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Assessment of Pathogenic Potential of Two Indian H5N1 Highly Pathogenic Avian Influenza Virus Isolates by Intravenous Pathogenicity Index Test

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Abstract: Intravenous Pathogenicity Index (IVPI) test was conducted to assess the pathogenicity of two H5N1 (A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06) AIV isolates. Both the H5N1 virus isolates were isolated from natural outbreaks during 2006, both isolates caused death of all birds with in 48 h after inoculation experimentally, birds showed typical clinical signs, gross and microscopic lesions of Avian influenza. IVPI of A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06 was 2.96 and 2.95 respectively. This test showed that both H5N1 isolates are highly pathogenic. Virus could be re-isolated from all the organs of infected chickens and it was reconfirmed by RT-PCR using WHO and Lee primers.

Key words: HPAI, ivpi, india, rt-pcr and pathology

INTRODUCTION

Notifiable avian influenza is defined by the World Organization for Animal Health as "an infection of poultry caused by any influenza A virus of the H5 or H7 subtypes or by any avian influenza virus with an Intravenous Pathogenicity Index (IVPI) greater than 1.2 (or as an alternative at least 75% mortality)". The terminology "Highly pathogenic avian influenza" was officially adopted in 1981 at the first International Symposium on Avian Influenza. Influenza viruses are segmented, negative-sense, single-stranded RNA viruses of the family *Orthomyxoviridae* and are divided into three genera *Influenza virus* A, B and C types, based on antigenic difference in their nucleoprotein and matrix protein (Lamb, 1989). However, only type A influenza viruses have been known to cause natural infections of birds. Classification of an avian influenza virus based on the Intravenous Pathogenicity Index (IVPI) and the amino acid sequence of the HA cleavage site, the viruses are sub-classified into two pathotypes of Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI) viruses (Swayne, 1997; Wood *et al*., 1993). HPAI viruses cause severe systemic disease that is associated with high rates of morbidity and mortality, which often approaches 100% in chickens and turkeys. To date, all HPAI isolates have been of the H5 or H7 subtypes, although not all H5 or H7 subtype viruses cause HPAI (OIE, 2005).

Till January 2006 India was free of highly pathogenic avian influenza. In February 2006 and subsequently till April 2006 four outbreaks of H5N1 virus were noticed in Maharashtra, Gujarat and Madhya Pradesh. In the present study we isolate the H5N1 viruses from natural cases and pathogenicity of first two virus isolates was assessed by IVPI test, following OIE protocols.

MATERIALS AND METHODS

The influenza virus H5N1 isolates (A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06) were isolated from Navapur, Maharastra during the period of this study, were subjected to Intravenous Pathogenicity Index (IVPI) test. To determine the pathogenicity of the two H5N1 virus isolates (A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06) the IVPI test was carried out according to the recommendation of the *Office International Des Epizooties* (OIE, 2005). Four to six week old chickens were obtained from government poultry farm, Bhopal; the chickens were tested negative for antibodies to avian influenza virus. Eight birds in each group were injected intravenously through jugular/wing vein with 0.2 mL / bird of inoculum containing 1:10 dilution of fresh allantoic fluid having a HA titre > 4 HAU with sterile 1X PBS. The control groups were inoculated with 1X PBS. Birds were examined daily for 10 days and scored, 0 if normal, 1 if sick, 2 if very sick, and 3 if dead. Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: Respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and head, nervous signs. Dead individual birds must be scored as 3 at each of the remaining daily observations after death. Examine the birds for clinical signs at intervals of 24 h over a 10 days period. At the end of 10 days of the observation period, the sum of the observations in each category was totaled and divided by the total number of observations. The results were interpreted as described in WHO/OIE manual. Total score 0 means all the birds are normal up to ten days. If the score is 3 all the birds are dead within 24 h.

Before inoculation the blood samples were collected and checked by both AGID and HI for presence of antibodies against both AIV and NDV. Tissue samples

were collected from chickens that died during the experiment. Tissue samples were also collected from uninoculated control chickens after the experiment. The following tissues samples were collected: brain, trachea, lung, heart, liver, spleen, intestine, pancreas, proventriculus, kidney and cloaca. A part of tissue sample was fixed in 10% neutral buffered formalin solution and was later used for histopathological studies. Another part was cryopreserved at -80°C and later used for reisolation of virus. Tissue samples (10% neutral buffered formalin fixed) were processed for histopathology by routine paraffin embedding technique and 5-6 microns thick sections where stained with hematoxylin and eosin (H and E) for light microscopic examination. The reisolation of the virus from tissues collected from various groups of IVPI tested chickens was done as per the procedures explained below to study the tissue invasiveness of the avian influenza virus isolates.

One hundred milligram of tissue was ground in 1mL with 1X PBS in a sterile mortar and pestle, making a 10% suspension. The suspension was transferred to an eppendorf tube and centrifuged at 400 x g for 10 min to remove extraneous materials. The supernatant was removed and after the addition of 1/10 volume of 10X antibiotic mixture, incubate at 37°C for 1 h. The innoculum was filtered with 0.45 µM filter and inoculated into 9 to 11 day old embryonated eggs *via* allantoic and amniotic routes. Examine the 9 to 11 day old eggs with an egg Candler, place the eggs with blunt end up into egg trays and label each egg with a specific identification number (3 eggs per specimen). Wipe the tops of the eggs with 70% ethanol and punch a small hole in the shell over the air sac. Three eggs per specimen are usually inoculated. Aspirate 1 mL of processed clinical specimen into a tuberculin syringe with a 22 gauge, 1½ inch needle. Holding the egg up to the Candler, locate the embryo and insert the needle into the hole of the egg. Using a short stabbing motion, pierce the amniotic membrane and inoculate 100 µL into the amniotic cavity. Withdraw the needle about ½ inches and inoculate 100 µL of the specimen into the allantoic cavity, remove the needle. Inoculate the two other eggs in the same manner. Seal the holes punched in the eggs with a drop of glue. Incubate the eggs at 33-34°C for 2-3 days. Note down embryos which are dead in every 24 h by candling. 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°C overnight or for 4 h before harvesting. Clean off the top of each egg with 70% ethanol. With sterile forceps, break the shell over the air sac remove the cell membrane and push aside the allantoic membrane with the forceps. Using a 10 mL pipette, aspirate the allantoic fluid in a labeled plastic tube. Then using a syringe and needle, pierce the amniotic sac and remove as much amniotic fluid as possible. Centrifuge harvested fluids at 3000 rpm / 5 min to remove blood and cells. The clear supernatant was aseptically transferred into sterile and labeled vials. These vials were then stored at -70°C until further use.

The subtypes of the virus isolates were confirmed by Haemagglutination inhibition test and RT-PCR. QIAamp Viral RNA Mini Kit (Qiagen, Germany) was used to extract viral RNA from allantoic fluids. 560 µL of the prepared buffer AVL containing carrier RNA was pipetted into a 1.5 mL microfuge tube. 140 µL of allantoic fluid was then added to the buffer AVL/carrier RNA in the microfuge tube. To mix properly pulse vortexing was done for 15 sec. Tubes were then incubated at room temperature 22-25°C for 10 min. Pulse centrifugation was done to collect drops from inside lid. To the sample 560 µL of pure ethanol was added and mixed by pulse vortexing for 15 sec. After mixing, the eppendorf tubes were briefly centrifuged to collect drops from inside the lid. 630 µL of the solution from previous step was applied to the QIAamp spin column with collection tube. The column was placed in the centrifuge and spun at 8000 rpm for 1 min. This step was repeated for one more time. The flow through was discarded and 500 µL of buffer AW1 was added to the column. Centrifugation was done at 8000 rpm for 1 min. The QIAamp column was then transferred to a fresh collection tube and the tube containing the filtrate was discarded. Five hundred micro liter of buffer AW2 was added to the column and centrifuged at 14,000 rpm for 3 min. The QIAamp column was then carefully transferred to a fresh 1.5 mL eppendorf tube and the collection tube was discarded. Sixty micro liter of buffer AXE pre equilibrated to room temperature was added to the center of the column and incubated at room temperature (22-25°C) for 1 min. Centrifugation was performed at 8000 rpm for 1 min. The flow through collected is viral RNA and was stored at -70°C until further use.

One-Step Reverse Transcriptase Polymerase Chain Reaction was conducted by using RNA form all 11 organs of experimental birds. HA gene was amplified using QIAGEN® One Step RT-PCR) Kit (Germany) as per the protocol given below by using standard primers for Avian influenza diagnosis ie WHO (219bp) and Lee (545bp) primers (Table 2). The following reaction mixture of 25 µl (for one reaction) was prepared in a 0.5 mL tube on ice:

Component	Volume	Final Concentration
5X Buffer:	5.0 µL	1X
Q Solution:	5.0 µL	1X
10mM dNTPs:	1.0 µL	200 µM
5 U/µl RT Enzyme:	1.0 µL	5 U
Primer HSAIV 1F:	1.0 µL	20 pmol
Primer HSAIV 1R:	1.0 µL	20 pmol
Template (RNA):	4.0 µL	
Nuclease free water:	7.0 µL	
Total volume:	25.0 µL	

The same reaction composition as indicated above was used for the other RT-PCR reaction with different sets of primers (WHO, 2002 and Lee *et al*., 2005).

The reactions were carried out in a thermal cycler (EPPEDORF, HYBAID, USA) as per the following program:

Step I: Reverse Transcription:	50°C for 30 min (1 cycle)	
Step II: Initial denaturation:	95°C for 15 min (1 cycle)	
Step III: Denaturation:	94°C for 30 sec	(35 cycles)
Annealing of primer:	50°C for 30 sec	
Extension:	72°C for 40 sec	
Step IV: Final extension:	72°C for 10 min (1 cycle)	

Similar reaction and cycling conditions were used for RT-PCR amplification with both WHO and Lee primers. The HA gene RT-PCR products were subjected to agarose gel electrophoresis using submarine electrophoresis system (Thermo Orion). Agarose gel (1.5% w/v) was prepared in 1X TAE was melted in microwave oven and ethidium bromide was added to a final concentration of 0.5 µg /mL. It was poured in a gel casting tray with comb and then allowed to solidify at 10°C for 20 min. Ten micro liter of each PCR product was mixed with 2 µL of 6X loading dye and loaded in to the well. Along with the products a 100 bp DNA molecular size marker (Fermentas, USA) was run. Electrophoresis was carried out at 10 Volts/cm and 500 Amp, for 1 h. After the run, the gel was observed under long range UV light.

RESULTS AND DISCUSSION

Both the H5N1 avian influenza virus isolates (A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06) isolated earlier were passaged in 9-11 day old embryonated chicken eggs after neutralizing with 1:10 dilution of 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 and subtype was confirmed by HI with specific reference serum. The HA titers of the isolates A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06 used in the intravenous pathogenicity index test are 128 and 256 respectively.

More than four HA units of virus isolates were diluted in the ratio of 1:10 with 1X sterile PBS as per the recommended procedures of WHO/OIE. Birds were inoculated with two Indian H5N1 avian influenza virus isolates A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06. The A/Ck/Ind/7966/06 virus caused death of six birds within 24 h (75% of mortality) (Fig. 1); on the other hand the A/Ck/Ind/7972/06 virus caused death of five birds within 24 h (62.5% of mortality) (Fig. 2). Most of the birds were died by per acute infection without showing any signs/symptoms. All the birds died Within 48 h of post inoculation (100% mortality). The birds showed typical symptoms and gross lesions of avian influenza. Control birds were healthy throughout the observation period

Fig. 1: Birds showing huddling, dullness and death in stainless steel Isolator at one day after intra vinous inoculation with H5N1 virus isolate A/Ck/India/7966/06

Fig. 2: Birds showing severe symptoms and death in stainless steel Isolator at one day after intra vinous inoculation with H5N1 virus isolate A/Ck/India/7972/06

and were sacrificed after 10 days. The Intravenous pathogenicity index was calculated based on OIE/WHO guide lines (Table 1). The serum samples collected from all the birds before inoculation were negative for influenza antibodies.

Birds infected with the A/Ck/Ind/7966/06 virus isolates caused death of six birds in one-day post infection. In that two birds died with per acute infection without any clinical signs. Another four birds were severely affected, with swollen head, respiratory distress with rales, gasping and off feed and Paralysis was noticed. Another two birds were less severely affected and they were dull and depressed. Within 48 h these birds were also died with typical clinical symptoms like dull and depressed, off feed, stretched legs, swollen hock joint, edema and hemorrhages of footpad, respiratory distress, rales with nervous disorders. On postmortem, all six birds dead by

Table 1: Intravenous pathogenicity index test for AIV isolates A/Ck/India/7966/06 and A/Ck/India/7972/06

Clinical signs	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	Total	Score
Isolate A/Ck/India/7966/06												
Normal	0	0	0	0	0	0	0	0	0	0	0×0	= 0
Sick	1	0	0	0	0	0	0	0	0	0	1×1	= 1
Paralyzed	1	0	0	0	0	0	0	0	0	0	1×2	= 2
Dead	6	8	8	8	8	8	8	8	8	8	78×3	= 234
Total												= 237
Index												2.962
Isolate A/Ck/India/7972/06												
Normal	0	0	0	0	0	0	0	0	0	0	0×0	= 0
Sick	1	0	0	0	0	0	0	0	0	0	1×1	= 1
Paralyzed	2	0	0	0	0	0	0	0	0	0	2×2	= 4
Dead	5	8	8	8	8	8	8	8	8	8	77×3	= 231
Total												=236
Index												2.950
Uninoculated control												
Normal	8	8	8	8	8	8	8	8	8	8	80×0	= 0
Sick	0	0	0	0	0	0	0	0	0	0	0×1	= 0
Paralyzed	0	0	0	0	0	0	0	0	0	0	0×2	= 0
Dead	0	0	0	0	0	0	0	0	0	0	0×3	= 0
Total												= 0
Index												0.0000

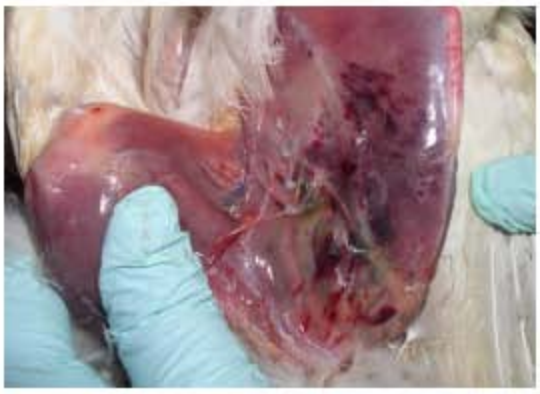


Fig. 3: Breast muscles of H5N1 virus infected chicken showing extensive hemorrhages at two day post inoculation

Fig. 4: Thigh muscles of H5N1 virus infected chicken showing hemorrhagic streaks at two day post inoculation

one-day post inoculation showed swollen head, mucopurulent discharge from nostrils, hemorrhages on thigh muscles (Fig. 4) and some birds showed congestion of liver, spleen and lungs. The birds, which were died at 48 h, showed consistent gross lesions, like swollen head, slight enlargement and congestion of liver and spleen. Congestion of lungs with cloudy air sac was noticed. Severe congestion of kidney, hemorrhages on thigh muscle was recorded. Heart showed hydropericardium and pinpoint hemorrhages on the pericardium (Fig. 8). Serosal vessels congested severely, severe intra cranial hemorrhages and congestions were noticed.

The birds infected with the A/Ck/Ind/7972/06 virus isolates caused death of five birds in one day post infection. All the five birds were died with per acute infections without showing any clinical signs. The severely affected birds showed clinical signs like off feed, severe respiratory distress, unable to walk, stretched legs with paralytic symptoms. Other birds are showed mild respiratory distress. On second day post infection all the birds died exhibiting both respiratory and nervous symptoms. On post mortem examination birds showed swollen head with mucopurulent discharge from nostrils. Other PM lesions were hemorrhages on the thigh muscle and breast muscles (Fig. 3), congestion of lung and cloudy air sac are with slight enlargement of spleen. The proventricular lumen contains mucus and the wall showed pinpoint hemorrhages (Fig. 6). Serosal vessels congested. Patchy hemorrhages were observed in intestine and pancreas in few birds. The birds which died at two-day post infection showed enlarged hock joint with edema and hemorrhages in the shank and footpad (Fig. 7). Swollen head, hemorrhages on forehead (Fig. 5) skin, hydropericardium and pinpoint hemorrhages on spleen were also noticed.

Table 2: Sequences of oligonucleotides used in the study

Name	Gene	Sequence (5'-3')	Reference
H5-1F	HA	GCCATTCCACAACATACACCC	WHO, 2002
H5-3R		CTCCCCTGCTCATTGCTATG	
H5-F	HA	ACACATGCYCARGACATACT	Lee *et al*., 2001
H5-R		CTYTGRTTYAGTGTTGATGT	

On microscopic examination birds inoculated with the A/Ck/Ind/7966/06 showed lesions in the lungs and spleen at 24 h. The lesions were characterized by mild congestion and hemorrhages of the lungs with infiltration of lymphocytes beneath the bronchiolar epithelium leading to thickening of the bronchiolar wall and alveolar ducts were filled with significant amounts of RBCs. In most of the birds the alveolar ducts were filled with mucus exudates (Fig. 13). In the spleen the red pulp was highly congested and hemorrhages were seen extending to the other areas (Fig. 12). In brain, trachea, heart, liver, proventriculus, pancreas, intestine and kidney no microscopic lesions were observed. Birds died at two-day post infection had severe congestion of the lungs, spleen and kidney. The lungs and spleen lesions were similar to those described for the lesions at one-day post infection, in lung hyperactivity of mucus glands in the larger bronchiolar lumen and alveolar ducts with infiltration of lymphocytes were noticed (Fig. 9 and 11). In brain glial nodules with small areas of perivascular cuffing were observed (Fig. 10). In the kidney the intertubular capillaries were congested and in many cases hemorrhages were noticed, focal areas of congestion, necrosis and lymphocytic infiltration were noticed in two birds. In some birds the mucus glands of the trachea were distended with the mucus and the subepithelial region showed mild to heavy infiltration of lymphocytes. Histopathological lesions in birds infected with A/Ck/Ind/7972/06 had similar to the birds infected with A/Ck/Ind/7966/06. However in the pancreas of one bird showed degeneration and necrosis of pancreatic cells with mild infiltration of heterophils. In brain small areas of perivascular cuffing were noticed (Fig. 14). In control birds lesions were not observed.

Avian influenza virus reisolation was attempted from all the organs of infected and control groups. The organs included were brain, trachea, lung, heart, liver, spleen, proventriculus, intestine, pancreas, kidney and cloaca, which were collected from dead birds. The inoculums prepared from these organs (1:10 concentration) were inoculated in to 9-11 day old chick embryos. All the H5N1 virus infected embryos died with in 48 h. Reisolation of virus could be achieved from all the organs of H5N1 virus inoculated birds. On HA test the allantoic fluid gave HA titer ranging from 1:64 to 1:2048 (Table 3) and the HI titer with the subtype specific reference serum ranged from 1:64 to 1:2048 (Table 3), from the control birds virus could not be isolated.

RNA was extracted from the allantoic fluids obtained from 9-11 days old chick embryo inoculated with the

Fig. 5: Skull of chicken infected with H5N1 virus showing hemorrhagic spots at two day post inoculation

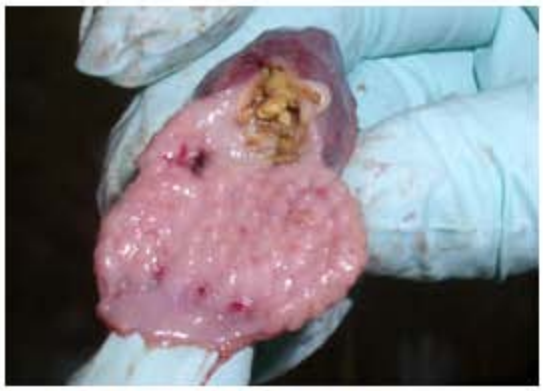


Fig. 6: Proventriculus of chicken infected with H5N1 virus showing pinpoint hemorrhages at two day post inoculation

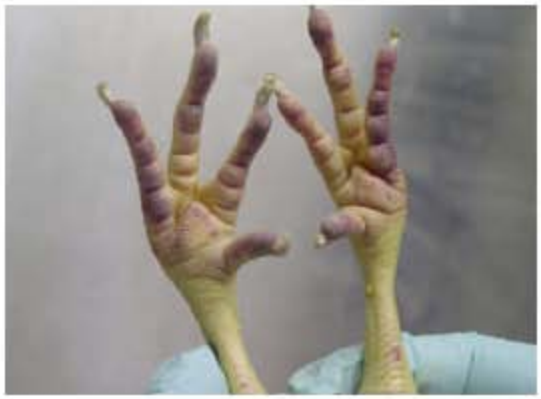


Fig. 7: Chicken showing swollen and cyanosis of the foot pad at two day post challenge

inoculums prepared from the tissue samples from all the birds including controls (1:10 dilution with 1XPBS). The inoculum was prepared from all the organs separately. All the organs from H5N1 virus isolates

Fig. 8: Heart of chicken infected with H5N1 virus at two day post inoculation showing hemorrhages on the pericardium

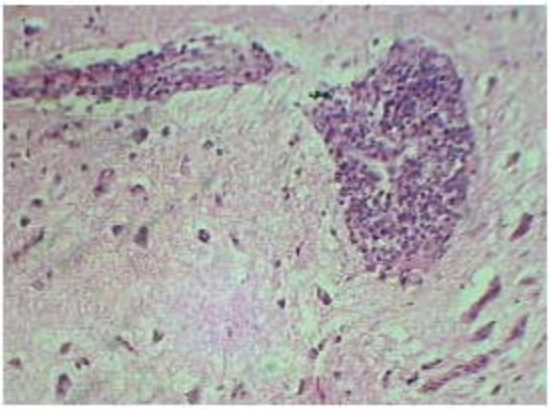


Fig. 9: Cerebrum of chickens infected with H5N1 virus showing neuronal degeneration, perivascular cuffing and gial nodule (H & E; 200X)

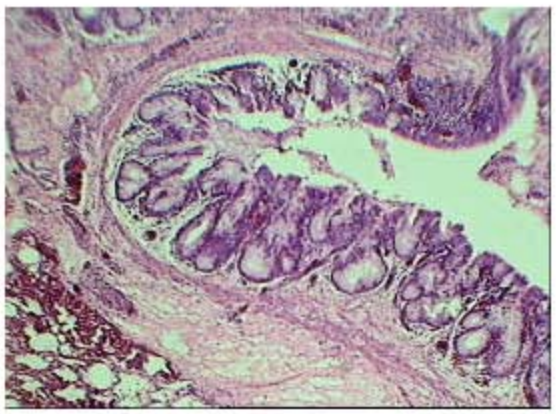


Fig. 10: Lung of chicken infected with H5N1 virus showing hyperactivity of mucus glands in the larger bronchiolar lumen and alveolar ducts with infiltration of lymphocytes at two day post inoculation (H & E; 200X)

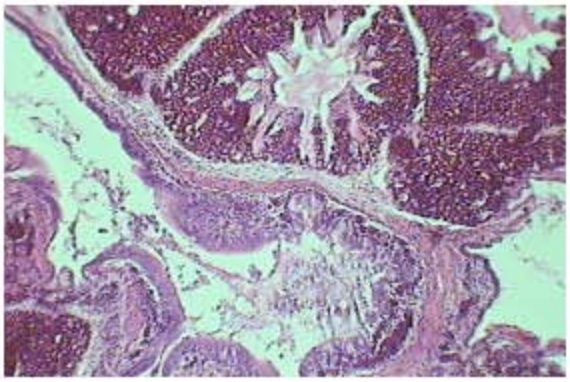


Fig. 11: Lung of chicken infected with H5N1 virus showing marked congestion and deposits of exudates in the bronchiolar lumen and alveolar ducts with mild to moderate infiltration of lymphocytes in the bronchiolar wall at two day post inoculation.(H & E; 200X)

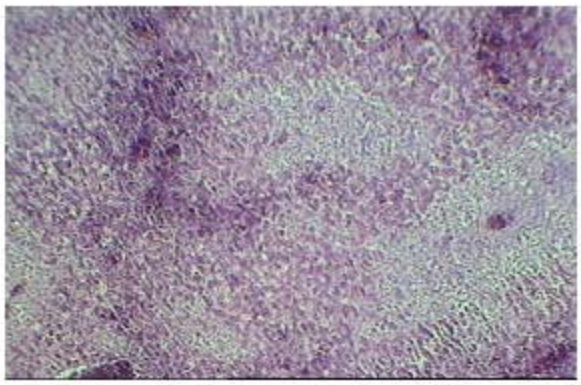


Fig. 12: Spleen of chicken infected with H5N1 virus showing congestion and hemorrhages in the red pulp extending into other areas at two day post inoculation (H & E; 200X)

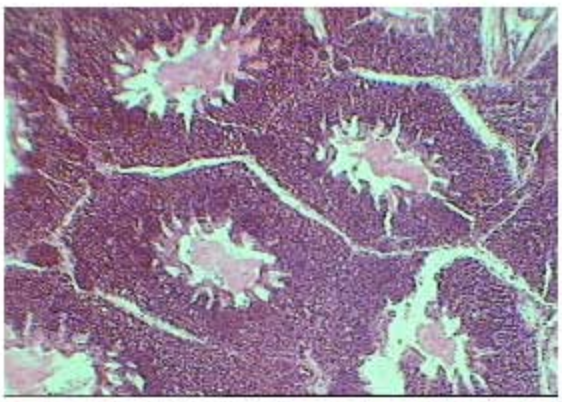


Fig. 13: Lung of chicken infected with H5N1 virus showing congestion and mucus exudates in the alveolar ducts at two day post inoculation (H & E; 200X)

Table 3: Comparison of diagnostic tests conducted on samples from IVPI tested birds (A/Ck/India/7966/06 and A/Ck/India/7972/06 virus isolates)

Virus isolates	A/Ck/India/7966/06			A/Ck/India/7972/06		
Organs	HA titer	HI titer	RT-PCR	HA titer	HI titer	RT-PCR
Brain	1:512	1:1024	+	1:2048	1:1024	+
Trachea	1:256	1:512	+	1:512	1:256	+
Lung	1:128	1:512	+	1:1024	1:512	+
Heart	1:128	1:256	+	1:1024	1:256	+
Liver	1:128	1:256	+	1:1024	1:256	+
Spleen	1:128	1:256	+	1:512	1:512	+
Intestine	1:128	1:256	+	1:1024	1:256	+
Pancreas	1:128	1:128	+	1:64	1:64	+
Proventriculus	1:128	1:128	+	1:1024	1:256	+
Kidney	1:256	1:512	+	1:1024	1:1024	+
Cloaca	1:1024	1:1024	+	1:2048	1:1024	+
Control birds samples	Nil	Nil	-	Nil	Nil	-

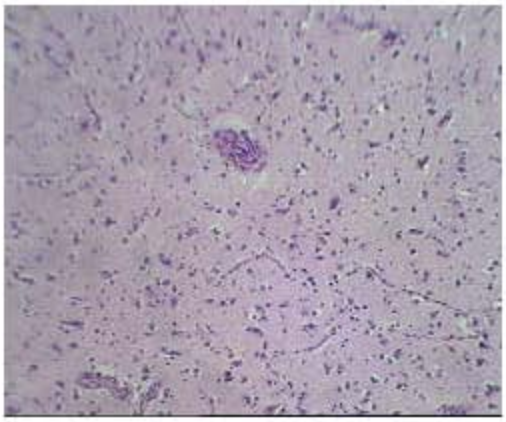
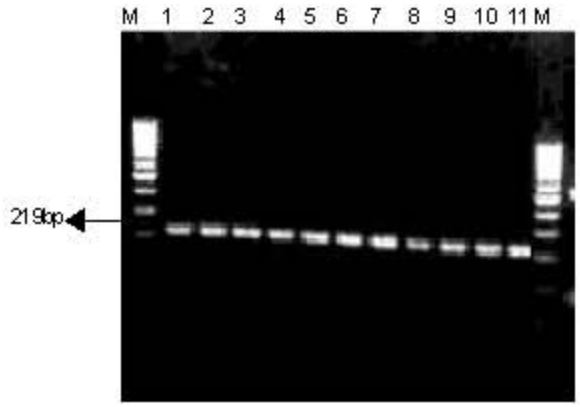


Fig. 14: Cerebrum of chicken infected with H5N1 virus showing mild neuronal degeneration and perivascular cuffing at two day post inoculation (H & E; 200X)

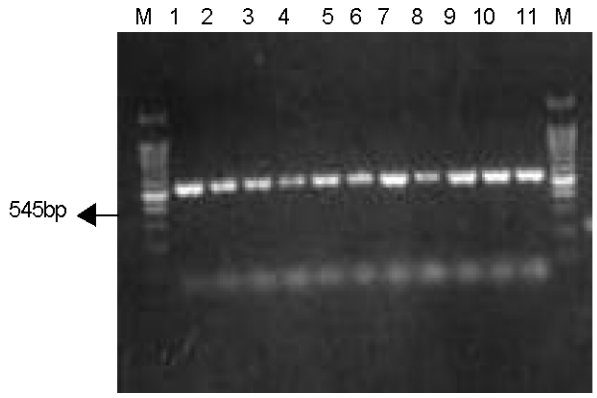


M: 100 base pair ladder (Fermentas)
Lane 1: Brain
Lane 2: Trachea
Lane 3: Lung
Lane 4: Heart
Lane 5: Liver
Lane 6: Spleen
Lane 7: Pancreas
Lane 8: Intestine
Lane 9: Proventriculus
Lane 10: Kidney
Lane 11: Cloaca

Fig. 15: Reisolation of virus and Subtyping by RT-PCR with WHO (219 bp) primers

(A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06) infected birds gave the specific amplification of 219 bp and 545 bp with H5 specific WHO and Lee primers, respectively. The control birds gave negative result (Table 3).

The intravenous pathogenicity index test was conducted for three different isolates. The experimental birds were observed for a period of 10 days and all the surviving birds were sacrificed, as proposed by WHO (2002). Avian influenza virus infections in domestic poultry produce different clinical syndromes (Yilmaz *et al*., 2004). In the present study two H5N1 virus isolates (A/Ck/Ind/7966/06 and A/Ck/Ind/7972/06) were used for IVPI test. The A/Ck/Ind/7966/06 virus isolate caused 75% mortality within 24 h, on other hand A/Ck/Ind/7972/06 virus isolate caused 62.5% mortality and both isolates caused 100% mortality in 48 h after infection. The typical clinical signs and gross lesions like swollen head, cyanosis of comb and wattles, severe respiratory distress, nervous system disorders, hemorrhages on thigh muscles, congestion and enlargement of kidney, spleen and liver were observed. The IVPI of both isolates were calculated according to WHO/OIE guidelines. The index of 2.96 (A/Ck/Ind/7966/06) and 2.95 (A/Ck/Ind/7972/06) were obtained. Similar observations were also recorded by Nguyen *et al*., 2005; Lee *et al*., 2005 and Tumpey *et al*., 2003. Thus both the H5N1 Indian isolates studied were found to be highly pathogenic according to the European Union definition (WHO, 2002). As per the OIE/WHO (2002) guide lines the IVP index of 1.2 to 3.0 is considered as highly pathogenic. In the present study both the H5N1 viruses had IVP indices of 2.96 and 2.95, hence they are highly pathogenic. Variations in the pathogenicity and transmissibility of influenza viruses for different hosts have frequently caused problems in diagnosis, definition



M: 100 base pair ladder (Fermentas)

Lane 1: Brain	Lane 2: Trachea
Lane 3: Lung	Lane 4: Heart
Lane 5: Liver	Lane 6: Spleen
Lane 7: Pancreas	Lane 8: Intestine
Lane 9: Proventriculus	Lane 10: Kidney
Lane 11: Cloaca	

Fig. 16: Reisolation of virus and Subtyping by RT-PCR with Lee (545 bp) primers

and the understanding of the influenza infection in poultry (Bankowski, 1982).

The organs collected from the experimental chickens which died within 24 h showed microscopic lesions mainly in lungs and spleen. In birds dead during 48 h the lesions were noticed in lung, trachea, spleen and kidney but there were no significant lesions noticed in other organs (heart, liver, proventriculus, pancreas and intestine). Because of the high virulence of the virus all the birds died within 48 h after inoculation, so there was no time to develop lesions in these organs. Lee *et al.* (2005) and Tumpey *et al.* (2003) also noticed similar histopathological lesion in chickens. Avian influenza virus reisolation was attempted from the both infected and control groups from brain, trachea, lung, heart, liver, spleen, proventriculus, intestine, pancreas, kidney and cloaca in embryonated chicken eggs. Virus could be reisolated from all the organs of chickens at 24 and 48 h died that were infected with H5N1 virus isolates. Lee *et al.* (2005) and Tumpey *et al.* (2003) were also reisolated H5N1 virus from all organs of infected birds. RT-PCR serves as a fast and effective alternative to virus isolation for the detection of influenza A virus (Claas *et al.*, 1998; Yuen *et al.*, 1998). Keeping this in mind RT-PCR was used to study the tissue tropism of avian influenza viruses isolated in experimentally infected chickens. All samples positive in HI are also gave the positive results in PCR. Thus RT-PCR results showed 100% correlation with virus isolation in embryonated chicken eggs.

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