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HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES FOR THE PATHOGENESIS OF A LOW PATHOGENICITY H9 AVIAN INFLUENZA VIRUS IN EXPERIMENTALLY INFECTED COMMERCIAL BROILERS

R. Aslam, A. Aslam, Y. Tipu, J. Nazir[*], A. Ghafoor[**] and S. Fatima[***]

Department of Pathology, [*]Department of Microbiology, [**]University Diagnostic Lab, [***]Quality Operations Lab, University of Veterinary and Animal Sciences, Lahore, Pakistan
Corresponding author email: jawad.nazir@uvas.edu.pk

ABSTRACT

Present study has been designed to check the pathological effects of a low pathogenicity H9 avian influenza virus on broilers following experimental infection through intranasal, ocular and oral route at 21 days of age. Moderate clinical signs were observed which started appearing at two days post infection (PI), became severe on five days while started subsiding from seven days onwards. Severity of the signs and lesions was highest in respiratory organs following intranasal route of infection. No mortality was observed except one bird each in the groups infected through intranasal and oral route of infection. Histopathology of the organs in the birds infected through intranasal route revealed severe hyperemic trachea and congested lungs. While moderate congestion of kidneys and mild necrosis in the liver tissue was seen in all treatment groups. Detection of virus antigen through immunohistochemistry was possible in trachea, lung, and kidney of few birds on two days PI while in all birds at five days PI. In some of the birds inoculated through either of the route, the virus was detected in liver and duodenum tissue at five days PI.

Key words: H9, AIV, IHC staining, Histopathology, Postmortem.

INTRODUCTION

Poultry industry is one of the energetic segments of agriculture sector in Pakistan which showed enormous growth in last decade and still has further potential of expansion. Infectious diseases are a major threat for the growth of the industry. Avian influenza (AI) devastated the industry several times in past throughout the world (Capua and Alexander, 2004). AI viruses (AIV) can be categorized into 144 subtypes on the basis of hemagglutinin and neuraminidase surface glycoprotein (Fouchier *et al*., 2005). All of the isolates of the same subtype do not have equal pathogenic potential. Some are of low pathogenicity (LP) while others are high pathogenicity (HP) isolates (Capua and Alexander, 2004). HP isolates belong to either H5 or H7 subtype (OIE, 2005) while all of H9 isolates are reported to be of LP. In Pakistan a number of AIV subtypes like H5N1, H7N3, H9N2 have been reported in past (Naeem *et al.,* 1999: Bano *et al*., 2003: Naeem *et al.*, 2007: Khan *et al.,* 2010). H9 AIV was first time isolated in 1998 from breeder flock in Mansehra (Naeem *et al*., 1999). Since then the viruses are circulating in domestic and rural poultry of the Pakistan. Despite extensive vaccination in the commercial poultry, complete eradication of the disease was not possible (Capua and Marangon, 2007). Although, H9 AIV do not cause high mortality in the infected birds but can also do so in immune-compromised population. LP AIV mostly affect the respiratory and gastrointestinal tract but less commonly can also affect liver, spleen and brain (Nili and Asasi, 2002, 2003). High pathogenicity AIV affects multiple organs in the body. Limited work has been done on the pathobiology of local AIV isolates in Pakistan. A recent study on the pathogenesis of a local LP H9 isolate showed the presence of virus in trachea, lungs and kidneys following five days of the experimental infection and the virus showed tissue tropism with respiratory and urinary system (Subtain *et al.,* 2011). That study and most of previous other studies used intranasal route to infect the birds but however, route of infection plays an important route in the disease pathology and virus excretion. The present study has been designed to assess the effect of various routes (intranasal, ocular, and oral) of infection on the gross and microscopic lesions as well as virus localization in various tissues (trachea, lungs, kidney, liver, proventriculus, duodenum, heart) of the broilers.

MATERIALS AND METHODS

Virus Propagation: A locally isolated and characterized H9 AIV was procured from quality operations lab (QOL), University of Veterinary and Animal Sciences (UVAS), Lahore. The virus was propagated in allantoic cavity of 9 days old embryonated chicken eggs by the standard protocol (Dennis, 2008) under sterile conditions. Following inoculation, the eggs were candled after 24 and 48 hours to check viability of embryos. All of the embryos were transferred to refrigerator for an overnight chilling so as to facilitate the harvesting of the allantoic-

amniotic fluid (AAF). Virus propagation was checked by spot haemagglutination test of the AAF. Confirmation of the virus was done by hemagglutination inhibition (HI) assay using mono-specific serum against H9 AIV (Stephan and Charles, 2008). Virus quantitation was performed by calculating embryo infectious $dose_{50}$ (EID_{50}) through a previously described method (Dennis, 2008). Virus stocks were stored in sterile 15 ml plastic tubes at -70°C till further use.

Experimental procedure: A total of 40 day old chicks were reared in the experimental poultry shed of the UVAS, Lahore. The chicks were reared as per standard managemental conditions and vaccinated against Newcastle disease at 4 and 14 days of age and infectious bursal disease at 10 days of age via drinking water while none of birds were vaccinated with any of the AIV vaccine. At the age of 21 days all of the birds were divided into four groups (n = 10 per group) named as A, B, C, and D. The virus stock was diluted in sterile phosphate buffered saline (PBS) to have a final concentration of $10^{6.3}$ $TCID_{50}$/ml to be used for the infection purposes. Serum samples were collected from five randomly selected birds prior to virus inoculation to ensure that the birds were serologically negative for H9 as determined by HI assay. An amount of 0.10 ml of the virus was administered through oral, ocular, and nasal route to the birds of the groups A, B, and C, respectively. Birds of the group D were kept un-inoculated as negative control and reared in a separate room. From each group at 2, 5, and 10 days post-infection (PI) 3, 3, and 4 sick or dead birds, respectively were processed to collect various organs (trachea, lungs, intestine, proventriculous, heart, kidney and esophagus). The organs were preserved in 10 % neutral buffered formalin for histopathological and immunohistochemical studies.

Histopathology and Immunohistochemistry: Unstained paraffin-embedded 4 µm sections from each of the organ were prepared and stained with eosin and haematoxylin. These processed tissue sections were studied under light microscope for pathological findings. Tissue sections of 4 µm were immunohistochemically stained by using SuperPicture™ 3rd Gen IHC detection kit (Invitrogen, NY, USA) according to the manufacturer instructions. Briefly, paraffin sections were de-paraffinized with xylene and rehydrated in a graded series of ethanol. Antigen retrieval was done by treatment of tissue sections with quenching solution for 5 minutes followed by rinsing with PBS. Primary antibodies were applied and incubated for 60 min. Afterwards, 2 drops of horse radish peroxidase (HRP) polymer conjugate was applied and incubated for 10 minutes. For staining 3,3'-Diaminobenzidine (DAB) chromogen solution (1 drop of DAB chromogen mixed with 1 ml of the DAB substrate) was poured onto the tissue sections. At the last step, the tissue sections were counterstained with hematoxylin dye.

Statistical Analysis: The data thus collected was statistically analyzed by chi squared test using SPSS software for windows, Rel.13.00 (IBM Corporation, Chicago, USA).

RESULTS

Clinical signs and gross lesions: It was observed that at one day PI none of the bird was sick or died among all of the groups. While at 2 day PI, some of the birds in groups A, B, and C were lethargic and showed respiratory signs (respiratory sounds, nasal discharge, facial swelling) with diarrhea. During whole of the experimental period after receiving infection, one bird was found dead in group A and C while no mortality was observed in group B and D. During postmortem examination, grossly hyperemic trachea, congested lungs, and swollen kidneys were observed (Figure-1 and 2). Five randomly selected birds from control group were also slaughtered at 5 and 10 days PI but no gross lesions were seen on postmortem examination. It was observed that during experimental period within the virus inoculated groups about 30 % (9 out of 30) of the birds became clinically sick. Comparative distribution of lesion in various organs at 2, 5, and 10 days PI is given in the Figure 3.

Histopathological findings: Histopathological examination revealed inflamed trachea characterized by sloughing off of the epithelium (Figure 1-B). Severe infiltration of inflammatory cells, severe hemorrhages in the bronchioles and engorgement of the interstitial capillaries were seen in the lungs tissue (Figure 2-E and 2-F). Severe congestion of glomeruli of kidney was also observed (Figure 1-C). Necrotic foci were seen in the sections of liver. Generally, more severe histopathological changes were observed in respiratory organs and kidney. Other organs (proventriculous, small and large intestine) have shown mild to moderate microscopic lesions except heart where no microscopic changes were observed. Detailed description of the histopathological findings is given in the Table-1. It was evident that 47 % of the lungs and 43 % of the kidney tissue sections were positive for pathological changes while all of the birds of the control group did not shown any of such lesions.

Immunohistochemical staining: Localization of the virus particles in various body organs as revealed by immunohistochemical staining is shown in Table-2. In lungs, kidneys, and tracheal tissues approximately 77, 67, and 63 %, of the samples were positive by the immune reaction. None of the heart sample was positive for immunological staining. Some of the samples obtained from clinically healthy birds with no apparent

microscopic lesions were found positive by immune reaction. No positive immune reaction was observed in the control group slides. Distribution of virus antigen in various organs at 2, 5, and 10 days PI is given in Figure-4. Table-3 shows that immunohistochemistry is more sensitive technique for the confirmatory diagnosis of H9 AIV in comparison to histopathology.

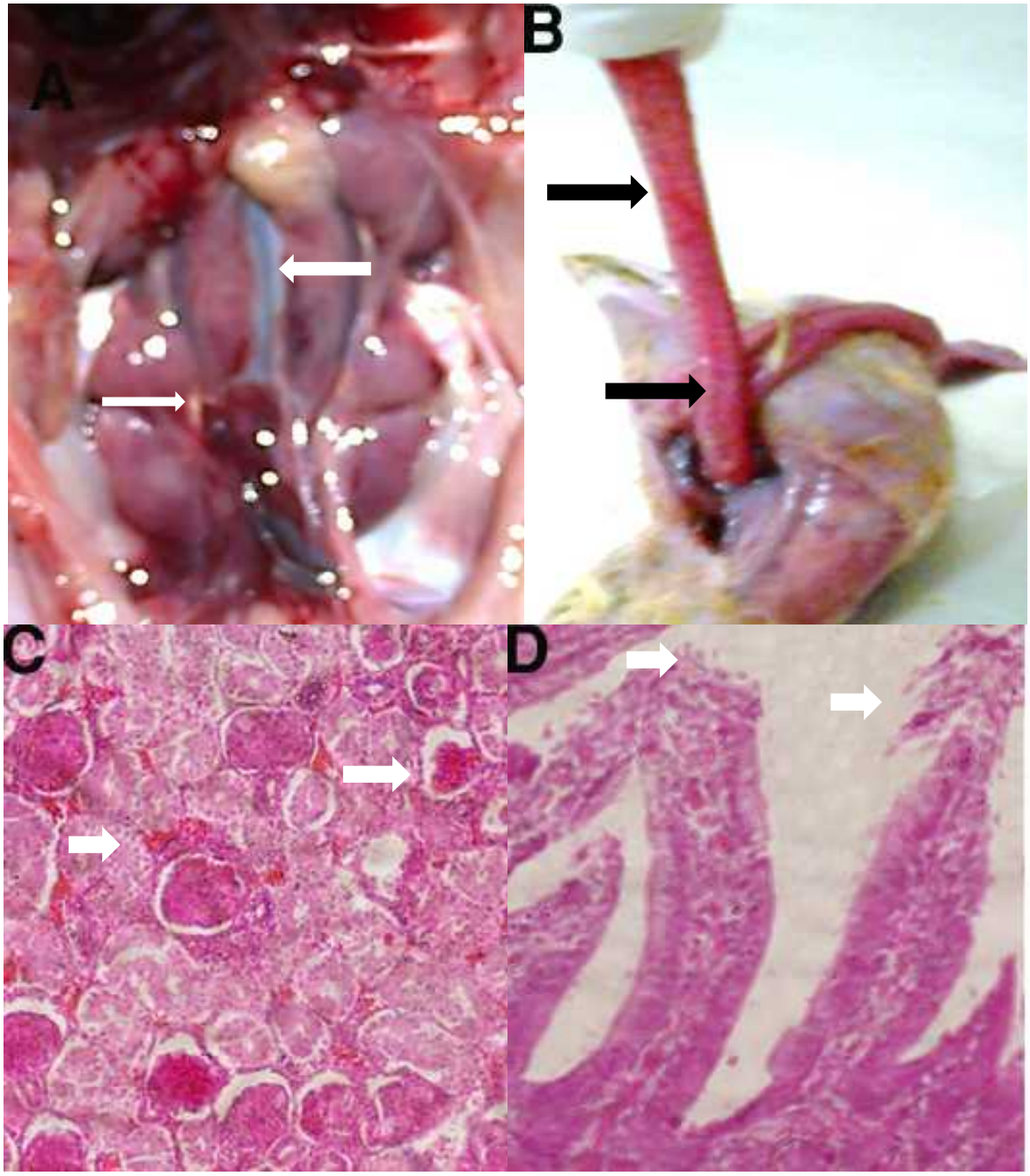

Figure 1: (A): Kidney of 23 days old chicken inoculated with H9 AIV showing severe congestion and swelling. (B): Trachea of 26 days old chicken inoculated with H9 AIV showing Hyperemia. (C): Kidney of 26 days old chicken inoculated intranasally with H9 AIV showing congestion in glomerulus and in interstitial spaces (H&E×40). (D): H&E×40 Histology Section from duodenum showing degeneration and sloughing of villi in broiler birds infected with H9 avian influenza virus.

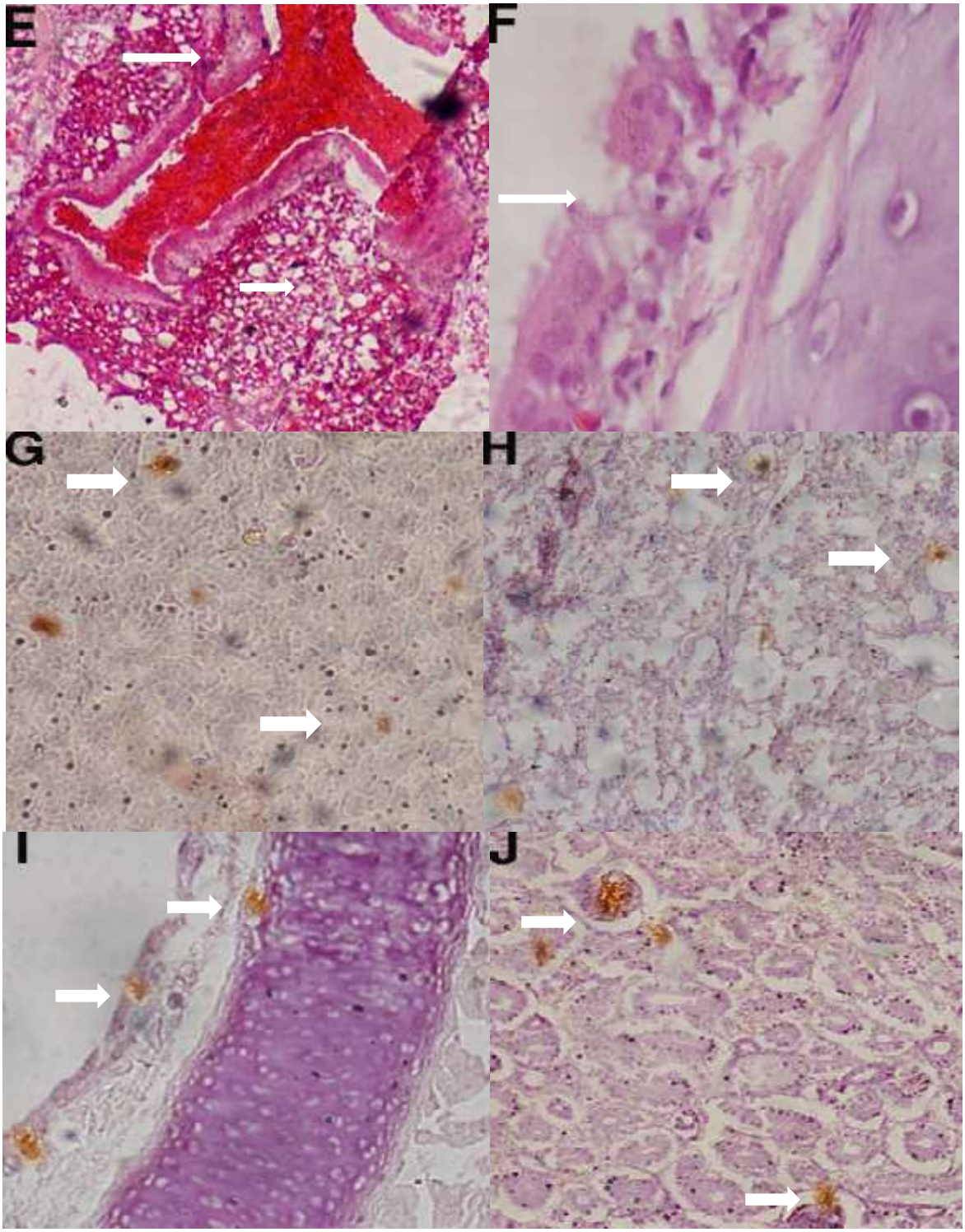

Figure 2: (E): H& E ×40 photomicrograph of lung section showing severe hemorrhages in the bronchioles and engorgement of the interstitial capillaries of a bird infected with H9 avian influenza virus on day 2nd post-infection. (F): Trachea of 26 days old chicken inoculated intranasally with H9 AIV showing sloughing of the epithelium. (G): Liver of 26 days old chicken inoculated with H9 AIV showing the immunolabelling of antigen within hepatocytes and intralobularseptum. (H): Lung section of a bird at 2nd day p.i. showing H9 antigen in the bronchioles and alveolar sacs. (I) Immunohistochemical detection of H9 antigen in broiler bird after experimental infection. Arrow showing the immunolabelling of antigen within the cartilage of trachea and epithelium. (J): Section of kidney of 26 days old chicken inoculated with H9 AIV showing the immunohistochemical staining in glomerulus and convoluted tubules.

Table-1: Histopathological findings in the organs of H9 avian influenza virus infected birds following oral, ocular and nasal routes of the virus infection.

DPI	Groups	No.of birds	Trachea	lungs	Liver	Kidney	Heart	Proventri-culous	Intestine
	A	3_{eu}	1	1	0	1	0	1	2
2	B	3_{eu}	1	1	0	0	0	1	1
	C	3_{eu}	1	2	0	2	0	1	1
	A	$1d=2_{eu}$	2	2	1	2	0	1	2
5	B	3_{eu}	1	2	1	2	0	1	2
	C	$1_{d}+3_{eu}$	2	2	0	2	0	2	2
	A	4_{eu}	1	1	0	1	0	0	0
5	B	4_{eu}	0	1	0	1	0	0	1
	C	4_{eu}	1	2	0	2	0	1	1
Total	A,B,C	30	10/30	14/30	2/30	13/30	0/30	8/30	12/30
%age		100%	33%	47%	7%	43%	0%	27%	40%

No. of positive samples/total samples eu= euthanized d=dead birds

Table-2.Virus localization through immunohistochemical staining of various tissue samples obtained from broilers inoculated with H9 avian influenza virus through oral, ocular and nasal routes.

DPI	Groups	No. of birds	Trachea	lungs	Liver	Kidney	Heart	Proventri-culous	Intestine
	A	3_{eu}	2	3	1	2	0	2	2
2	B	3_{eu}	0	2	1	3	0	1	1
	C	3_{eu}	3	3	1	2	0	2	2
	A	$1d=2_{eu}$	2	3	2	2	0	1	2
5	B	3_{eu}	2	2	1	2	0	1	3
	C	$1_{d}+3_{eu}$	3	3	2	3	0	2	2
	A	4_{eu}	2	2	1	3	0	1	2
5	B	4_{eu}	2	2	2	1	0	0	2
	C	4_{eu}	3	2	2	2	0	1	1
Total	A,B,C	30	19/30	22/30	13/30	20/30	0/30	11/30	17/30
% age		100%	63%	77%	43%	67%	0%	36%	57%

No. of positive samples/total samples eu= euthanized d=dead birds

Table-3: Comparison of histopathological and immunohistochemical techniques for the confirmatory diagnosis of H9 avian influenza virus infection in various organs.

Organs	Histopathology (%)	IHC (%)
Trachea	33	63
Lungs	47	77
Liver	6	43
Kidney	43	67
Heart	0	0
Proventriculous	27	36
Intestine	40	57

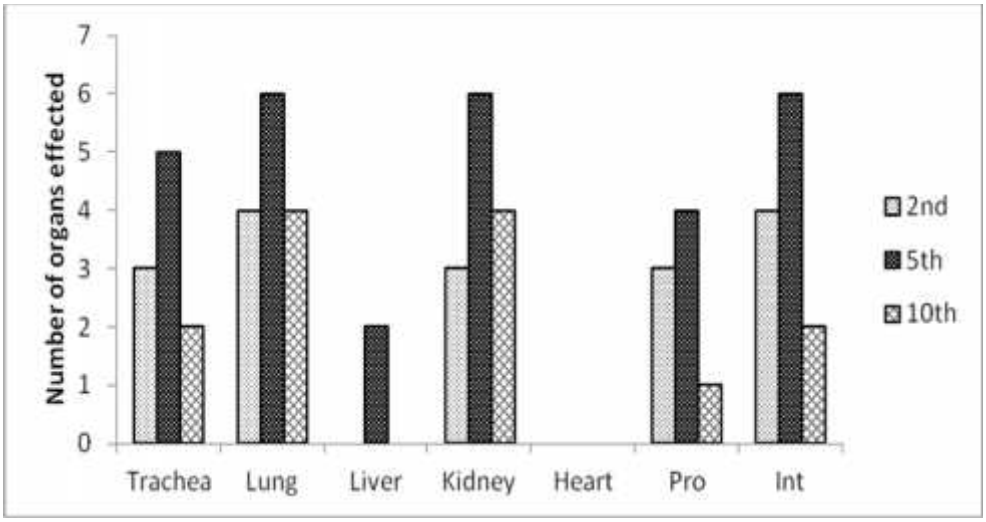

Figure-3: Distribution of pathological effects in various organs of broilers following challenge with H9 avian influenza virus at different days post infection

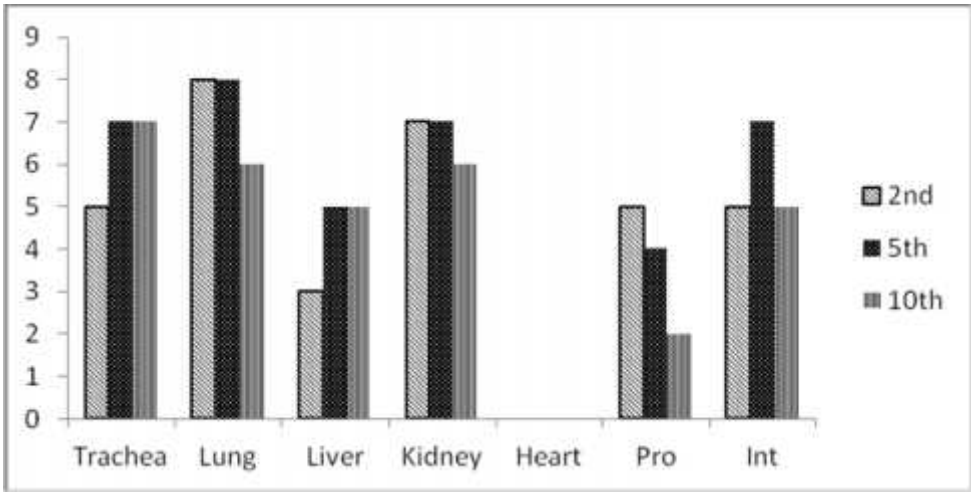

Figure-4: Distribution of virus in various organs of broilers as detected by immunohistochemistry following challenge with H9 avian influenza virus at different days post infection

DISCUSSION

AIV subtype H9 is a LP virus that usually causes low mortality in the broiler birds. In present study, an overall 6 % mortality was observed following experimental infection that corroborates with the findings of Nili and Asasi (2002) who detected 5 % mortality in broilers after infecting them with H9N2 AIV. Several previous studies have variable findings regarding the mortality pattern that ranges from no mortality (Shalaby *et al*., 1994; Banks *et al*., 2000) to up-to 65 % mortality in the field cases (Vasfi and Bozorgmehrifard, 1999). Although virus pathogenicity is a major factor in the disease severity but multiple factors like age, sex, environment, species, existing bacterial infections, and route of infection may contribute to the outcome of the AIV infection (Taubenberger and Morens, 2008). The birds infected through intranasal route developed clinical signs earlier than the ones inoculated through intranasal or oral route but generally no significant difference exist between the involvements of various organs in the birds infected through various routes (Table-1). Also the respiratory organs showed more prominent gross lesions in comparison to other organs. Similar findings were observed by Taubenberger and Morens (2008) and Capua and Alexander (2009) where more cell injuries in the tissues of respiratory system and GIT were recorded after LP AIV infection in birds. More severe signs in the lungs and trachea could be attributed to the presence of trypsin like enzyme in respiratory mucosa which cleaves the HA protein into HA0 and HA1 subunits of LP AIV while HA0 proteins of HP AIV can be cleaved by furin like protease which are present in all body tissue and hence explains the presence of the virus in multiple organs

(Swayne, 2006). Severity of signs was highest at five days PI which is in line with the findings of Kwon *et al.* (2008) who observed that following infection with a LP AI H9N2 virus, the disease was at its peak on 6 days PI. In present study, diarrhea and dullness of the birds was observed after 2 days of infection, reached to its maximum at 5 and started decline at 7 days which is in line with the findings of Vasfi *et al.* (2000).

Immunohistochemical findings revealed the presence of virus in respiratory and GIT tract but no virus was detected in the heart tissue (Table-2). However viral antigens were detectable in the kidney which confirms the findings of Mosleh *et al.* (2009) who stated that an H9N2 AIV was nephropathic in nature. Another report showed that in low severity, LP AIV subtype infect respiratory system and kidney while in high severity it infect cardiac system (Swayn *et al.*, 1997). Presence of low severity as revealed by low mortality in our study might be a reason for the absence of virus in the cardiac tissue. A number of studies are available that shows the detection of various LP AIV isolates in trachea, lung, kidney, and spleen etc of the commercial broiler and layer chicks by IHC staining or immunoperoxidase assay (Slemons *et al.*, 1990; Swayne *et al.*, 1992; Shinya *et al*., 1995; Hablolvarid *et al*., 2003).

A comparison of histopathology and IHC staining showed that viral antigens could be detectable as early as 2 days PI while tissue changes were evident only at 5 days PI. Histopathology is helpful to detect the damage to various organs and tissue at cellular level but cannot pin point the etiology of the damage. While IHC staining has more diagnostic importance and can locate the virus antigen in the tissue specimen (Hablolvarid *et al*., 2003). It can be concluded from the results of present study that in case of H9 LP AIV, all of the natural routes of infection may lead to clinical disease but the severity is relatively higher after intranasal inoculation and the viruses primarily affects respiratory and GI system.

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