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Purification of Ani-Ns1 Mab and its use in competitive ELISA for DIVA

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Abstract
Avian influenza viruses are of high economic importance because of incurred losses caused, so need for rapid and accurate diagnois and also differentiation of vaccinated and naturally infected bird is required. The study highlights the purification of already produced Anti-NS1 MAb and its application in standardization of competitive ELISA for DIVA strategy.

Keywords: DIVA, NS1, avian influenza, bird flu

Introduction
Influenza viruses have gained importance in past few years because of its widespread infections and variable nature. It has caused heavy economic losses to poultry industry and is cause of pandemics threatening human life. Avian influenza (AI) is the most important reportable poultry diseases worldwide [1]. Both are highly contagious viral diseases affecting a wide range of bird species. It belongs to family orthomyxoviridae and has segmented genome rather than singe stretch. They are RNA viruses with three types. Type A influenza viruses are of prime importance as it includes all mammalian viruses e.g. swine flu, bird flu etc. Conversely, the AIV genotypes differ greatly in their immunogenicity; there is no cross-protection between viruses that differ in their haemagglutinin (HA) surface glycoprotein [2].
Migratory birds and the trade in live birds are believed to be the main sources of transmission of those two diseases globally, although for AIV, backyard birds, particularly water fowl (duck and geese), are important risk factors for transmission within endemic areas [3]. Backyard poultry is the main poultry production sector that suffers from the two diseases, particularly in developing countries [4]. This is due to the low level of biosecurity measures and the low rate or lack of vaccination. High mortality in birds is caused by highly pathogenic avian influenza (HPAI). Infection of birds with AIV can be of varying clinical severity, ranging from 100% fatality to a silent [3]. In addition, both have zoonotic potential. In the case of AIV, this is especially true for viruses with haemagglutinin surface antigens H5, H7 and H9 [4].
In past few years the AIV outbreaks had devastating effects on the poultry industry causing heavy economic losses to farmers with prevailing danger of zoonotic infections. To avoid these losses and reduce risk to human lives it is crucial to have vaccination, rapid diagnosis and also detection of silent carriers in flocks. Mostly kits are Nucleoprotein and Matrix protein based which not able to differentiate between vaccinated and infected birds (DIVA).NS1 protein is suitable for that. NS1 based Monoclonal Antiboby against NS1 (10E2) are required for DIVA and has already being produced discussed in previous paper [5].
The present study focuses on characterization of already produced anti-NS1 mAb and its use in standardization of competitive ELISA to differentiate between vaccinated and infected field samples.

Material and Methods
The anti-NS MAbs were isotyped with antigen-mediated indirect ELISA using isotype specific antibodies available in Mouse Isotyping Kit (Cat. no-ISO-2) from SIGMA according to the kit protocol with minor modification. The and a reactivity of different MAbs was tested in western blot with [5]. Briefly, the viral protein was run on SDS-PAGE (12.5%) and the proteins were transferred to nitrocellulose membrane using semi-dry transfer system. The nitrocellulose membranes containing viral proteins were blocked with BSA and followed by incubation with different MAbs produced against NS1(1:2 diluted in blocking buffer) for 2 hrs and the reaction was detected by Anti-Mouse IgG (Fab specific) Peroxidase conjugate (1:3000) from SIGMA.

The monoclonal antibodies were purified using Protein G column (Amersham Biosciences) which was first equilibriated with 2-5 volumes of binding buffer A (1.5M Glycine/NaOH 3M NaCl, pH-7) avoiding trapping of any air bubble then prefiltered cell culture supernatant was mixed with equal volume binding buffer and is passed through the column slowly with speed of 1-2ml/min. The flow through is collected after discarding first 1-2 ml solution from the column. Subsequently different fractions are collected by passing 2-5 volumes elution buffer B2 (0.2M Glycine/HCl pH-2.5) at slow speed as elutes E1 (500ul) –E5 with E2 having maximum volume (1-2 ml) in eppendorfs having 130ul of neutralization buffer C (1M Tris/HCl pH-9). The process can be repeated several times for larger volumes. For reuse, the column is again equilibriated with binding buffer, for storage it is washed with 20% ethanol and sealed and kept at 4^0 C. The anti NS1 monoclonal antibodies being purified were checked in 15% SDS PAGE gel for presence of heavy and light chain bands, after digesting it with 2X sample treatment buffer in equal volume. Bands being seen after staining gel in commassie brilliant blue stain for half an hour and subsequently destaining it with destain I and preserving gel in destain II.

Standardization of ELISA

A competitive ELISA was standardized following the basic protocols described by [5]. Selection of anti-NS1 MAb for use in competitive ELISA. The available MAb was used for titration of antigen against MAb. (b) Titration of recombinant NS1 antigen against selected anti-NS1 MAb:Varying dilutions of purified preparation of recombinant NS1 antigen were coated on the ELISA plate and were allowed to react with excess of MAb (ascitic fluid) to standardize the optimum quantity of NS1 antigen to be used in competitive ELISA.(c) Titration of monoclonal antibody against fixed quantity of antigen. The ELISA plates were coated with a fixed quantity of gag antigen as standardized earlier and varying dilutions of selected anti-NS1 Mab were allowed to react with the gag to optimize the dilution of Mab to be used in competitive ELISA. (d) Mab based competitive ELISA for testing serum for antibodies against BIV: Using the concentrations of recombinant gag antigen and dilutions of anti-NS1 Mab and test serum, the competitive ELISA was performed as per the basic protocol described [5] with minor modifications. The rNS1 antigen was diluted (dilution as standardized above) in carbonate-bicarbonate buffer (pH 9.6) and 50 µl of the diluted antigen was added to each well of the plate. The plate was kept at 4ºC for overnight. The antigen was discarded and the wells were washed once with PBS. The test serum was diluted 1:5 in diluent and 50 µl was added in duplicate wells. 50 µl of the 1:320 (diluted in diluent) anti-NS1 Mab was added in each well and the plate was kept for 2 hours at 37ºC. The plate was washed thrice with washing buffer (PBS with 0.1 % Tween20). 50 µl of the anti-mouse HRPO conjugate diluted at 1:5000 was added to each well and the plate was kept at 37ºC for an hour. The plate was washed thrice as before and 50 µl of the ready-to-use TMB (from Sigma) was added and the plate was kept in dark at room temperature. After 15 mins the reaction was stopped by adding 50 µl of stop solution (1N H2SO4) in each well. The plate was read at 450nm and the mean percent inhibition values for each test serum were calculated by the formula given as under:

Percent Inhibition (%) = (1- Mean OD of test serum/ Mean OD of the negative control) X 100

Using the protocol optimized as above, 51 sera (which were confirmed in indirect ELISA and HI) were tested and inhibition values were calculated. The cut-off value of inhibition was set for discriminating positive samples from negatives on the basis of formula,

Cut-off value = Mean % inhibition of negative sera (n=50) + 2X standard

Results and Discussion

After subcloning, the cell culture supernatant was subjected to indirect-ELISA using Mouse Immunglobulin isotyping kit (ISO-2 from Sigma). Only 1 MAb (10E2) was found to be IgG type hence was selected, MAb 4C4 was IgA, while rest showed no OD. Further, subclones of IgG type 10E2 were also isotyped and were confirmed to be IgG2b type. The subclone 10E2E7 showed higher OD and hence was further subcloned to obtain its monoclones 10E2E7A12 and 10E2E7G3. 10E2E7G3 was selected for subsequent work with comparatively higher OD to its counterpart 10E2E7A12. Western blot analysis was done with rNS1 antigen of SDS-PAGE gel (15%) of protein rNS1 of size 28KD. The protein is tagged with Penta His-Tag hence reactivity is confirmed with anti-His tag conjugate (QIagen) in the blot shown in picture fig. The anti-NS1 monoclone cells diluted in sterile 1x PBS were injected in mouse and were watched for bulging of belly region due to growth of cells and then ascitic fluid was collected, producing bulk amount of MAb and was checked for its absorbance at 450 nm in ELISA.

Standardisation of MAb based competitive ELISA for detection of antibodies against NS antigen of AIV was done by MAb based competitive ELISA was being attempted for detection of antibodies against NS1 in AIV infected chickens in order to differentiate between vaccinated and infected birds. Total of 51 samples were tested out of which 7 positives, (3 field samples and rest post challenge sera were from birds reared under containment facilities provided) and 44 were negative including 2 fields negative and 41 were sera collected from healthy birds reared in HSADL, animal houses.

***Percent (%) Inhibition=** (1-OD of the MAb with +ve sera/ OD of MAb without serum) x100

But MAb was not able to differentiate between positive and negative serum samples.

The purified recombinant NS1 antigen was titrated with excess of MAb (undiluted ascitic fluid) and graph was plotted between absorbance value and different dilution of NS1 antigen. Using standardized quantity of antigen and MAb cELISA was done. Sera samples at 1:5 dilution were checked for percent inhibition in presence of anti-NS1 MAb. MAb was not able to differentiate between positive and negative samples and percent inhibition was too high for negative sera samples.

The anti-NS1 monoclonal antibodies was being purified using Prosep “G” spin column kit (Milipore),and confirmed by analysing it on 15% SDS PAGE gel. The most purified mab was obtained in third fraction collected as elute 2. Analysis of different fractions of mab purification, heavy (just below albumin) and light chain bands(lower side) along with albumin band on top predominant in flowthrough while only two bands of light and heavy chains clearly visible in elute 2 (lane 3).

An attempt was made to design NS1 based competitive ELISA to distinguish between vaccinated and naturally infected as a diagnostic tool for DIVA strategy. For this 10E2E7G3 Mab was used as a competitor Mab based on its reactivity with recombinant NS1 antigen (OD 0.389). The titration experiments conducted in this study established the optimum dilution of rNS1 antigen as 1:200 and the optimum dilution of MAb 10E2E7G3 (Ascitic fluid preparation) as (1:320). The optimum dilution of MAb serum was established as 1:5. Using th concentrations of rNS1 antigen and dilutions of MAb and test serum a protocol of competitive ELISA for detection of antibodies against BIV was developed as per the format suggested by [5] with minor modifications. Total of 187 serum samples were tested which constitutes 98 positives (in AGID and HI) and 87 negative samples (in HI).The percent inhibition of positive sera samples ranged from 22-87% while for negative samples showed higher percent inhibition in disagreement to previous studies done [6]. It can be presumed from the results that the monoclonal antibodies might not be against the commonly found field epitopes of NS1 protein. Another reason can be inability of anti-NS1 MAb to differentiate negatives from positives.

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