., 2009). Cloning and expression of ScFv antibodies against H5N1 avian influenza virus hemagglutination have also been reported (Hao et al., 2009). The ScFvs developed previously against avian influenza proteins have not been explored for developing a diagnostic test. The present study reports the development of RAbs in the ScFv format against the recombinant NP (rNP) derived from the Indian isolate-A/chicken/India/8824/2006(H5N1) of AIV followed by development of a competitive inhibition ELISA (CI-ELISA) for sero-diagnosis of avian influenza virus in chicken using the rNP and the anti-NP RAb. The RAb based CI-ELISA has been evaluated against AGID and HI test, the gold-standard for AIV serology.

## 2. Materials and methods

### 2.1. Animals

Balb/c mice were obtained from the National Centre for Laboratory Animal Sciences (NCLAS), National Institute for Nutrition (NIN), Hyderabad, India and were kept in cages in the Biocontainment Animal Facility of HSADL, Bhopal. The mice were fed on autoclaved feed pellets and water ad libitum. Experiments using mice were conducted with approval from the Institute's Animal Ethics Committee (IAEC) in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

### 2.2. Reference sera

A panel of reference sera consisted of 15 HA sub-type specific (H1–H15) sera obtained from National Veterinary Services Laboratory (NVSL), Ames, IA, USA. Besides, 5 sera from chickens vaccinated with inactivated H5N1 virus and 5 sera from known healthy flock of chicken were included in the reference panel.

### 2.3. Production and purification rNP from AIV-NP expression clone

An expression clone of NP gene of AIV in pET 28b+ expression vector (Novagen, USA) was used for production of the rNP antigen. The expression clone carried an insert of 1594-bp DNA fragment of the NP gene (GenBank Acc. No. EF010524.1) amplified from the Indian isolate of H5N1 (A/chicken/Jalgaon/India/8824/2006) virus. The size of the recombinant fusion protein was estimated to be approximately 63-kDa with 68 amino acids provided by the vector including the six molecules of histidine as fusion tag. Using the pET-NP clone, a 63-kDa rNP was expressed in *Escherichia coli* (BL21) cells upon induction with 1 mM IPTG (isopropylthio-β-D-galactoside). The inclusion body fraction of the induced culture was purified using the Bugbuster extraction reagent (Novagen, EMD Biosciences, Inc, Madison, WI, USA) as per the manufacturer's instructions. Briefly, the bacterial cell pellet of induced culture was resuspended in 1× Bugbuster reagent at room temperature. Volume of the Bugbuster reagent was 1/5 of the total culture volume. Lysozyme (1 KU/ml final concentration) was added to the cell suspension by gentle mixing and the cell suspension was kept at room temperature on rocker for 20 min. After 20 min, 6 volumes of 1:10 diluted 1× Bugbuster reagent was added and the suspension was gently mixed on a vortexer. The Suspension was centrifuged at 5000 × *g* for 15 min at 4 °C and the supernatant was removed. The inclusion body pellet was washed twice with half the original culture volume of 1:10 diluted Bugbuster reagent. The suspension was again centrifuged as described previously. The pellet was resuspended once more in 1:10 diluted Bugbuster reagent and centrifuged at 16,000 × *g* for 15 min at 4 °C. Final pellet of the inclusion bodies was dissolved in 1× solubilization buffer (Novagen, EMD Biosciences, Inc, Madison, WI, USA). Sodium lauryl sarcosine (SLS) (0.3% final concentration) was added as preservative. The 6× His-tagged expressed protein (rNP) in the solubilized inclusion body was purified by nickel affinity chromatography using the Ni-NTA spin columns (Qiagen, GmbH, Hilden, Germany) as per the protocol recommended by the manufacturer. The purity of rNP in the inclusion body preparation was verified in 12.5% SDS-PAGE. The concentration of the purified protein was estimated using UV spectrophotometer. The expression of 6× His-tagged rNP protein was confirmed by Western blotting using anti-His HRPO conjugate (Qiagen, GmbH, Hilden, Germany). The purified rNP was checked for its immunological reactivity with the reference AIV antibody positive sera by indirect ELISA and Western blotting. The purified rNP protein was used as antigen in ELISA and was used to immunize mice for RAb production.

### 2.4. Generation of ScFv DNA fragment from spleen of NP-immunized mouse

Five Balb/c mice (6–8 wks old) were immunized with 10 μg of the rNP antigen per mouse with equal volume (100 μl) of the Freund's complete adjuvant (FCA) intraperitoneally. Boosters were given with 10 μg of the rNP antigen in the Freund's incomplete adjuvant (FIA) on 22nd day, 33rd day, 41st day and 49th day. Sera from the immunized mice were tested for anti-NP antibodies by indirect ELISA using 100 ng/50 μl/well of the rNP antigen. One of the immunized mice, which showed high titre (>3.00 OD at 1:100 dilution of serum) in the rNP based indirect ELISA, was sacrificed for collection of spleen. The cells were harvested from the spleen and were centrifuged at 150 × *g* for 10 min. The mRNA, extracted from the spleen cells by QuickPrep mRNA purification kit (Amersham Biosciences, Uppsala, Sweden) was used for cDNA synthesis using reverse transcriptase (RT) enzyme and oligo dT primers. The variable heavy ($V_H$) and the light chain ($V_L$) gene fragments were amplified from the cDNA using the primers provided in the kit as per the kit protocol (RPAS ScFv module, Amersham Biosciences, Uppsala, Sweden). After purification and quantification, the heavy and the light chain DNA products were assembled into a single gene using a DNA linker fragment provided in the kit through an extension overlap PCR. In a second PCR, the assembled ScFv DNA was amplified and restriction sites were added using the restriction site primers (RS primers) having *Sfi1* (forward primer) and *Not*1 (reverse primer) restriction sites. The amplified ScFv DNA with restriction sites was gel purified and quantified by comparing band intensities with the standard ScFv DNA on agarose gel.

### 2.5. Cloning and expression of ScFv

The ScFv DNA was digested with the *Sfi 1* and *Not 1* enzymes and ligated into the similarly digested phagemid vector pCANTAB 5E (Amersham Biosciences, Uppsala, Sweden). The *E. coli* TG1 cells (Amersham Biosciences, Uppsala, Sweden) were transformed with ligation mixture by standard heat-shock method (42 °C for 2 min). The colonies obtained were scraped and diluted with 2× YT medium till the OD reached to 0.3 at $A_{600}$. Appropriate antibiotics