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ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Pathogenicity for Chickens of Avian Influenza Virus Strain H9N1 Isolated from Water Coot in India

B.P. Shankar[1], R.N.S. Gowda[1], B.H. Manjunath Prabhu[1], B. Pattnaik[2],
S. Nagarajan[2], S.S. Patil[2] and H.K. Pradhan[2]
[1]Department of Pathology, Veterinary College, Hebbal, Bangalore - 24, Karnataka, India
[2]High Security Animal Disease Laboratory, IVRI, Bhopal, India

Abstract: Avian Influenza (AI) is caused by Type A Influenza virus belonging to the family *orthomyxoviridae*, which is classified into 16 HA and 9 NA subtypes based on two surface glycoprotein's Haemagglutinin (HA) and Neuraminidase (NA). Influenza A viruses are divided into 2 distinct pathotypes on the basis of their virulence, highly pathogenic and low pathogenic. Highly pathogenic AI viruses are restricted to H5 and H7 subtypes and these are capable of causing severe respiratory disease and high mortality in infected chickens and can be transmitted directly to humans. In the present study one H9N1 (A/Wc/India/5844/05) Avian Influenza virus was isolated from Water Coot sample. Virus isolate showed HI titer of 1:128 with H9 subtype specific serum. RT-PCR, using HA gene specific primers yielded specific amplicons of 488bp. Intravenous Pathogenicity Index (IVPI) test was conducted by inoculating 0.2 mL of 4HA unit of 1:10 diluted virus to 3 week old chicks and observed for 10 days. Two birds were showed mild respiratory distress on 3rd and 5th day after inoculation, recovered on 7th day. All birds were sacrificed after ten days. The H9N1 virus showed an IVP index of 0.05/3.0, it indicates the present H9N1 virus isolated in India is of low pathogenic. Grossly 2 birds were showed thigh muscle hemorrhages with mild congestion of spleen, liver and lung. Microscopically hyperactive mucus glands, ballooning, infiltration of lymphocytes with deciliation in trachea, congestion with swollen neurons in brain, secondary lymphoid follicles in spleen, congestion, hemorrhages with heavy infiltration of lymphocytes in lung, necrosis of pancreatic gland, fibrous replacement and secondary lymphoid follicles were noticed in pancreas.

Key words: Avian influenza, H9N1, IVPI, water coot

INTRODUCTION

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 in birds. Type A Influenza viruses are further divided into subtypes based on antigenic relationships of the Hemagglutinin (HA) and Neuraminidase (NA) surface glycoproteins. To date, 16 unique HA subtypes (H1-H16) and nine unique NA subtypes (N1-N9) have been recognized. Viruses of all HA and NA subtypes with most possible combinations have been isolated from feral aquatic birds and from a wide range of domestic avian species, such as chickens, turkeys, pheasants, geese and ducks. The natural reservoirs of Influenza A viruses are the aquatic wild birds, in which the viruses appear to be in evolutionary stasis (Webster *et al*., 1992).

Based on the Intravenous Pathogenicity Index (IVPI), the viruses are sub-classified in to two pathotypes of Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI) viruses. Influenza virus has been isolated from variety of animals, including humans, pigs, horses, sea mammals and birds (Webster *et al*., 1992). Historical evidence suggests that Southeast Asia is an epicenter of influenza pandemic. H5N1 is of particular concern because of the propensity to infect humans. Its ability to cause severe disease in humans has now been documented on three occasions. In 1997, 18 people became infected which included 6 deaths with an H5N1 virus during an outbreak of HPAI in Hong Kong. In February 2003, 2 new human cases of H5N1 were documented in Hong Kong. In December 2003, an outbreak of H5N1 initiated in poultry in South Korea and in January 2004, a quick spread was seen in Japan, Taiwan, Viet Nam, Thailand and China. In Viet Nam five cases were documented in humans due to H5N1 virus infection. In addition, H5N1 viruses have a propensity to reassort with influenza viruses circulating in other animal species.

The massive HPAI epidemic of H5N1 virus in Asia caused severe effect on poultry population, more than 100 million birds have either died or been killed to contain the outbreak. Therefore, it is important to have regular AIV surveillance to formulate strategies to control the disease in domestic poultry and prevent human illness and death.

MATERIALS AND METHODS

AIV isolates of H9N1 subtype isolated from migratory bird in India (A/Wc/India/5844/05) was used in this study. This virus was isolated in SPF or AIV antibody negative embryonated chicken eggs (ECE) from the Water Coot tissue samples submitted to High Security Animal Disease laboratory for AIV diagnosis and used in the form of chorio-allantaic fluid (CAF). AIV subtype of the isolate was serologically determined by AGID, HA, HI and NI assays following standard procedure (4. Gamma irradiated whole virus antigen for the HA subtypes 1-16 and NA subtype 1-9 and reference positive serum for all the 16 HA subtypes and 9NA subtypes obtained from NVSL, USA were used in type and subtype identification of the isolate. In addition the isolate was also subjected to RT-PCR using partial HA gene specific primers (Lee *et al*., 2001). Specific 488bp amplicons was obtained with only HA-H9 specific primer pair.

Eleven-day-old embryonated chicken eggs originating from SPF or AIV antibody negative flock were commercially procured. Three to four weeks old white leghorn chickens were obtained from Madhya Pradesh State Government Poultry Farm, Bhopal, were used in this study. The birds were housed in negative pressure (-10 mm water column) stainless steel isolators in a high containment facility (Biosafety Level 4) at HSADL, Bhopal. Feed and water were provided *ad libitum*.

One hundred milligram of tissue was ground in 1 mL 1x PBS in a sterile mortar and pestle, making a 10% suspension. The suspension was transferred to an eppendorf tube and centrifuged at 400 xg 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 filters.

Examine the 9-11 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. Three eggs per specimen are usually inoculated. Aspirate 1 mL of processed clinical specimen into a tuberculin syringe and inoculate 100 µL into the amniotic cavity. Withdraw the needle about ½ inches and inoculate 100 µL of the inoculums into the allantoic cavity, remove the needle. Inoculate the two other eggs in the same manner with the same syringe and needle, finally seal all eggs. Incubate the eggs at 37°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. The following day chorio-allantaic fluid was collected by aspiration and stored at -70°C.

Intravenous Pathogenicity Index (IVPI) test was carried out in stainless steel isolators. The test procedure as recommended by the OIE (2R was followed. The Influenza H9N1 isolate (A/Wc/India/5844/05) isolated from a migratory bird sample from Hariyana during the period of study, were subjected to Intravenous Pathogenicity Index (IVPI) test. AIV isolate was inoculated I/V (16 HA unit) into a group of 8 birds, 8 birds were included as control. Observed for any clinical signs up to 10 days and recoded. On 10th Day Post Inoculation (DPI) blood samples were collected from all chickens and all birds were sacrificed on day 10 and organs were collected separately for virus reisolation and for histopathology in 10% formalin. IVPI was calculated as per the OIE guidelines.

For histopathology tissue samples were processed by routine paraffin embedding and 5-6 microns thick sections where stained with Hematoxylin and Eosin (H and E) for light microscopic examination.

Reisolation of the virus from tissues was accomplished in ECE as mentioned above. Presence of virus in CAF was confirmed by HA, HI and NI test.

RESULTS AND DISCUSSION

The virus isolate examined in the present investigation showed HI titer of 1:128, only with H9 subtype specific serum indicating that they belong to H9 subtype. Reference H9 antigen had an HI titer of 1:64. RT-PCR, using partial HA gene specific primers yielded specific 488bp amplicons which confirm that the isolate belonging to H9 subtype of AIV. In neuraminidase inhibition assay, the isolates were found to be of N1 subtypes. In IVP testing group of 8 birds were inoculated with the H9N1 virus (A/Wc/India/5844/05). One bird showed mild respiratory distress on 2nd day and another one on 5th day. One bird showed mild respiratory distress on 7th day of post inoculation and recovered next day and remained birds were normal throughout the period of the study. All the birds were observed for a period of 10 days and all the birds including control birds were sacrificed and observed for gross lesions, if any. Mild congestion and slight enlargement of liver and spleen were observed in four birds. Only one bird showed mild hemorrhages in thigh muscle, with mild congestion in the liver, spleen and lungs. Other inoculated and uninoculated control bird's dint showed any clinical signs and gross lesions. Serum samples were collected from these birds were showed positive for H9N1 by AGID and HI.

On 10th day post infection all birds showed characteristic microscopic lesions in all organs except in intestine. In brain congestion with swollen neuronal cells were noticed. Hyperactive mucus glands, ballooning, mild to heavy infiltration of lymphocytes and deciliation in trachea were noticed (Fig. 1). In proventriculus there were mild to moderate infiltration of lymphocytes below the epithelium resulting thickening, the junction between the proventriculus and gizzard showed lymphocytic infiltrations (Fig. 2). Spleen showed secondary lymphoid follicles (Fig. 4). Lungs showed

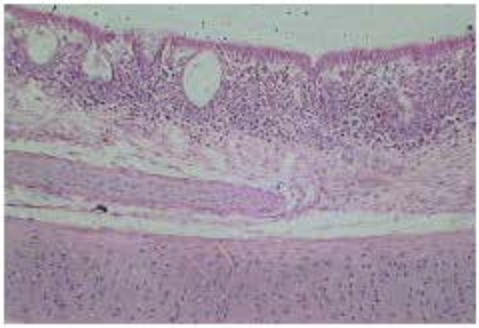

Fig. 1: Trachea of chicken infected with AIV (H9N1) showing ballooning of the mucus glands and lymphocytic infiltration resulting in thickening of the mucosa (H & E; 200X)

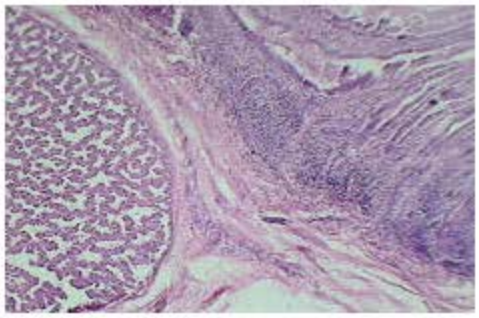

Fig. 2: Proventriculus of chicken infected with AIV (H9N1) showing lymphocytic infiltration in the mucosa (H & E; 200X)

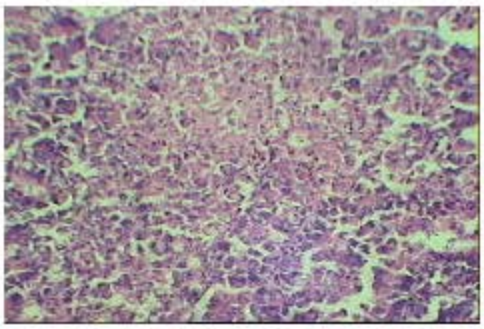

Fig. 3: Pancreas of chicken infected with AIV (H9N1) showing necrosis of the acinar cells resulting in decreased of glandular area (H & E; 200X)

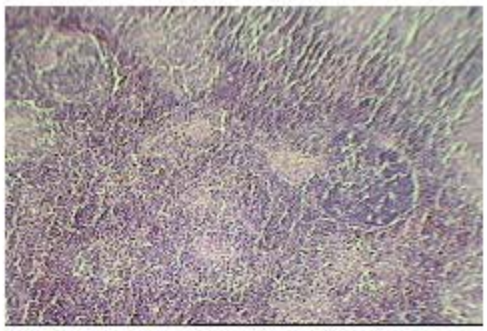

Fig. 4: Spleen of chicken infected with AIV (H9N1) showing secondary lymphoid follicles (H & E; 200X)

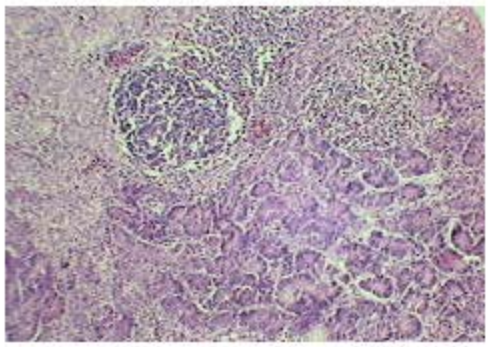

Fig. 5: Pancreas of chicken infected with AIV (H9N1) showing necrosis of the acini and secondary lymphoid follicles (H & E; 200X)

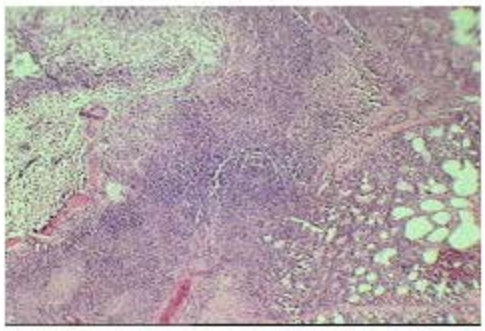

Fig. 6: Lung of chicken infected with AIV (H9N1) showing marked infiltration of lymphocytes in the perilobular areas (H & E; 200X)

congestion of blood vessels and hemorrhages and mild to heavy infiltration of lymphocytes in perilobular spaces (Fig. 6). Bronchiolar glands were hyperactive in all the birds. Heart showed pericarditis with focal infiltration of lymphocytes and in some places plasma cells were also noticed (Fig. 8) but it was not consistent. In some birds pericardial blood vessels were congested. In liver focal infiltration of lymphocytes were noticed (Fig. 7). The lesions in the pancreas were same in all the birds but in some birds necrosis of the pancreatic gland, fibrous tissue replacement and formations of lymphoid follicles were recorded (Fig. 3, 5). Lesions were not observed in control birds.

For virus reisolation only one passage was gives on ECE and presence of the virus in CAF was confirmed by HA, HI and NI assay. No virus could reisolated from control birds. A detail of virus reisolated from different organs of H9N1 infected birds is mentioned in Table 1. IVP index was calculated according to the protocol of OIE (2004) and mentioned in Table 2.

Table 1: Details of virus reisolated from different organs of experimental H9N1 infected birds

	Brain	Trachea	Lung	Heart	Proventiculus	Liver	Spleen	Kidney	Intestine	Pancreas	Cloaca
5844/05 H9N1 virus isolate											
HA Titer	1:64	1:128	1:256	1:128	1:64	1:128	1:128	1:256	Nil	1:128	1:128
HI Titer	1:64	1:64	1:256	1:256	1:128	1:128	1:128	1:128	Nil	1:128	1:256
Uninoculated control											
HA Titer	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
HI Titer	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Table 2: Intravenous pathogenicity index test for H9N1 AIV isolates (A/Wc/India/5844/05)

Clinical signs	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}	Total	Score
H9N1 Virus Isolate (A/Wc/India/5844/05)												
Normal	8	7	8	8	7	8	7	7	8	8	76×0	=0
Sick	0	1	0	0	1	0	1	1	0	0	4×1	=4
Paralyced	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												=4
Index												0.05
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
Paralyced	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												0.000

Pathogenicity for Chickens of Avian Influenza Virus strain H9N1 Isolated from Water Coot in India

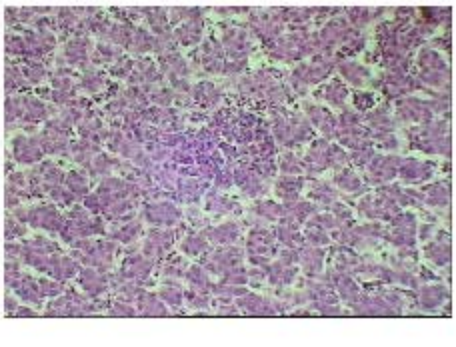

Fig. 7: Liver of chicken infected with AIV (H9N1) showing normal architecture of the parenchyma with focal infiltration of lymphocytes (H & E; 200X)

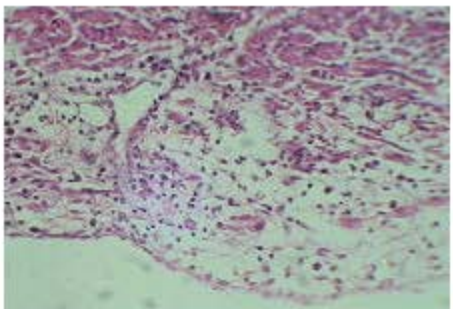

Fig. 8: Pericardium of chicken infected with AIV (H9N1) showing infiltration of lymphocytes and plasma cells (H & E; 200X)

The present study was carried in order to isolate and evaluate the pathogenicity of Indian H9N1 AIV isolates by IVP test in chickens. The H9N1 virus isolate not kill any birds up to 10 days observation period and elicited HA H9 specific immune response in experimentally infected chickens with out any mortality (IVPI-0.05/3.0) and mild clinical signs indicating of AI and it showed histopathological lesions in most of the organs. These observations indicated that the H9N1 AIV isolated from water coot in India is of non-pathogenic type. According to OIE Guidelines any Influenza virus showing more than 1.2/3.0 IVPI is belongs to highly pathogenic avian influenza. Low virulent AIV isolate failed to cause mortality in experimental infected chickens (Lee *et al*., 2005).

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