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COMPARATIVE PATHOLOGY OF EXPERIMENTALLY INDUCED LOW PATHOGENIC AVIAN INFLUENZA (H7N3) INFECTION IN CHICKEN, DUCKS AND QUAILS

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ABSTRACT

Low pathogenic Avian Influenza Viruses (LPAIVs) are a persistent threat to poultry and have zoonotic potential. Wild birds such as Ducks and Quails may serve as not only carrier but could be intermediate host for mutation of viruses into highly pathogenic forms. Present study compares pathogenesis of LPAIV H7N3 in chicken, ducks and quails. Thirty-two birds of each species were grouped according to route of infection i.e. Intravenous (IV), Oculo-nasal (OCN) and Oral (OR) and a control group with each comprising eight birds. Birds were challenged with LPAIV H7N3 through IV, OCN and OR routes at dose of 0.1ml/bird (1×10^9 EID_{50} particles) while control group was inoculated with sterile PBS. Birds were monitored daily for development of clinical signs, mortality and mean death time (MDT). Serum samples were collected before infection and days 7, 14 and 21 post-infection. Dead birds were necropsied and surviving birds were euthanized and dissected at the end of experiment for recording gross and histopathological studies. Pathogenicity indices of virus through different routes were calculated. Results revealed cyanosis of comb (75%) and wattles (75%) as the major clinical signs in chicken infected through intravenous route. Chickens infected through OCN route had swollen heads (87%) respiratory disturbance (75%). Mild clinical signs such as depression (2%) and torticollis (1%) were observed in quails. No clinical signs of infection were observed in ducks. Highest mortality was recorded in chicken inoculated through OCN (75%), followed by IV (50%) and OR (38%) routes. MDT was higher (13.6days) in OR route. Quails showed only 25% in IV infection whereas no mortality occurred in ducks. Intravenous pathogenicity index (IVPI) was higher (1.019) in chicken as compared to quails (0.544). Within chicken species higher pathogenicity score was found in OCN (1.419) group as compared to IV (1.019) and OR (0.45) routes. Gross pathological findings included nephritis as predominant lesion in chickens inoculated IV (78%) and OC (50%) routes, whereas OR route showed hypertrophied bursa (30%) and nephritis (35%). Congested lungs and livers were also observed in 25 % of dead birds. Oculo-nasal infection caused lesions in trachea (25%). No gross pathological lesions were observed in ducks and quails. Histological changes such as moderate infiltration of neutrophils cells, necrosis in the renal tubules, and urate

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deposition and non-suppurative focal interstitial nephritis were observed in IV group where as in OCN, mostly lung tissues showed edematous changes, hemorrhages, and severe congestion. The antibody titers after intravenous inoculation increased from day 7 p.i to day 14 but then declined at day 21. Trend observed was similar in chicken, quails and ducks. It is concluded from the results that chicken are more susceptible to LPAIV H7N3 while ducks and quails are resistant but do seroconvert.

Keywords: chicken, duck, influenza virus, low pathogenicity, pathology, quail

INTRODUCTION

Avian influenza is caused by genus Influenza Virus A, belonging to the family Orthomyxoviridae (Huang *et al*., 2012). Low pathogenic avian influenza viruse (LPAIVs) cause mild respiratory disease, reduction in egg production, and moderate increase in mortality (Marche *et al*., 2010). The avian influenza virus (AIV) is distributed throughout the world in many domestic birds, including chickens, turkeys, quails, geese and ducks and in wild water fowls, gulls and shore birds. It has various sub-types including H5, H7 and H9. Majority of the H5 and H7 and all of H9 subtypes are of low pathogenicity. These strains of LPAI virus are of major importance may impose high economic loss to poultry industry. Presence of these viruses in wild bird hosts poses potential threat to domestic poultry as well as human beings due to their zoonotic transmission and possible antigenic shifting into high pathogenicity strains (Alexander, 2007). Due to such serious threat, LPAIV surveillance programs have been implemented in many countries (Gonzales *et al*., 2010).

Pathogenicity AIVs vary considerably depending upon host species, routes of infection and infective doses (Spackman *et al*., 2010, Cagle *et al*., 2011, 2012). Carrier bird such as quails and ducks may shed LPAIV as well as facilitate their adaptation to chicken (Bertran *et al*., 2013). Since LPAIV may produce variable pattern of disease in different species of birds, present study was conducted to compare the susceptibility along with pathological changes occurring in chicken, ducks and quails infected with a local isolate of LPAIV H7N3.

MATERIALS AND METHODS

Preparation of stock virus and titration

The LPAIV (H7N3) isolated from chickens at Sindh Poultry Vaccine Centre Karachi namely A/Chicken/Pakistan SPVC 26/03 was inoculated into the allantoic cavity of 10 days old embryonated SPF chicken eggs. After inoculation, eggs were incubated at 35^{o}C for 72 hours. Eggs were candled and chilled before harvesting allantoic fluid (AAF). The AAF was harvested and tested for presence of virus by Micro Hemagglutination Assay (MHA), which was performed to determine HA titer (OIE, 2014). Moreover, virus titer in terms of Egg Infectivity Dose (EID50) was also determined.

Experimental infection of LPAIV in chicken, quail and duck

Housing and experimental infection

Day-old broiler chicks of chicken, quails and ducks were purchased from the hatchery in Karachi. The birds were screened for presence of anti-AIV antibodies

by hemagglutination inhibition (HI) test. The birds were raised till they achieved the optimum age of 06 weeks in broiler, 05 weeks in ducks and 05 weeks in quails. Birds were provided commercial feed and water ad libitum. The hygienic and bio safety measures were strictly adopted during the course of the experiment. For experimental infection, chickens, ducks and quails were grouped into three treatment groups according to different routes of administration i.e. Oral (OR), (Oculo-nasal (OR) and Intravenous (IV) each containing eight birds, total (n=24). Each bird was administered with 0.1 ml inoculum having1 x 10^9 EID_{50} viral particles. Control group having eight birds was inoculated with phosphate buffered saline (PBS).

Serological examination

All the birds were bled before infection and on days 7, 14 and 21 post-infection (p.i) through wing vein. Sera were separated and stored at -20°C till used for serological testing by HI test (OIE, 2014).

Clinical findings and determination of pathogenicity indices

All the birds were monitored twice daily for twenty days. Mortality and morbidity were recorded. The clinical signs/lesions appearing during the course of disease were recorded. For determination of pathogenicity indices for LPAIV H7N3 through Intravenous, oral and oculo-nasal routes of infection in chicken, quails and ducks, birds were scored as 0, 1, and 2 for normal, sick and dead (OIE, 2014).

Gross and histopathology

Dead birds were necropsied for evaluation of gross pathological lesions. Tissue samples from trachea, proventriculus, lungs, spleen, liver, kidneys pancreas, and intestine of dead birds were collected and fixed in 10% neutral buffered formalin for histopathological examination. Formalin fixed samples were washed twice in PBS. These were then processed using automatic tissue processor (HT- UK) pre-programmed for timing in increasing grades of ethanol as 75, 85, 95, 95, 100 and 100 percent for 1 hour in each dilution. Tissues were then cleared by two changes of pure xylene for 30 min each and then infiltrated with melted paraffin wax at 65^0C for 01 hour twice. Paraffin wax blocks of tissues were prepared by embedding tissues in paraffin wax using HT-Wax Embedding System. Sections of 3µm were cut and mounted on frosted slides. Slides were heated for 24 hrs at 42^oC for fixation and then stained with Hematoxylin and Eosin (Merk). Dewaxing, rehydration and staining was performed in automatic tissue stainer (HT- UK). Slides were cover-slipped using DPX (Merk) and examined at 10X and 40X magnifications for histopathological evaluation.

RESULTS

Clinical findings

Birds were observed for clinical signs, such as depression, respiratory involvement, neurological involvement, diarrhea, edema or swelling of face head, hemorrhages on shanks, cyanotic comb and conjunctivitis. Frequency of clinical lesions is shown in Table 1.

Results depicted in Table 1 show cyanosis of comb/wattles as the major clinical sign that appeared in chicken infected through IV route. Chicken infected through OCN route had greater percentage of upper respiratory signs i.e. swollen heads (87%), followed by respiratory disturbance (75%). Depression and hemorrhages were recorded in 65% birds. Cyanosis of comb/wattles and hemorrhages on shanks were the major clinical findings with oral route. Neurological signs were observed in very few birds infected through IV route. Diarrhea occurred only in birds infect by OCN route. Mild clinical sign such as depression, and neurological sign i.e. torticollis was observed in few quails. No clinical sign of AIV was observed in ducks.

Mortality (%) and mean death time (MDT)

The mortality rate and MDT in chicken, quail and ducks are shown in Table 2. Results reveal that mortality was higher in chicken than in quail. Highest mortality occurred in chicken infected through OCN route, followed by IV and OR routes. The MDT was higher in chicken infected orally than oculo-nasally and intravenously. No mortality was recorded in ducks.

Pathogenicity indices

Susceptibility of three species to LPAIV H7N3 in three species was evaluated by determining pathogenicity indices with three routes of inoculation which are shown in Table 3. Results reveal that intravenous pathogenicity index is higher (1.019) in chicken as compared to quails (0.544), whereas neither clinical signs nor mortality was observed in ducks. Pathogenicity indices in oculo-nasal group (1.419) are higher than intravenous (1.019) and oral (0.45) groups. The indices were higher in intravenous group (0.544) of quails.

Gross and histopathology

Gross pathological lesions and their relative frequencies are depicted in Table 4. Highest frequency of lesion i.e. nephritis were recorded in chicken inoculated through intravenous (78 %) and oculo-nasal routes (50%), where as in oral route highest frequency of lesion was nephritis (35%), followed by hypertrophy in bursa (30%). Lung and liver congestion was also observed. Oculo-nasal infection showed lesions in trachea (25%). There were no lesions in trachea of birds infected through oral route. No gross pathological lesions were observed in any of the organs in ducks and quails.

Histopathological lesions and their relative frequencies are depicted in Table 5. Highest frequencies of lesion were observed in tissue samples of kidneys obtained from chicken infected through intravenous route (80%), followed by oral route (35%). Predominant lesions were moderate infiltration of neutrophils, necrosis in the renal tubules urate deposition and non-suppurative focal interstitial nephritis. Lung tissue showed edema, hemorrhages, and severe congestion. Severity of lesions was variable from moderate multifocal to severe inflamed lesions with moderate infiltration of heterophils in the lumen of para-bronchioles epithelium. Histopathological examination of trachea revealed that epithelial cells of trachea lost their cilia from several places, with desquamatation and slightly infiltration of leukocytes.

Table 1. Clinical signs in chicken inoculated with AIV H7N3 by various routes

Clinical Signs	Frequency of Clinical Signs (%)		
	Intravenous	Oculo-nasal	Oral
Depression	50	65	10
Respiratory involvement	20	75	00
Neurological signs	02	00	00
Diarrhoea	00	00	10
Cyanotic Comb/Wattles	75	62	50
Haemorrhages of Shank /Claw	50	65	40
Sinusitis	25	62	00
Conjunctivitis	38	50	00
Swollen head/face/orbital swelling	25	87	00

Table 2. Mortality and mean death time in chicken, quails and ducks inoculated with LPAIV H7N3 by various routes

Dose and Route of Inoculation	Mortality and Death Time					
	Chicken		Quail		Duck	
	Mortality (%)	MDT (day)	Mortality (%)	MDT (days)	Mortality (%)	MDT (days)
IV	50	5.25	25	7	0	0
OCN	75	7.6	0	0	0	0
OR	38	13.6	0	0	0	0

Table 3. Pathogenicity indices in chicken, quails and ducks inoculated with LPAIV H7N3 by three routes

Routes of Inoculation	Poultry Birds		
	Chicken	Quails	Duck
IV pathogenicity	1.019	0.544	0
OCN pathogenicity	1.419	0.35	0
ORpathogenicity	0.45	0.281	0

Table 4. Frequency of gross pathological lesions in different organs inoculated with LPAIV H7N3 in chicken through various routes

Organs	Lesions	Frequency of Lesions in Various Routes of Inoculation		
		IV	OCN	OR
Kidney	Inflammation	78	50	35
Bursa	Hypertrophy	50	20	30
Lung	Congestion/Pleuritis	25	25	13
Liver	Ischemia	25	0	0
Trachea	Congestion/hemorrhage	13	25	0
Proventriculus	Hemorrhage	13	0	0
Intestine	Hemorrhage	13	13	0
Pancreas	Hypertrophy	5	0	0

Table 5. Frequency of histopathological lesions in different organs of chicken inoculated with LPAIV H7N3 through various routes

Organs	Predominant lesions	Frequency of Lesions in Various Routes of Inoculation		
		IV	OCN	OR
Kidney	Necrosis and inflammation	80	5	35
Bursa	Lymphoid atrophy	40	30	38
Lung	Necrosis/pneumonia	35	38	13
Liver	Lymphocyte infiltration	26	5	0
Trachea	Necrosis/ Tracheitis	15	32	0
Proventriculus	Lymphocyte infiltration	7	5	5
Intestine	Hemorrhage	7	13	5
Pancreas	Lymphocyte infiltration	5	3	5

Serology

Serological response against LPAIV H7N3 was detected through HI test. Geometric means $\log_2$ values of HI at days 7, 14 and 21 p.i were recorded (Figure 1). Results revealed that antibody titers after intravenous inoculation increased from day 7 p.i (8.54) to day 14 (9.0) but then declined at day 21 (8.32). Similar trend was observed in chicken, quails and ducks. Antibody titers after OCN inoculation in chicken were higher (8.46) as compared to quails (4.31) and ducks (1.36). Similar results were observed in oral inoculation as well. Quails and ducks inoculated oculo-nasally showed increase in antibody titers from day 7 to day 21 p.i, whereas in chicken titers decreased at 21 days p.i. In all birds infected through oral route seroconversion rate increased slightly until day 21 p.i. Intravenous inoculation showed highest antibody titers at day 7 p.i.

DISCUSSION

LPAIVs cause low mortality in poultry, however they produce losses in the form of decreased productivity, immunosuppression and deaths due to secondary bacterial infections. Although, occasional outbreaks of HPAIV have been reported in Pakistan most prevalent AIV infections are caused by LPAIVs. In a recent sero-surveillance study, the prevalence of H9 subtype in commercial poultry farms of Thatta, Karachi and Mirpurkhas districts of Sindh was found to be 97%, 86% and 89%, respectively as compared to 31%, 41% and 53% for H7N3 subtype (Shahab, 2015). The LPAIVs in poultry remain a persistent threat to commercial poultry due to involvement of wild birds such as ducks and quail which may become carriers, where viruses may mutate into highly pathogenic forms. Present study was therefore conducted to study comparative pathology of LPAIV H7N3 in chicken, ducks and quails.

Results of present studies showed marked species differences in development of clinical disease. Chicken showed classical clinical signs and necropsy findings whereas no typical findings were observed in ducks and quails. In chicken, cyanosis of comb/wattles was the major clinical sign when infected through intravenous route. Whereas oculo-nasal infection caused upper respiratory signs like swollen heads and respiratory disturbance. Neurological signs were observed in very few birds which were infected through intravenous

route. Diarrhea occurred in birds which were infected orally. Shahab (2015) has also reported depression, cyanosis of comb and wattles along with diarrhea as major clinical signs in H7 infection in field conditions. Similarly, edema of face and comb region, vesicles and necrosis of comb has been observed by Cheema *et al*. (2011). Several studies have reported that pathogenicity of AIV infection may vary depending on the host species, routes of infection and doses of inoculated virus (Cagle *et al*., 2011; 2012). Spackman *et al*. (2010) have also reported that H7 LPAIV subtypes produce mild or sub-clinical infection in ducks. Bertran *et al*. (2013) reported that H7N2/ LPAIV did not develop either clinical signs or pathological lesions in infected quails. Findings of present study are therefore in accordance with these reports. In present study, quails and ducks did not show any clinical signs, but there was probably a sub-clinical infection as shown by seroconversion in both species. Studies suggest that infectivity depends on the adaptation of virus into any species. Variability in pattern of infection and pathogenicity may be due to different isolates and host susceptibility. One possible reason of variable pathogenicity of H7N3 in three species could be that the isolate was from a chicken outbreak while it may not have been adapted to quails and ducks. Transmission AIV to new host requires some process of adaptation that improves viral replication and transmission. Mundt *et al.* (2009) compared the replication and pathogenesis of low-pathogenic H5N1 (wild bird isolate), H5N2 and H5N3 (isolates from chicken) AIVs in duck and chicken. It was observed that H5N1 replicated superior in duck than in chicken; whereas, chicken showed greater infectivity with H5N2 and H5N3. The results of this study revealed that H5N1 being isolated of wild bird was more adapted to duck.

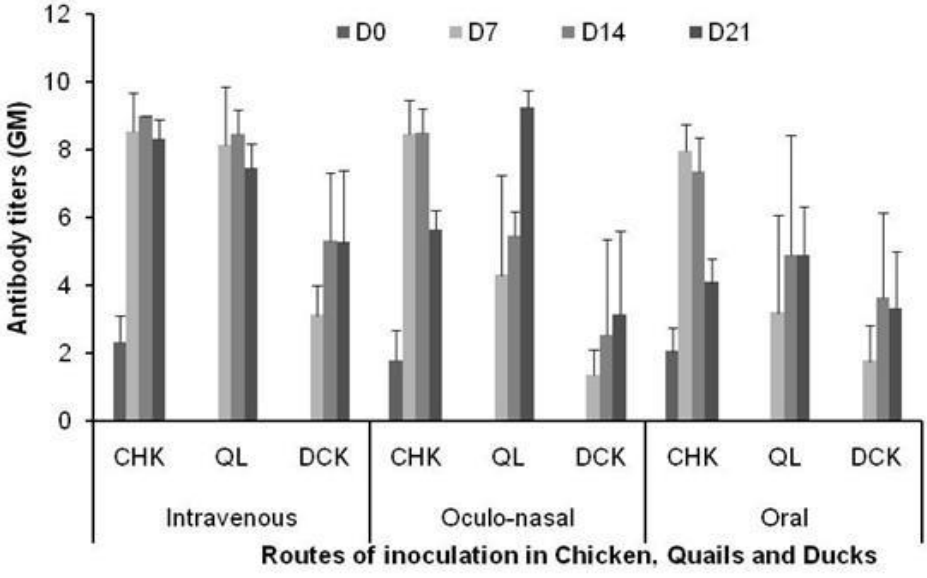

Figure 1. Anti-AIV H7N3 antibodies in infected chickens (CHK), quails (QL) and ducks (DCK) D = Day

Present study showed variation in mortality percent in chicken and quails infected through different routes. No mortality was recorded in ducks. In chicken, oculo-nasal inoculation caused highest mortality followed by intravenous and oral infection. Mortality in quails was higher in intravenous route as compared to Oculo-nasal and oral infection. Spackman *et al.* (2010) have reported that MDT of chicken infected with LPAIV H7N3 by oral route was higher i.e. 13.6 days as compared to oculo-nasal and I/V, which was 7.6 and 5.25 days.

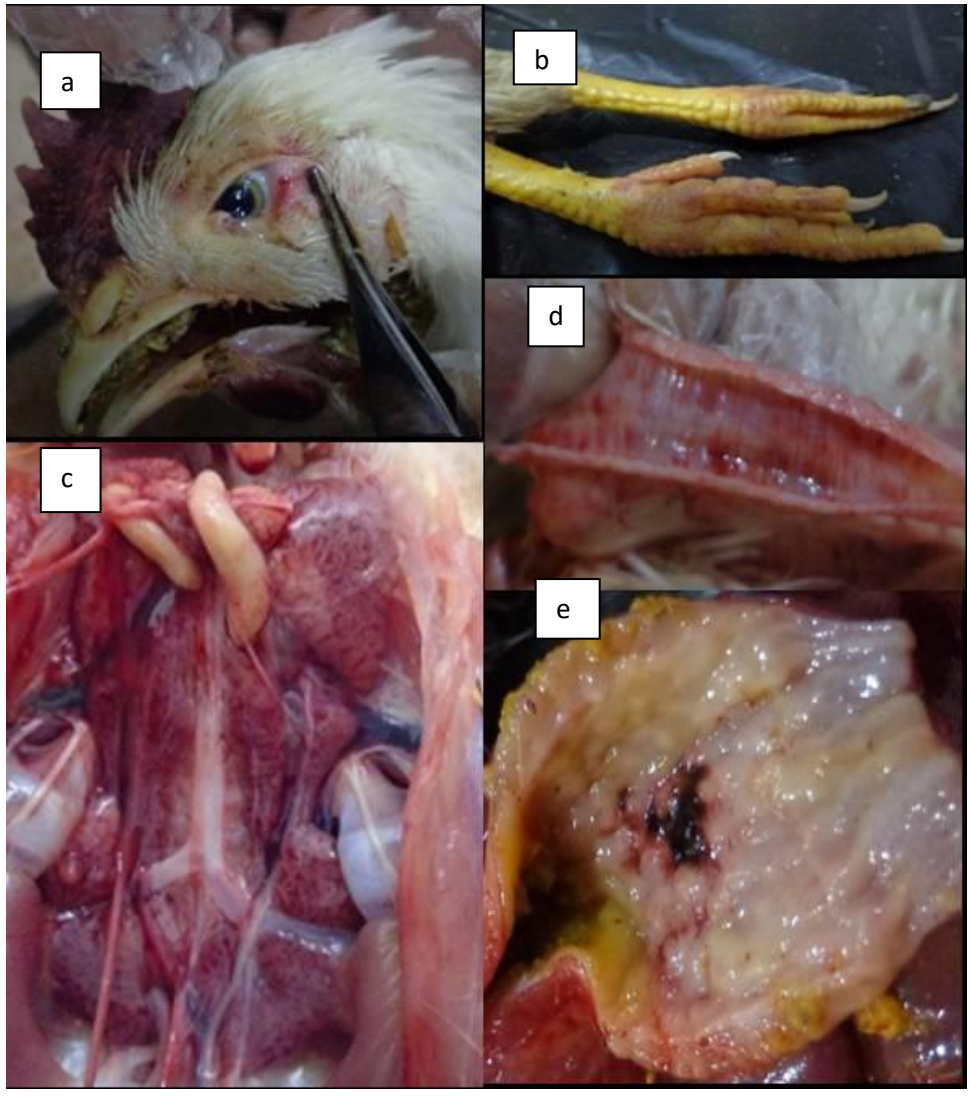

Plate 1. Gross pathological lesions on body and viseeral organ. Conjunctivitis and (a) cyanotic comb, (b) cyanotic claws (c) inflamed kidneys and (d) haemorrhagic proventriculus

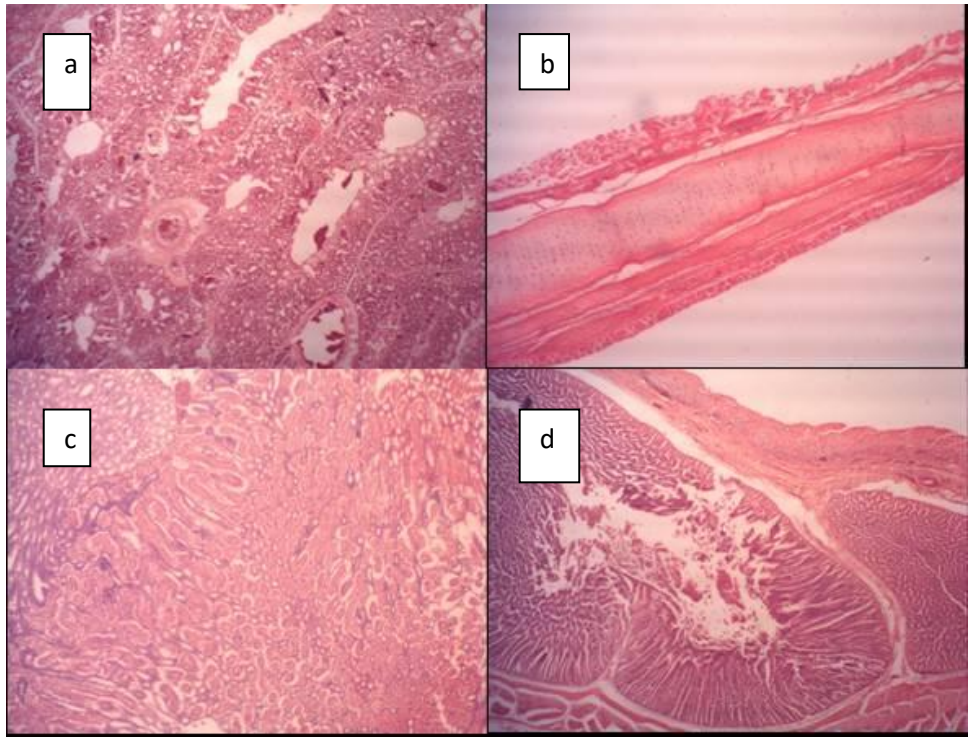

Plate 2. Histopathological lesions in different organs (a) Necrotic lung, (b) epithelial degeneration and necrosis in trachea (c) inflamed kidney with necrotic areas and (d) haemorrhagic foci in proventriculus

In the present study, pathogenicity of chicken derived LPAIV sub-type H7N3 was assessed in three species infected by three different routes. Oculo-nasal route is the natural route of infection for AIVs, therefore pathogenicity was higher by this route while intravenous route results in a direct viremia which also produces moderate lesions. Kwon *et al.* (2010) suggested that upper respiratory route of infection results high virus infection and transmission than oral exposure which requires a higher infection dose. Alexander *et al*. (2008) had also assessed pathogenicity of eight isolates of H5 inoculated through intramuscular, intra-nasal route as well as contact pathogenicity. Results of present study revealed that intravenous pathogenicity index H7N3 in chicken was 1.02 where as in quail it was 0.5 and none in duck. As per WHO standards for intravenous pathogenicity, the isolate A/Chicken/Pakistan SPVC 26/03 (H7N3) is of low pathogenicity. Shahab (2015) determined the IVPI of an isolate of H7N3 AIV obtained from field samples of Karachi and found that it was low pathogenic as its IVPI in 4 weeks-old SPF chicken was 0.49. Previously Khanum *et al*. (2008) reported IVPI of H7 isolate (K-03), isolated Karachi as 0.48. Probably circulating H7 viruses in Karachi are largely of low pathogenicity. However, results of present study show marginally higher score IVPI that is 1.0, could be due to continuous passage in embryonated eggs that might had developed adaptation

of this virus isolate for chicken. The isolate showed remarkably less pathogenicity in quails and no pathogenicity in ducks.

Nephritis was the major gross pathological finding in chicken infected through IV, OCN and OR routes. However, highest frequency of lesion appeared in intravenous route, followed by bursa, lung and trachea. Whereas, trachea and lungs were second to kidneys, in oculo-nasal route. Also reported that LPAIVs have tissue tropism for kidney and respiratory system. We could not observe any gross pathological change in any organ of duck and quail. Similarly, Costa *et al.* (2011) reported that mallard ducks and three other avian species i.e. redheads (*Aythya americana*), wood ducks (*Aix sponsa*), and laughing gulls (*Leucophaeus atricilla*) infected with two three different mallard origin LPAIV i.e. H5N2, H7N3 and H3N8 did not show any gross pathological lesions. Studies have reported that Japanese quails are highly susceptible to HPAIV where as LPAIV are colonized in respiratory tract but no typical clinical signs of AIV appear (Betran *et al*., 2013). Despite not showing clinical signs in ducks and quails, it cannot be ruled out that these avian species may remain carrier of LPAIV and pose threat of transmitting infection to chicken population.

Anti-AIV antibody titers varied with species, time and route of inoculation in the present study. Spackman *et al*. (2010) have also reported the differences in sero-conversion after infection with LPAIV H7 in chicken, duck and turkey. Highest antibody titres were found in chicken which suggests that chickens were more susceptible to infection than quails and ducks. It was further observed that sero-conversion was higher in intravenous route and oculo-nasal route that shows that route of infection has impact on severity of infection.

CONCLUSION

It is concluded from the results of present studies that susceptibility to LPAIV H7N3 varies with species and route of inoculation. Chicken are more susceptible to LPAIV H7N3 while ducks and quails are resistant. Susceptibility is higher in oculo-nasal route, followed by intravenous infection and is lowest in oral route. Cyanotic comb and Hemorrhagic shanks are typical clinical signs nephritis is most common necropsy finding in H793 infection. Serocon version in ducks and chicken shows that they may harbor subclinical infection.

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