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Molecular studies on avian influenza virus isolates from Indian poultry

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Abstract

The present study was carried out to characterize the avian influenza virus of Indian origin by detailed molecular techniques. For this purpose, 1,423 number of tissue samples were processed for virus isolation in avian influenza virus antibody negative chicken embryos of 9-11 day old. RT-PCR was conducted from the RNA extracted from the allantoic fluid of avian influenza virus inoculated chicken embryos. A total of 515 samples were tested by RT-PCR and positive bands of specific amplification of 488 bp of HA gene were obtained by one step RT-PCR with HSAIVH9F and HSAIVH9R subtype specific primers from the three samples. A 555 bp product which included HA gene cleavage site was obtained using the primers HSAIV47F and HSAIV47R from the cDNA synthesized from the allantoic fluids of all the three isolates. The H9 subtype was also confirmed by amplifying another region of HA gene using primers H9HA 692-714 and H9HA 1011-988 which gave rise to 319 bp amplicon. The 488 bp, 555 bp and 319 bp fragments of three isolates were cloned into pGEM-T easy vector and sequenced. The results indicated species variation among sequences of H9N2 isolates.

Keywords: avian influenza virus; Indian origin; isolate; molecular techniques; RT-PCR.

Contents

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1. Introduction

Guan et al. 1999. Crossing the species barrier to mammals highlights the pandemic potential of H9N2 virus (Peiris et al. 1999). The outbreaks due to H9N2 subtypes have been reported in Germany, Italy, Ireland, South Africa, USA, Korea, China, Middle East, Saudi Arabia and Pakistan during the later half of last decade (Alexander 2000; Banks et al. 2000). Highly pathogenic avian influenza has not been detected in India so far. Recently H9N2, non-pathogenic subtype of avian influenza virus, was isolated from poultry at High Security Animal Disease Laboratory (HSADL), Indian Veterinary Research Institute, Bhopal, India. But there is a constant threat posed by migratory birds that might bring pathogenic strains along with them to our country (Adam 2005).

The key to influenza pandemic preparedness is good surveillance for influenza viruses (Guan et al. 1999). Today, modern laboratory techniques, clinical and epidemiologic knowledge, and global communication provide the opportunity to monitor the evolving outbreak and act on it (Hien et al. 2004). Thus, continuous screening of the samples from Indian poultry and identification of the subtype of the virus isolated is imperative. Also the pathogenic potential of the isolated subtypes remains to be recognized.Keeping in view the paucity of information on Avian Influenza in India the present work was envisaged with the objectives to isolate and characterize avian influenza virus from Indian poultry, to study the pathogenicity of the virus isolates by intravenous pathogenicity index (IVPI) test, to determine the amino acid sequence in the cleavage site of HA gene of the avian influenza virus isolates and to study the localization of virus in the target tissues by immunoflourescence test, RT-PCR and Real Time-PCR.

2. Materials and Method

Samples were from indigenous birds and 2,977 samples were from imported grand parent stocks from countries like Netherlands, France, UK, etc. Also total of 1,423 number of tissue samples were received from various parts of the country were analyzed. The influenza virus isolated from field samples were characterized and four of the H9N2 isolates already available (virus lab accession no. 2424/04, 2543/04, 2544/04, 2317/04) at HSADL, Bhopal were used in this study.γ(gamma)-irradiated H7N7 whole virus antigen imported from CSIRO Laboratory, Geelong, Australia was used for AGID test. γ-irradiated whole virus antigen imported from NSDL, USA for HA subtypes 1-15 were used for HA subtyping. γ-irradiated whole virus antigen for NA subtypes 1-9 imported from NSDL, USA were used for NA subtyping.

Agar gel immunodiffusion test positive hyperimmune serum was used. Reference positive serum was procured for HA subtypes 1-15 were used for HA subtyping. Reference positive serum for NA subtypes 1-9 imported from NSDL, USA were used for NA subtyping. Anti-chicken IgG (whole molecule) FITC conjugate was procured from Sigma, USA. 3-4 weeks old white leghorn chickens were obtained from Madhya Pradesh State Government Poultry Farm, Bhopal, and were housed in negative pressure (-10 mg water) stainless steel isolators in a high containment facility (Biosafety Level 4) at HSADL, Bhopal. Feed and water were provided *ad lib*. Chicken Embryo Fibroblast (CEF) primary culture and Madin-Darby Canine Kidney (MDCK) cell line maintained in the laboratory were used in the study. The sequences of oligonucleotides used in this study are presented in the Table 3.1. Agar Gel Immunodiffusion Test (AGID), Hemagglutination (HA) and Hemagglutination Inhibition (HI), Neuraminidase Assay (NA), Neuraminidase Inhibition Assay (NAI) were performed as per standard methods as described by Cruickshank et al. (1975).

Processing of tissues

100 mg tissue was ground 1ml with 1X PBS in a sterile mortar and pestle, making a 10% suspension. The innoculum was filtered with 0.45 µM filter and inoculated in to the embryonated eggs *via* allantoic and amniotic routes. An aliquot was stored at -70^{o}C for use if the need arise. 100 µl of the specimen was inoculated into the allantoic cavity. The embryos that died within 24 h after inoculation were discarded. After 72 h incubation period the embryos were removed from the incubator and chilled at 4^{o}C. Eggs were chilled at 4^{o}C overnight or for 4 h before harvesting. These vials were then stored at -70^{o}C until further use. The confluent MDCK monolayer cells were resuspended in GMEM medium with 10% FBS and 1X antibiotics keeping the split ratio as 1:4. Infection of MDCK Cell Line, preparation of chicken embryo fibroblast culture and infection of chicken embryo fibroblast culture were performed as per standard procedures of Sambrook et al. (1989).

Reverse transcription polymerase chain reaction (RT-PCR)

One-step and Two Steps Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) were performed as per manufacture's protocol mentioned in Wizard RT-PCR Kit (Promega, USA). Cloning followed by extraction of plasmid was done as per standard procedure using the Wizard DNA Purification Kit (Promega). The protocol was as per the kit guidelines as described below:

Restriction endonuclease digestion

Sanger's dideoxy method was followed for nucleotide sequencing (Sanger et al. 1977).

Analysis of sequencing results

The sequencing results were analyzed using GenBank Software. The sequences obtained from the isolates were aligned with HA gene sequences from various isolates from India and other countries using the MegAlign software through clustral method.

Intravenous pathogenicity index (IVPI) test

The influenza virus isolates (accession no. 2424/04, 2543/04, 2544/04, 2317/04) along with an isolate (accession no. 3722/04) isolated from Gujarat during the period of study, were subjected to intravenous pathogenicity index (IVPI) test.

Immunoflourescent test

The immunoflourescent test was performed as per procedure described by Cruickshank et al. (1977).

3. Results and Discussion

Screening of serum samples

Among the samples from indigenous birds 473 (7.21%) were tested positive by AGID test for the presence of the antibodies against avian influenza virus. None of the samples from imported grandparent stock tested positive by AGID test.

Subtyping of the serum antibody

All the serum samples showed no HI with the H5 and H7 subtype specific reference antigens but gave a HI titer of 1:16 to 1:128 with H9 subtype specific reference antigen indicating that the serum contained antibodies to influenza A H9 virus subtype. The serum samples that gave less than 1:16 HI titer with 4 HAU of reference antigen were considered negative.

Screening of tissue samples

A total of 1,423 number of tissue samples were received from various parts of the country. The tissue samples were processed for virus isolation in avian influenza virus antibody negative chicken embryos of 9-11 d old after dilution of 1:10 with 1X PBS. The allantoic fluid from the infected embryos was harvested at 72 h after infection and a total number of three samples (3722/04, 3738/04 and 3709/04) were positive by HA test and the titer of the virus isolates ranged from 1:8 to 1:64. The virus isolates were neutralized with 1:10 dilution of NDV hyperimmune serum and then passaged further in embryonated chicken eggs to rinse out the contamination with NDV. Even after neutralization with NDV serum, all the three isolates showed HA activity.

Subtyping of influenza virus

HI test: The virus isolates showed no HI with the H5 and H7 subtype specific reference positive serum but gave a HI titer of 1:32 with H9 subtype specific reference positive serum indicating that the virus isolates were influenza A H9 subtype. The reference H9 subtype specific antigen gave an HI titer of 1:64. The virus isolates were also subjected to HI test with NDV hyperimmune serum and all samples gave negative result indicating that the isolates were free of ND virus contamination.

Neuraminidase inhibition assay

The virus showed inhibition of the neuraminidase activity up to the dilution of $10^{-1.0}$ as indicated by white color of the reaction mixture when compared with other subtypes against N2 subtype antiserum. This indicated that the virus belong to N2 subtype.

Cytopathic effect in cell culture

The cytopathic effect including rounding of cells, coalescing of the cells, formation of plaques and finally detachment of cells from the surface of culture flask were noticed in both CEF and MDCK cell lines on the fourth day onwards when cultured in the presence of trypsin. No CPE was observed in the cell control, trypsin control and cells infected with virus without trypsin.

Reverse transcription PCR

A 555 bp product which included HA gene cleavage site was obtained using the primers HSAIV47F and HSAIV47R from the cDNA synthesized from the allantoic fluids of all the three isolates. H9 subtype was also confirmed by amplifying another region of HA gene using primers H9HA 692-714 and H9HA 1011-988 which gave rise to 319 bp amplicon.

Cloning of PCR products

The 488 bp, 555 bp and 319 bp fragments of three isolates (3722/04, 3712/04 and 3697/04) products obtained were purified using Wizard PCR Purification Kit (Promega, USA) and cloned into pGEM-T easy vector. The ligated products were transformed into competent JM109 *E. coli* cells and the cells were allowed to grow in SOC. Upon plating the growth on AIX plates, blue and white colonies were seen. Fifteen recombinant white colonies from each transformation were picked up and grown in LB medium with ampicillin for extraction of plasmids. The presence of insert in the recombinant plasmids was confirmed in 10 colonies by plasmid PCR and restriction endonuclease digestion with *Not I* and *Eco RI* enzymes and plasmids with insert were chosen for sequencing.

Sequencing

Three colonies with inserts were sequenced for each isolate by Hoofer Manual Sequencer using γ^{33}P ATP and γ^{32}P ATP labeled M13 forward and M13 reverse primers. Since it is difficult to read more than 250 bases using forward or reverse reaction in autoradiogram, an internal PCR was done for 319 bp spanning 692-1011 bases of the HA gene of AIV. Cloning and sequencing were done for the internal PCR products of all the three isolates. The sequencing results obtained were aligned by MegAlign software tool of DNASTAR® LASERGENE program with sequences from H9N2 isolates from various parts of the world. The results indicated that the 488 bp sequences of the HA gene of the isolates aligned with a similarity of 98.4% with the sequences of Indian isolates and 92-93% similarity with various isolates of H9N2 in chicken throughout the world

Intravenous pathogenicity index test

Passaging of virus isolates in embryonated chicken eggs

The influenza H9N2 virus isolates (accession no. 2543/04, 2544/04, 2317/04, 2424/04, and 3722/04) isolated earlier were passaged in 9-11 d old embryonated chicken eggs after neutralizing 1:10 dilution with NDV hyperimmune serum. The allantoic fluid from the infected embryos was harvested at 72 h after infection and tested by HA test for the presence of the virus. The HA titers of the isolates used in the intravenous pathogenicity index test. The virus isolates were diluted in the ratio of 1:10 with 1X PBS as per the recommended procedures of WHO/OIE. The test was conducted in three phases. Two birds inoculated with the virus isolate 2543/04 and 2544/04 showed mild respiratory distress on the 6 and 4 d, respectively. The birds recovered next day and remained normal throughout the remaining period of study. The birds were observed for a period of 10 d and all the surviving birds were sacrificed and observed for gross lesions if any. Both the control and inoculated birds did not show any clinical signs and lesions.

Serology

Avian influenza virus group specific precipitating antibodies where present in the serum samples collected from the entire five groups, on 4th and 10th day, which where inoculated with the virus. There was no evidence of precipitating antibodies against AIV.

Immunofluoroscence test

The results of immunoflourescence test are presented in Table 2.

Virus reisolation

Avian influenza virus reisolation was attempted from the both infected and control groups from liver, kidney, proventriculus, brain, trachea, spleen, intestine and heart which were collected on 10 d of post infection. The inoculums prepared from these organs were used for inoculation of embryos. Reisolation of virus could be achieved from three groups (of isolates with accession no. 2543/04, 2544/04, and 2317/04) and not from two other groups (of isolates with accession no. 2424/04, and 3722/04) and control group. On HA test the allantoic fluid gave HA titers ranging from 1:2 to 1:1024 (Table 1).

Reverse Transcription-PCR

The RNA was extracted from the allantoic fluid collected from various groups and RT-PCR was done. The RT-PCR test done with H9 subtype specific primers HSAIV H9F and HSAIV H9R and nucleoprotein primers NP1F and NP1R. The virus isolates gave specific amplification of 488 bp and 443 bp, respectively. All the samples which gave positive amplification by H9 subtypes specific primers also gave amplification of nucleoprotein gene. RT-PCR gave positive results from most of the organs from three groups (of isolates with accession no. 2543/04, 2544/04, and 2317/04) and from none of the organs of the remaining two other groups (of isolates with accession no. 2424/04, and 3722/04) and control group.

Real-Time PCR

Real-time PCR assays were data generated in analyzed to calculate “threshold cycle” (Ct value) which is inversely proportionate to the initial template concentration in the samples, and dissociation temperature (Tm; temperature melting) which differentiate between specific and non-specific amplifications. Detection of AIV genome in different organs of chickens experimentally infected with Indian isolates of H9N2 AIV by RT-PCR is represented in Tables 3, 4 and 5. A typical amplification and dissociation curve obtained from different concentrations of AIV NP cDNA plotted against Ct value. The Tm of target was calculated to be 81.1-81.6^{o}C. A typical dissociation curve of the nucleoprotein amplicons, where fluorescence was plotted against temperature and complete dissociation was observed at Tm 81.6^{o}C indicating specificity of the amplification reaction. In chicken infected with H9N2 AIV isolate no. 2317/04, the virus could be detected in trachea, lung, intestine, liver, proventriculus and brain. No virus could be detected in spleen and kidney. Tissue invasion of H9N2 AIV isolate 2543/04 was detected in trachea, lung, intestine, liver, proventriculus, kidney and brain; whereas virus could not be detected in spleen. In case of isolate 2544/04, the virus could be traced in liver, lung, intestine, proventriculus, kidney and brain, whereas virus could not be detected in spleen and trachea. Virus could not be detected in any of the organ / tissues of infected with the AIV isolate 3722/04 and 2424/04 which indicated non invasiveness of the virus. Absence of specified amplification from uninfected healthy from any of the organs of chicken indicates specificity of the primers and test.

Avian influenza virus reisolation was attempted from the both infected and control groups in embryonated chicken eggs as the avian Influenza viruses are very stable in allantoic fluid because the presence of protein protects the viruses, making this the most universally used method for isolation of Avian Influenza viruses (Easterday et al. 1997). In a similar study for rapid detection of influenza virus in humans a real time-PCR was applied targeting matrix gene (Stone et al. 2004). Based on their findings and our results it is now possible to target either the matrix gene or NP gene for real time PCR assay for diagnosis of AIV.

The results of tissue tropism results obtained by real-time PCR were same as those of virus isolation in 9-11 day old embryonated chicken eggs and RT-PCR studies. Thus, it can be concluded that real-time PCR is a trustworthy alternative to virus isolation in embryonated chicken eggs and RT-PCR. This further strengthens the claim of Spackman et al. (2003), who concluded in a study conducted by them, that real-time PCR result is a reliable alternative to virus isolation in embryonated chicken eggs. In addition to the almost "0" indices of IVPI tests, the absence of CPE in cell cultures and absence of multiple basic amino acid at the cleavage sites of HA gene, clearly indicated that the H9N2 viruses circulating in India are non-pathogenic to poultry. The present study also indicated that the country is free from highly pathogenic avian influenza as none of the serum sample and virus isolated from poultry belonged to H5 or H7 subtypes. Though the country is fortunate in not having highly pathogenic avian influenza, continuous surveillance and monitoring of avian influenza status in our country is required.

4. Conclusion

The serological incidence of avian influenza in Indian poultry is very less i.e. 7.21%, Only H9N2 subtypes were found to be present in the Indian poultry, all the isolates subjected to intravenous pathogenicity index (IVPI) test were found to be non-pathogenic, the Indian isolates of avian influenza virus have originated from a common ancestor and have not undergone much change in their sequence, the results of immunoflourescence test showed that the organs from experimentally infected birds failed, with few exceptions, to give immunoflourescence, the results of the localization of virus in the target tissues were similar for RT-PCR and Real Time-PCR.

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