

Study on Pathogenesis of Low Pathogenic Avian Influenza Virus H9 in Broiler Chickens

S. M. Subtain,* Zafar Iqbal Chaudhry, Aftab Ahmad Anjum, Azhar Maqbool and Umer Sadique
University of Veterinary and Animal Sciences, Lahore

Abstract.- Avian influenza viruses (AIV) are placed in the family of Orthomyxoviridae having three influenza genera known as A, B and C. The viruses which are responsible for infections in birds, belongs to genus *influenza virus A* which can produce two different types of diseases on bases of their virulence and pathogenicity viz., highly pathogenic avian influenza virus (HPAIV) and a low pathogenic avian influenza virus (LPAIV). Frequent outbreaks of LPAIV H9N2 in Asian countries such as in Pakistan and China revealed that this subtype has become endemic in poultry industry. In this study pathogenesis of virus H9 has been studied in broiler chickens under field for which, challenge strains (H9 virus was isolated from field samples and experimental inocula were prepared for study of pathogenesis of H9 virus out under controlled environmental conditions. The virus prevailing in Pakistan was found non pathogenic with Intravenous Pathogenicity Index (IVPI) 0.12/3, produced significant reduction in body weight and had tissue tropism for kidney and respiratory system. Immunohistochemical detection of viral antigen and rapid molecular detection of virus from tissues by using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) revealed that RT-PCR is efficient method than immunohistochemistry. In conclusion, the virus subtype H9 circulating in Pakistan is low pathogenic in nature with affinity for respiratory system and urinary system in broiler chickens.

Keywords: H9N2 virus, pathogenicity, avian influenza virus, low pathogenic avian influenza virus.

INTRODUCTION

Poultry industry in Pakistan has emerged as an important sub sector of agriculture as it has taken a quantum shoot from backyard poultry to decidedly specialized industry during the near past (Mushtaq, 1994). This industry is now the second biggest industry after textile and is playing imperative role in economy of the country (Economic survey of Pakistan 2008-2009). On the other hand, due to intensive farming, semi-vertical integration system and lack of adaptation of biosecurity measures, this country has become an ideal place for infectious diseases like avian influenza. Avian influenza (AI) is an historical viral infection in poultry industry causing huge economic losses through out the world. The infection is widely distributed in commercial poultry and migratory wild/shore birds while aquatic birds especially ducks, act as natural host for virus (Stallknecht and Shane, 1988). The virus can be transmitted from these natural hosts to highly prone species such as chickens and turkeys (Davidson *et al.*, 1999). In natural reservoirs, the

virus replicates in intestinal tract and is considered as non pathogenic as no evident clinical signs of disease are manifested (Selmons and Easterday, 1977). The virus belongs to Orthomyxoviridae family (Calnek *et al.*, 1997) which is classified into three genera named as types A, B and C on the bases of antigenic difference of the NP and M1 protein (Wright and Webster, 2001). Among these genera, only influenza A viruses are further classified into different subtypes. This classification is based on antigenicity of two transmembrane glycoproteins on the surface of virus which were named haemagglutinin (H / HA) and neuraminidase (N on NA) (Hinshaw *et al.*, 1981). Thus viruses are separated into sixteen haemagglutinin (H1 to H16) and nine neuraminidase (N1 to N9) antigenic subtypes (Fouchier *et al.*, 2005).

AIV are known to produce two different types of diseases in poultry birds on the bases of their virulence and pathogenicity. Due to this unique characteristic, the viruses are further classified into two types known as a highly pathogenic avian influenza virus (HPAIV) and a low pathogenic avian influenza virus (LPAIV) (Capua and Alexander, 2004). HPAIV are responsible for rapid and fatal systemic infection inducing mortality upto 100% in broilers, layers and breeders while LPAIV produce asymptomatic infection. All HPAI viruses

* Corresponding author: Office of the District Livestock officer Rahim Yar Khan, Punjab. smsubtain@yahoo.com
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belong to H5 and H7 subtypes, even though not all H5 or H7 viruses are truly pathogenic but most of them are non pathogenic also (OIE, 2005). Pathogenicity of HPAI viruses is associated with polybasic amino acids (arginine and lysine) at their HA cleavage site motif (Wood *et al.*, 1993) which enable them to replicate and damage all the vital organs and tissues ultimately resulting in death of the infected birds (Rott, 1992). In contrast, LPAI viruses contain monobasic amino acid (arginine) at cleavage site and are capable to replicate only in limited tissues of respiratory and digestive systems. That is why these subtypes produce unapparent disease and are considered non pathogenic (Mo *et al.*, 1997; Alexander, 2000).

Pathogenicity of influenza viruses also depends upon the role of surface HA antigen (Klenk and Garten, 1994; Fouchier *et al.*, 2005) as its enzymatic cleavage into HA1 and HA2 is compulsory for efficient viral entry and replication into host cells (Byrum and Slemons, 1995). Here HPAI viruses differ from LPAI viruses in cellular proteases as ubiquitous furin and PC 6 (proprotein convertases) are essential for cleavage of HA of HPAI viruses while HA of LPAIV is cleaved only by trypsin-like proteases secreted from cells in respiratory and intestinal tracts (Klenk and Garten, 1994; Swayne and Suarez, 2000).

In recent years, H9N2 virus has attained a great importance as its infection has reached in panzootic proportions (Cameron *et al.*, 2000) In Pakistan, first outbreak was reported in 1994-95 (Muneer *et al.*, 1995) while in 1998, an outbreak of disease in breeder flock was diagnosed as avian influenza and subtype was confirmed as H9N2 virus (Naeem *et al.*, 1999). Another outbreak due to this subtype was reported during 1999-2000 (Anjum, 2000). Importantly frequency of outbreaks of this subtype is increasing noticeably in Iran, Pakistan and United Arab Emirates (Nili and Asasi, 2002; Bano *et al.*, 2003; Capua and Alexander, 2004; Aamir *et al.*, 2007).

At present there is a meager and insufficient data on the pathogenicity of H9N2 viruses. The present project was designed to reproduce disease as observed in field conditions, to evaluate and understand pathogenesis of LPAIV H9 in broiler chicken under controlled experimental conditions

and to calculate intravenous pathogenicity index (IVPI) of H9 viruses circulating in poultry industry in Pakistan.

MATERIALS AND METHODS

Isolation and identification of H9 virus

Thirty samples for isolation of H9 virus were collected from 10 different broiler flocks (3 samples/farm) showing mild respiratory signs with mortality up to 5-10%. All birds had the history of already vaccination against Newcastle disease, Infectious bursal disease, Infectious bronchitis and Marek's disease. Samples were collected by flushing trachea from freshly dead birds with 2ml phosphate buffered saline (PBS) or by direct tracheal swabs from live birds and brought to laboratory (Nili and Asasi, 2002), centrifuged for 30 min at 1500 rpm and the collected supernatant mixed with antibiotics (Penicillin 10,000 IU/ml, Gentamycin 1mg/ml, Streptomycin 10,000 µg/ml) and antifungal agents (Amphotericin B). Inoculum was prepared by passing through a 0.45 µm filter and incubating at 37°C for 1 hour. Virus isolation was performed by inoculating 10 days old embryonated chicken eggs with 0.2ml of inoculum according to the protocol adopted by OIE (2005). Harvesting of eggs was done 48 hours post inoculation (PI) and CAF was collected with a sterile syringe and centrifuged at 3000 rpm for 5 minutes to remove mixed blood and tissues. Presence of virus was confirmed by haemagglutination (HA) and haemagglutination inhibition (HI) tests as described by WHO (2005). The viral stock for challenge was prepared by 1:10 dilution of fresh chorioallantoic fluid having 4HA unit with sterile PBS (Shanker, 2009).

Experimental design

Forty day old broiler chicks were procured and reared during the month of March-April, 2009 under standard housing conditions which were as similar as farm conditions, fed with commercially prepared feed and water *ad libitum* and were vaccinated against Newcastle disease and Infectious bursal disease. All the birds were tested negative for antibodies to avian influenza virus H9 at 30th day of age. The birds randomly divided into two groups (A

and B) on 20th day of age with twenty birds in each group and housed in separate rooms. Birds in group A were inoculated with sterile phosphate-buffered saline (PBS) and kept as unchallenged controls while birds in group B were challenged intravenously (IV) through brachial vein with a volume of 0.2 ml of H9 virus inoculum at 31st day of age. Birds were monitored daily for 14 days for their general conditions, clinical signs of disease and mortality and all observations were recorded. On 5th, 9th and 14th day postinfection, three birds from each group were randomly selected, slaughtered and all gross lesions recorded. Organs such as trachea, lungs, liver and kidney were collected for histopathological and immunochemical staining.

Indicators of pathogenicity

Clinical signs

During experimental period all the birds were examined once daily for development of clinical signs and mortality and all the observations were recorded (Calnek, 1997).

Intravenous pathogenicity index (IVPI)

According to the clinical signs and number of dead birds during 10 days postinfection (PI), intravenous pathogenicity index (IVPI) of H9 virus was calculated in birds of group B (H9 virus infected) and compared with the birds of group A (Control) as per standard procedure (WHO, 2002). The clinical signs established were: depression, respiratory involvement, diarrhea and edema or swelling of face and head. It was also established that normal birds scored 0, sick bird (one of these signs) 1, severely sick bird (more than one signs) 2 while dead birds were scored maximum (3 score) at each of the remaining daily observations. IVPI was calculated on the bases of this scoring. At the end of observation period, sum of observations in each category was totaled and divided by total number of observations. Results were interpreted as described in WHO/OIE manual. Total score 0 means that all birds were normal while 3 score means that all birds were died within 24 hours (Shanker *et al.*, 2009).

Gross pathology and histopathological studies

On day 5, 9 and 14, five birds from each

group were slaughtered and all gross lesions recorded (Easterday and Beard, 1984). Samples like trachea, lungs, kidney and liver were fixed in 10% neutral buffered formalin and processed in standard techniques for fixation, dehydration, clearing, embedding, sectioning and staining as described by Drury and Wallington (1980).

Effect on weight gain

Birds in group A (Control) and group B (H9 infected) were weighted daily postinfection and all readings were recorded (Gharaibeh, 2008).

Immunohistochemical staining

Unstained paraffin-embedded sections were prepared and immunohistochemically stained by using polyclonal antibody against nucleoprotein of type A influenza virus subtype H9N2 (Cuello, 1993). Briefly, after removal of paraffin and rehydration of the tissue sections, enzyme retrieval was done with 1% trypsin in PBS for 20 minutes at 37°C, inactivation of endogenous Peroxidase done by 3% hydrogen peroxide, incubation with primary polyclonal antibodies for 60 minutes in humidifier chamber, incubation with Biotinylated anti-rabbit antibody for 30 min, peroxidase-conjugated streptavidin and incubated for 10-15 min and finally counterstaining was done with a bath of Mayer's hematoxylin for 2-3 min and sections were examined under microscope.

RESULTS

H9 virus

H9 virus isolated from field samples was confirmed by Haemagglutination Inhibition (HI) test. First, collected CAFs were tested by Spot HA test for the presence of virus. Out of 30 samples 12 (40%) samples were positive. The virus titers determined by using HI test were ranged from 1:64 to 1:512. Out of 12 positive samples, 4 samples showed titer 1:64, three samples showed 1:128, two samples showed titer of 1:256 while three samples represented titer of 1:512.

Indicators of pathogenicity

Intravenous pathogenicity index

Intravenous Pathogenicity Index (IVPI) was

calculated in H9 virus infected group and compared with control group. None of the birds showed signs of sickness or respiratory involvement in the control group during observation period of 10 days. In infected group, 4 birds showed signs of diarrhea 2 days postinfection (PI) while 3 birds were depressed three days PI. These birds were recovered after eight and six days PI, respectively. The index was calculated as the mean score per bird per observation. Thus IVPI of the virus used in this study was calculated as $33/280 = 0.12$ (Table I)

WHO (2005) has set the criteria to define the pathogenic nature of AIV subtypes on the bases of IVPI indices as below:

| | |
|-------------------|----------------------------|
| Highly pathogenic | 2.0- 3.0 (range 1.74- 3.0) |
| Intermediate | 1.2- 1.4 |
| Non pathogenic | 0 (range 1.0) |

According to this criteria, it is evident that the virus used in this study was non pathogenic in nature.

Clinical signs

All birds were normal in control group while in the infected group most of the birds showed slight depression with low intake of feed and water between 2 to 7 days PI of H9 virus. Among these birds, 4 birds suffered with diarrhea while 3 birds revealed depression on 5th day PI. All the birds were recovered from depression after 7 days PI while diarrhea persisted up to 12th day PI. No mortality was observed among the birds.

Gross pathology

All visceral organs were found normal with no abnormal gross changes in control group while in infected birds only slight hyperemia and congestion was observed in trachea and lungs (Fig. 1A) in two birds each which were slaughtered on 5th and 9th day PI. Kidneys were swollen (Fig. 1B) in six out of 14 birds. The frequency of changes was 43 % in kidneys while only 10% in trachea and lungs (Table II).

Histopathological lesions

All the tissue sections were normal and no histopathological changes were observed in group

A. Slight changes were observed in form of deciliation, congestion and infiltration of leukocytes in trachea in group B. Lungs showed infiltration of leukocytes and congestion (Fig. 1C) on 5th day PI. The lesions were severe on 9th day PI. A significant

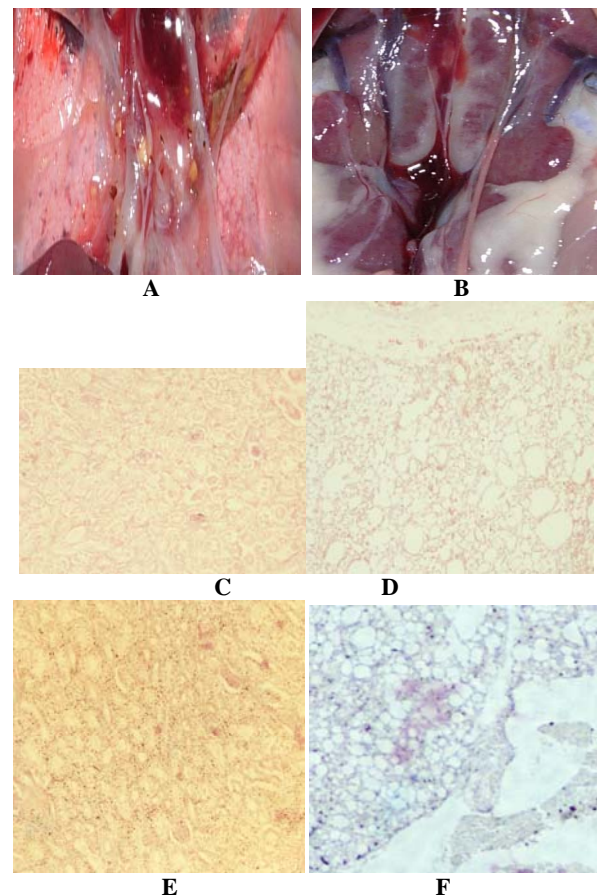


Fig. 1. Gross pathological and histopathological effects of low pathogenic avian influenza virus H9 in broiler chicken. A, congested lungs; B, swollen kidneys; C, lungs with infiltration of leukocytes; D, infiltration of leukocytes in kidneys; E, immunohistochemical staining in kidneys; F, immunohistochemical staining in lungs.

change was swelling of glomeruli and presence of leukocytes in tubular region in kidneys (Fig. 1D) which were in increasing intensity on 9th and 14th day PI. No significant changes were observed in liver except the presence of leukocytic infiltration. The frequency of changes was 25% in trachea/ lungs whereas 55% in kidneys (Table III).

Table I.- Intravenous pathogenicity index in H9 infected chickens.

| Groups | Clinical signs | Days | | | | | | | | | | | | | | Total | Score |
|--------|----------------|------|----|----|----|----|----|----|----|----|----|----|----|----|----|-------|-------|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | | |
| A | Normal | 20 | 16 | 13 | 13 | 13 | 16 | 16 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 247X0 | 0 |
| | Sick | 0 | 4 | 7 | 7 | 7 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 33X1 | 33 |
| | Severely sick | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0X2 | 0 |
| | Dead | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0X3 | 0 |
| | Total Index | | | | | | | | | | | | | | | | 33 |
| | | | | | | | | | | | | | | | | | |
| B | Normal | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 280X0 | 0 |
| | Sick | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Severely sick | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Dead | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Total Index | | | | | | | | | | | | | | | | 0 |

Table II.- Gross pathological lesions in experimental groups.

| Groups | Congestion | | | | Hypertrophy | | | | Fibrication | | | | Necrotic foci | | | | Frequency of changes (%) | | | |
|--------|------------|---|----|---|-------------|---|----|---|-------------|---|----|---|---------------|---|----|---|--------------------------|----|----|----|
| | T | L | Li | K | T | L | Li | K | T | L | Li | K | T | L | Li | K | T | L | Li | K |
| A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0 | 0 | 0 | 0 |
| B | + | + | - | + | - | - | - | + | - | - | - | - | - | - | - | - | 43 | 10 | 0 | 43 |

+, Present; -, Absent.
T, Trachea; L, Lungs; Li, Liver; K, Kidneys.

Table III.- Histopathological lesions in experimental groups.

| Groups | Congestion/ Haemorrhage | | | | Leukocytic infiltration | | | | Odema/ Swelling | | | | Necrosis/ Degeneration/ Sloughing | | | Frequency of changes (%) | | |
|--------|-------------------------|---|----|----|-------------------------|---|----|----|-----------------|---|----|----|-----------------------------------|---|----|--------------------------|-----|----|
| | T | L | Li | K | T | L | Li | K | T | L | Li | K | T | L | Li | K | T/L | Li |
| A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0 | 0 | 0 |
| B | + | + | - | ++ | + | + | - | ++ | + | - | - | ++ | + | - | - | 55 | 25 | 0 |

T, Trachea; L, Lungs; Li, Liver; K, Kidneys.

Effect on body weight

Body weight gain in group A (Control) and group B (H9 virus infected) was recorded daily for ten days post infection (PI). It was found that there was a significant difference (P< 0.05) between the mean body weights of the birds of group B from day 1st of PI to 10th day PI. Thus it is concluded that weight loss is associated with the presence of infection of H9 virus in birds.

Immunohistochemical detection of virus

Viral antigen was not evident in tissues of

birds in control group (Group A) while it was identified in kidneys and lungs tissues of infected birds (Group B). Positive immunohistochemical staining was obvious as dark brown deposits in the nuclei of pulmonary epithelial cells (Fig. 1E) and within nuclei or cytoplasm of necrotic renal tubular epithelium in kidneys (Fig. 1F). On day 5 PI, reaction found positive in trachea 16%, in lungs 50% while in kidneys 66%. On 9th day PI, reaction found positive in trachea 16%, in lungs 33% while in kidneys 50%. On 14th day PI, reaction found positive in lungs 16% while in kidneys 50% while

no viral replication detected in tracheal tissues (Table IV). This technique revealed that out of 18 samples 50% were positive for virus replication on day 5, 33% on day 9 and 17% on day 14 PI. The infection persisted in kidneys while decreased in lungs and trachea with the time. Comparison of results on different days PI is presented in Figure 2.

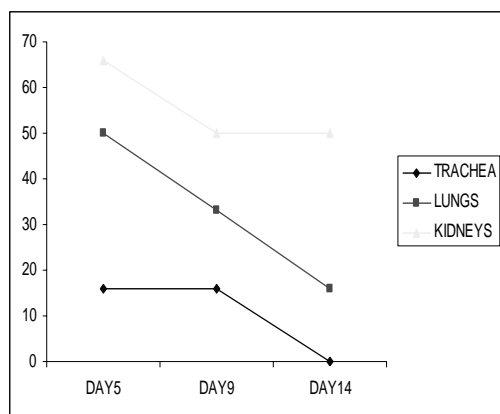


Fig. 2. Comparison of immunohistochemical detection on different days.

Table IV.- Immunohistochemical detection of H9 virus.

| Groups | Total no. of samples | Tissues | Positive on day 5 (%) | Positive on day 9 (%) | Positive on day 14 (%) |
|----------|----------------------|---------|-----------------------|-----------------------|------------------------|
| Control | 6 | Trachea | 0 | 0 | 0 |
| | 6 | Lungs | 0 | 0 | 0 |
| | 6 | Kidneys | 0 | 0 | 0 |
| Infected | 6 | Trachea | 1 (16) | 1 (16) | 0 |
| | 6 | Lungs | 3 (50) | 2 (33) | 1 (16) |
| | 6 | Kidneys | 4 (66) | 3 (50) | 2 (50) |
| Total | 18 | | 8 (50) | 6 (33) | 3 (17) |

DISCUSSION

Frequent outbreaks of low pathogenic avian influenza virus (LPAIV) H9N2 in Asian countries like Pakistan and China revealed that this subtype is now endemic in poultry birds (Swayne and Slemons, 1998; Naeem *et al.*, 1999; Aamir *et al.*, 2007). Infection results in involvement of respiratory and gastrointestinal system with varied clinical signs of variable severity (Mo *et al.*, 1997; Capua *et al.*, 2000) which ensue in great economic

losses (Nili and Asasi, 2003; Capua and Alexander, 2004). Interestingly, it was found that mortality rate in field conditions was high as compared to the experimental conditions (Vasfi Marandi *et al.*, 2000, 2003; Toroghi and Momayez, 2006).

The present study was intended to investigate the pathogenesis of LPAIV H9 under experimental conditions in broiler chickens. H9 subtype was isolated from field samples and confirmed by Haemagglutination Inhibition (HI) test. Out of 30 samples, 12 (40%) were positive for Haemagglutination test. Virus titer calculated by HI test ranged from 1:64 to 1:512. These findings are in line with the observations of Nagarajan *et al.* (2008) who reported the HI test positive with 1:16 to 1:512 titers for H9N2 virus isolates from India. On the bases of this comparison, it can be concluded that virus circulating in poultry industry in Pakistan could have the genetic homology with the Indian isolates.

IVPI test index was 0.12/3.0 which indicated that according to the criteria set by WHO (2005) this subtype belongs to LPAIV. Our findings are similar to that of Slemons *et al.* (1991) and Nagarajan *et al.* (2008) who documented that IVPI of H9N2 virus showed range of 0.0 to 0.49/3.0. Similarly Shanker *et al.* (2009) stated that the pathogenicity index of subtype H9N1 was calculated 0.05/3.0 in water coot in India. On the bases of this test it is possible to summarize that H9N2 viruses isolated from Pakistan are almost similar in their pathogenicity to the viruses mentioned by the above workers. These viruses contain monobasic amino acids in the HA cleavage site which enable them to replicate only in limited body tissues resulting in low pathogenicity. Whereas our results differ from some workers who reported that H9N2 viruses have become endemic in field conditions and are associated with high rates of outbreaks and mortality throughout the world since the mid 1990s (Claas *et al.*, 1998; Peiris *et al.*, 1999). One possible factor associated with high mortality may be the involvement of virulent sublineage of H9N2 virus. The genetic material of Asian H9N2 viruses revealed that there are three different sublineages, among these lineages isolates of first lineage (A/Chicken/Beijing/1/94,H9N2) can ensue 80% mortality, whereas viruses from second

and third lineages cannot produce any significant disease (Guo *et al.*, 2000; Tosh *et al.*, 2008). Here it can be stated that our results are comparable and agreeable at different spectrum to the above findings and it can be documented that virus subtype isolated and used as inoculum during present study were from low pathogenic sublineages.

During pathogenic study, it was seen that no obvious clinical signs and mortality were noted in control birds while some birds in group B (H9N2 infected) showed sign of diarrhea and slight depression. The findings of author are similar with the findings of the Davison *et al.* (1999) and Vasfi Marandi *et al.* (2000) who similarly reported diarrhea and depression in experimental birds. Thus it can be thought that the virus subtype used in these both studies have the same pathogenic potential. In author's study it was found that no clinical signs of respiratory involvement were noted but in these organs histopathological lesions were evident. The findings of Mutinelli *et al.* (2003) and Anonymous (2005) upto some extent did not agree with the findings of author who reported mild respiratory signs like mild tracheal rales, coughing and sneezing in broiler chickens. This could be due to difference in the sublineage of virus, strain of the birds, dose rate, geographic distribution and different climatic conditions from those of author's during experimental study. The frequency of gross pathological changes was 43% in kidneys while only 10% in trachea and lungs. During histopathological study, slight changes were observed in form of deciliation, congestion and infiltration of leukocytes in trachea. Lungs showed infiltration of leukocytes and congestion. A significant change observed was swelling of glomeruli and presence of inflammatory cells in tubular regions of kidneys. Frequency of changes was 25% in trachea and lungs whereas 55% in kidneys. These findings indicate that main target organs of virus are kidneys and lungs where lesions are more pronounced. Our findings in one part are in line with findings of Swanye and Slemons (1991) who reported nephritis and claimed that kidneys are the main target organ of H9 virus when infection is given by intravenous route. Here our study further explained the involvement of lungs with H9N2 virus replication in epithelial tissues of bronchi. This part

of result differs from the findings of Mutinelli *et al.* (2000), Slemons *et al.* (1990, 1991) who did not report the involvement of lungs when infection was given I/V. Similarly Habbolvarid *et al.* (2004) reported no significant lesions in lungs except only lymphocyte infiltrations under secondary bronchi on day 3 PI. But our findings to some extent are according to the findings of Pazani *et al.* (2008) who documented mild respiratory involvement by I/V route. This may happen due to different climatic conditions or subtype of virus or the virus has acquired the potential to replicate in respiratory system when it is disseminated throughout the body of the birds through the blood. As no mortality was seen in H9 infected birds, this finding is according to the findings of Mo *et al.*, (1997) and Banks *et al.* (2000) but differ from the results of Vasfi Marandi and Bozorgmehrfard (1999), and Kim *et al.* (2006) who reported 65%, and 30% mortality due to H9 subtype. This huge discrepancy may be due to use of different sublineage of H9 virus, route of inoculation and strain and health status of the experimental birds.

One significant observation noted that there was a significant difference ($P < 0.05$) between the mean body weights of the birds of group B from birds of group A from day 1st of PI to 10th day PI. Thus it is concluded that weight loss is associated with the presence of infection of H9 virus in birds. These findings are similar to that of Gharaibeh (2008) who reported a significant reduction in weight gain in experimental birds due to H9 infection. Similarly Vasfi Marandi *et al.* (2002) reported decrease in feed consumption due to H9N2 virus infection. The possible cause of reduction in weight could be effect of viral infection on pancreatic tissue which results in decrease production of pancreatic enzymes essential for efficient digestion (Silvano *et al.*, 1997; Shinya *et al.*, 1995). Here it can be documented that infection with LPAIV cannot be diagnosed on bases of spectrum of clinical signs, whereas it can be diagnosed by comparison of weight gain of the infected birds. Immunohistochemical staining showed that virus can be detected at the site of replication in while lungs and kidneys are the two main target organs for viral replication and infection persists in kidneys for longer time. These results of

author are in line with the results of Slemons *et al.* (1990) and Swayne *et al.* (1992). From this study, it seems reasonable to conclude that virus H9 prevailing in poultry industry in Pakistan belongs to non pathogenic Asian sublineage which did not produced mortality but produced significant reduction in weight gain of infected birds. The virus has tissue tropism for respiratory tract, gastrointestinal tract and urinary tract. Immunohistochemical staining is a good technique to detect viral replication responsible for lesions in infected tissues.

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