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Pathological Studies of A / Chicken / Tehran / ZMT - 173/99 (H9N2) Influenza Virus in Commercial Broiler Chickens of Iran

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Abstract: Avian influenza (AI) outbreaks due to H9N2 subtype of avian influenza virus (AIV) occurred in poultry industry in Iran, in 1998 and caused serious economic losses. The aim of this study was to investigate the pathogenesis, clinical signs, gross and histopathological findings of the chickens experimentally inoculated with A/Chicken/Tehran/ZMT-173/99 (H9N2) influenza virus, isolated from the kidney of the broiler chickens with 40% mortality. Two groups of 30-day-old, forty *Mycoplasma gallisepticum* positive (Mg+) and forty negative (Mg-) commercial broiler chickens were used. Each group subdivided into 10-membered two experimental and two control subgroups. One experimental subgroup inoculated intravenously (IV) and the other both IV and oculonasally (ON) with $10^{7.5}$ ELD50. Clinically, depression, crouching, huddling, ruffled feathers, coughing, sneezing, and sometimes gasping were observed. Mortality rate was 10% in each Mg+ experimental subgroup. The gross lesions in dead birds included exudative (fibrinous) casts in tracheal bifurcation, pulmonary congestion, thickened air sacs, swollen kidney with urate deposition, enlarged congested and hemorrhagic bursa of fabricious and thymus, petechial hemorrhages in epicardial fat and general congestion in the carcasses. The visceral organs were congested and edematous. Fibrinous tracheal casts in bifurcation were only observed in a chicken inoculated via both IV and ON routs. Histopathologic examination revealed extensive pulmonary hyperemia with infiltration of mononuclear inflammatory cells in lamina propria of bronchi and lungs, mild lymphocytolysis and atrophy of lymphoid follicles of the bursa of fabricious. Severe congestion, depletion of lymphocyte population and focal necrosis were seen in thymus lobules. Severe congestion, urate deposition and nonsuppurative focal interstitial nephritis was the predominant histological lesions. The results of the present study suggest renal lesions and tracheal casts are the principal causes of mortality and simultaneous inoculation play an important role in formation of tracheal casts. Also, immunosuppression due to depletion of lymphoid organs as well as concurrent infections such as *M. gallisepticum* might increase the pathogenic potential of H9N2 subtype to chicken.

Key words: Avian influenza, H9N2 subtype, chickens, pathogenic potential and pathology

Introduction

Influenza viruses belong to the family *Orthomyxoviridae* and to the genus *influenza virus*. These viruses are classified into three types A, B and C, on the basis of their internal nucleoprotein and matrix protein antigens. Both antigens are regarded as common to all strain of same type. Influenza viruses are further categorized into subtypes, according to their surface hemagglutinin (H) and neuraminidase (N) glycoproteins (Swayne and Halverson, 2003). Avian influenza viruses (AIVs) belong to type A and 16 H subtypes (H1-H16) and 9 N subtypes (N1-N9) have been reported (Fouchier *et al*., 2005). Based on the pathogenicity of AIVs to domestic poultry, these viruses are subclassified into two pathotype groups of highly pathogenic avian influenza (HPAI) viruses, causing rapid mortality in poultry which often approaches 100% and non - highly pathogenic avian influenza (nHPAI) viruses including mildly pathogenic (MP), low pathogenic (LP) and non pathogenic (NP) AIVs, causing unapparent diseases with mild respiratory signs, egg production losses and sometimes with slightly elevated mortality (Capua and Alexander, 2006). However, when exacerbation of this influenza infection is caused by other bacterial and viral organisms or environmental conditions, severe disease with high mortality may be seen, although the viruses isolated in these cases still produce little or no disease in experimentally infected chickens (Banks *et al*., 2000; Bano *et al*., 2003; Swayne *et al*., 1998). To date, all HPAI isolates have been of H5 and H7 subtypes, although viruses of these subtypes do not necessarily cause HPAI (Swayne *et al*., 1997; Zanella *et al*., 2001; Capua

and Alexander, 2006). These viruses are listed as A group diseases by the Office International des Epizooties (OIE) (OIE, 2002).

Avian influenza disease due to H9N2 subtype in poultry during later part of the 1990s has been noticeably increased worldwide. The H9N2 subtype outbreaks occurred in domestic ducks, chickens and turkeys in Germany during 1995 and 1998, in chickens in Italy in 1994 and 1996, in pheasants in Ireland in 1997, ostriches in South Africa in 1995, turkeys in the USA in 1995 and 1996 and in chickens in Korea in 1996 (Bano *et al.*, 2003; Capua and Alexander, 2004; Swayne and Slemons, 1998; Naeem *et al.*, 1999). More recently, H9N2 viruses have been reported in Middle Eastern countries and have been responsible for widespread and serious disease problems in commercial chickens in Iran, Pakistan, Saudi Arabia and United Arab Emirates (Aamir *et al.*, 2007; Alexander, 2003; Banks *et al.*, 2000; Capua and Alexander, 2004; Naeem *et al.*, 1999; Nili and Asasi, 2002; Vasfi Marandi and Bozorgmehri Fard, 2002).

Avian influenza due to H9N2 subtype was occurred in densely populated area of Tehran province of Iran and caused serious economic losses in poultry industry in 1998. Since these highly contagious viruses were spreading in poultry flocks of other provinces of country, a vaccination strategy by using inactivated H9N2 vaccine was adopted to control AI disease in poultry industry. However, some outbreaks continued to occur in several broiler flocks with great economic losses. All H9N2 subtype viruses isolated from vaccinated or unvaccinated chickens belonged to nHPAI till now (Vasfi Marandi *et al.*, 2003 and 2000; Vasfi Marandi and Bozorgmehri-Fard, 2002 and 1999). But, increasing mortality due to severe interstitial nephritis along with urate deposition and pulmonary congestion along with formation of exudative cast in tracheal bifurcation in some broiler flocks infected with H9N2 subtype pose a question about its pathogenicity. This study was designed to investigate the pathogenicity potential, clinical signs, gross and histopathological findings observed in the Mg+ and Mg- commercial broiler chickens infected by A/Chicken/Tehran/ZMT-173/99(H9N2) subtype of the AI virus.

Materials and Methods

Chickens: The Ross broiler chickens obtained from two different *Mycoplasma gallisepticum* positive (Mg+) and negative (Mg-) commercial broiler breeders vaccinated against H9N2 subtype of avian influenza were reared up to 30 days in two 40 member groups caged in separate places with similar conditions. They were housed up to 40 days, the end of the experiment.

Virus: A/Chicken/Tehran/ZMT-173/99 (H9N2) influenza virus isolated from the kidney of chickens in a broiler chicken flock, with 40% mortality was used in this study (Vasfi Marandi and Bozorgmehri-Fard, 2002). This virus was propagated in 10-day-old emberyonated chicken eggs using standard procedure (Swayne *et al.*, 1998). Amino allantoic fluid (AAF) was harvested and used as inocula.

Experimental design: A total of eighty 30-day-old chickens in two Mg+ and Mg- groups were used. Each group subdivided into four 10-membered subgroups, including two experimental and two control subgroups. The housing conditions were chosen as similar as farm condition. Feed and water were provided *ad libitum* during the time of experiment. Blood samples were taken to determine H9N2 antibodies with hemagglutination inhibition (HI) test before inoculation. Then, one experimental subgroup in each group was inoculated intravenously (IV) through brachial vein and the other was inoculated via both IV and oculonasal (ON) routs with a volume of 0.2 ml of a 1:10 dilution of infectious AAF with infectivity titer of $10^{7.5}$ ELD50 (Embryo Lethal Dose 50) as inocula. The four control subgroups were inoculated with sterile PBS as inocula. During 10 post inoculation days (PID), all subgroups were observed if they have clinical signs of disease or not and all observations were recorded. During this period, necropsy was done on dead chickens and all gross lesions were recorded. At the end of experiment at 40 days old, all survival birds were slaughtered for necropsy after blood sampling. No vaccinations were used in this experiment.

According to the clinical signs and the number of dead birds during 10 post inoculation days, Intravenous Pathogenicity Index (IVPI) of the virus in different control and experimental subgroups were calculated using European standard method described for calculation of IVPI of Newcastle disease virus upon which the experimental design was modified by Swayne *et al.* (1998).

For virus isolation, tissue samples of kidney, trachea, lung, thymus and bursa of fabricious collected from all dead and slaughtered birds were used. Standard procedures were used to reisolate the virus in emberyonated chicken eggs and to confirm the H subtype of isolates according to the procedure described by Swayne *et al.* (1998). Sera samples were obtained from all chickens at days 1, 7, 14 and 28 days and from any surviving chickens in each subgroup at tenth PID before being euthanized. Blood samples were tested by the HI test using H9N2 subtype antigen as described by Swayne *et al.* (1998). Rapid serum agglutination (RSA) test was done on all sera taken from Mg+ and Mg- subgroups on days 1 and 40 to see if they are positive or negative (Swayne *et al.*, 1998). The samples were tested by ND–HI on days 1, 7, 28 and 40 to follow up the Newcastle disease infection and by

IB–ELISA on days 1, 28 and 40 to follow up the infection with infectious bronchitis.
Tissue samples were collected from two dead birds in Mg+ experimental subgroups. The trachea, lung, kidney, liver, spleen, bursa of fabricious and thymus samples

were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 µm and stained with Hematoxylin and Eosin (H and E) for histopathologic examinations.

Findings
Clinical findings: No clinical signs were seen in control subgroups. In the experimental subgroups, the clinical sings were observed at second, third or fourth post inoculation days (PID). The clinical signs generally were depression, crouching, huddling, ruffled feathers and mild respiratory signs including coughing, sneezing and sometimes gasping especially in Mg+ subgroups. Two chickens died at 5 and 7th PID in Mg+ subgroups inoculated via both IV and ON routs and IV rout alone respectively.

Gross findings: At necropsy, carcasses were congested and severe congestion was noticed in visceral organs especially the liver. Congestion and subcapsular petechial hemorrhages were seen in spleen. Bursa of fabricious and thymus were congested, edematous and had subcapsular petechial to ecchymotic hemorrhages. The tracheal mucosa and lungs were severely congested and edematous. Formation of exudative (fibrinous) casts in tracheal bifurcation that extended to the lumen of secondary bronchi was observed only in the chicken that died at 5th PID from IV and ON routs inoculated Mg+ subgroups (Fig. 1). The air sacs were opaque and thickened (Fig. 1).The kidneys were severely swollen with urate deposition (Fig. 2). Numerous petechial hemorrhages in epicardial fat were present.

IVPI: Calculated Intravenous Pathogenicity index (IVPI) of the H9N2 subtype of AIV in all experimental and control groups were less than 1.

Serological findings: In serology, antibody against H9N2 subtype antigen was diagnosed in the sera samples of one-day-old chickens in HI test, but the samples taken before inoculation on day 30 were all negative in HI test. All sera samples taken on 10 PID in experimental subgroups were positive against H9N2 in HI test with high antibody titers. All sera from control subgroups taken on 1st and 10th PID were negative in HI test.
All sera taken on day 40 from Mg+ subgroups were positive and all sera taken from Mg- subgroups were negative in RSA test. All samples were positive in IB–ELISA and ND–HI tests on day one and were negative before inoculation. All sera were remained negative in IB–ELISA and ND–HI tests on day 40 too.

Virologic findings: In control subgroups, following inoculation of the allantoic cavity of the emberyonated chicken eggs with the tissue suspension including kidney, bursa of fabricious, thymus, trachea and lung obtained from slaughtered birds on the 10th PID, all embryos survived during 7 days post inoculation, and collected AAF of these samples, were negative in HA test.
In experimental subgroups, following inoculation of the emberyonated eggs with tissue suspension including kidney, bursa of fabricious, thymus, trachea and lung obtained from dead birds on 5th PID and 7th PID as well as slaughtered birds on 10th PID, some losses in embryos, were seen on 2 or 3 days after inoculation. The collected AAF of these eggs was positive in HA test and specific antiserum of H9N2 inhibited the hemagglutination activity of the allantoic fluids in HI test.

Histopathologic findings: Histopathologic examinations revealed pulmonary hyperemia with infiltration of mononuclear inflammatory cells in lamina propria of trachea, secondary bronchi, lungs and air sacs. Congestion, nonsuppurative focal interstitial nephritis, foci of hemorrhages and lytic necrosis and congestion and hypercellularity of glomeruli and urate deposition were present in kidney (Fig. 3, 4 and 5). Mild to moderate generalized lymphocyte depletion, atrophy of lymphoid follicles, corrugation of epithelium and severe interfollicular edema were observed in the bursa of fabricious (Fig. 6) and the reticular epithelium between the cortex and medulla of follicles were prominent (Fig. 6 and 7). Severe congestion, depletion of lymphocyte population and focal necrosis in cortex and prominence of reticuloepithelial cells and myocytes in medulla were seen in thymic lobules (Fig. 8). The spleen was congested with mild lymphoid hyperplasia. Lymphoid tissues including thymus and bursa of fabricious were more severely affected in the chicken that died at 7 PID inoculated by IV rout. The liver sinusoids were congested and hepatocytes granular degeneration was present.

Results and Discussion

During 1990-2007, outbreaks of nHPAI caused by H9N2 subtype have been reported from Germany, Italy, Ireland, South Africa, Bulgaria, Korea, China, Hong Kong, Iran, Pakistan and other middle east countries (Aamir *et al.*, 2007; Bano *et al.*, 2003; Guo *et al.*, 2000; Kim *et al.*, 2006; Swayne and Slemons, 1998). In Iran, Vasfi Marandi and Bozorgmehri-Fard (2002) isolated H9N2 subtype of AIV from the chicken flocks of Tehran province in an outbreak in 1998. This isolate didn't cause any losses in 30-days old inoculated chickens during

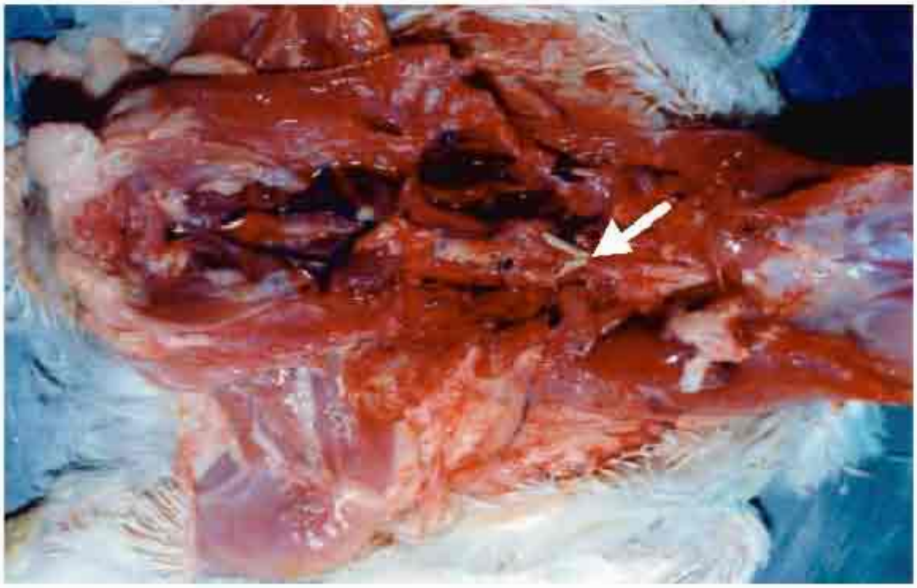

Fig. 1: A 35- day- old Mg+ chicken, was inoculated with a H9N2 subtype via IV and ON routs, and died 5th PID. Pulmonary congestion and exudative cast in tracheal bifurcation that extended to the lumen of secondary bronchi are present.

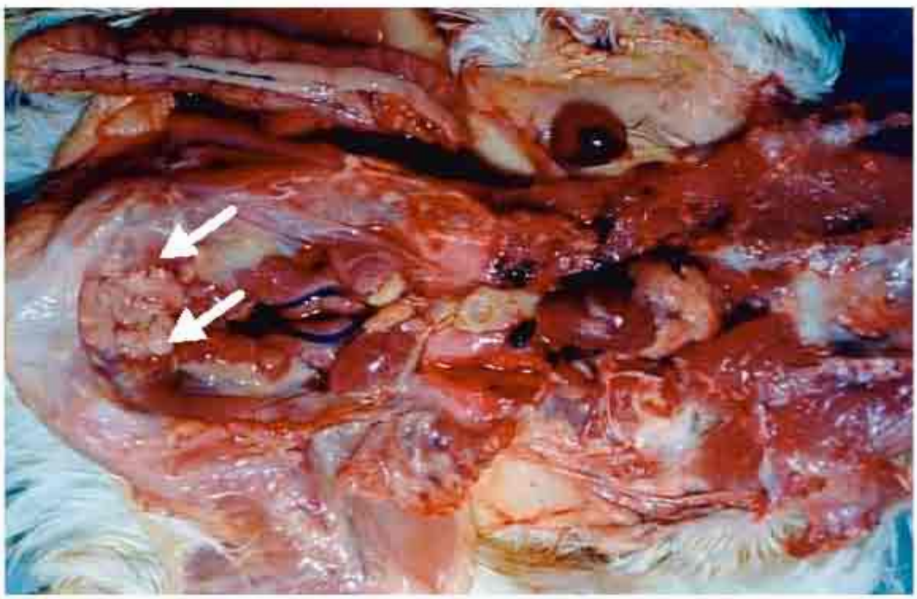

Fig. 2: A 37- day- old Mg+ chicken, was inoculated with a H9N2 subtype via IV rout, died 7th PID. Severely swollen kidneys and renal urate deposition are present.

experimental trials, but caused depression, crouching, listlessness and decreased feed consumption and sometimes diarrhea, coughing and sneezing. Therefore, this virus has been defined as non pathogenic (NPAI) or non-Highly pathogenic Avian Influenza (nHPAI). Subsequent serological and virological studies showed that H9N2 isolates are prevalent in the poultry farms almost in all provinces of Iran. These outbreaks caused great economic losses in poultry industry due to increased mortality and decreased egg production. Mortality rates increased up to 65% in chickens in an outbreak in 1998 (Vasfi Marandi *et al.*, 2003 and 2000; Vasfi Marandi and Bozorgmehri-Fard, 2002 and 1999). Besides, similar results were obtained by Toroghi and Mornayez (2006) and Pourbakhsh *et al.* (2000). This pathotype is recently named LPAI (Capua and Alexander, 2006).

However, lack of correspondence between pathogenicity

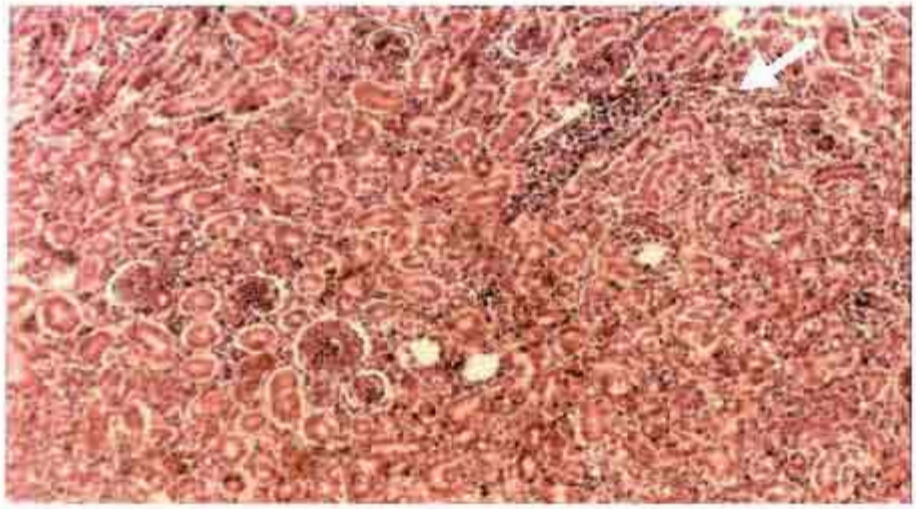

Fig. 3: Kidney of 37-day-old Mg+ chicken was inoculated with a H9N2 subtype via IV rout, died 7th PID. Congestion and hypercellularity of glomeruli and nonsuppurative focal interstitial nephritis (arrow) are present (H and E, × 100).

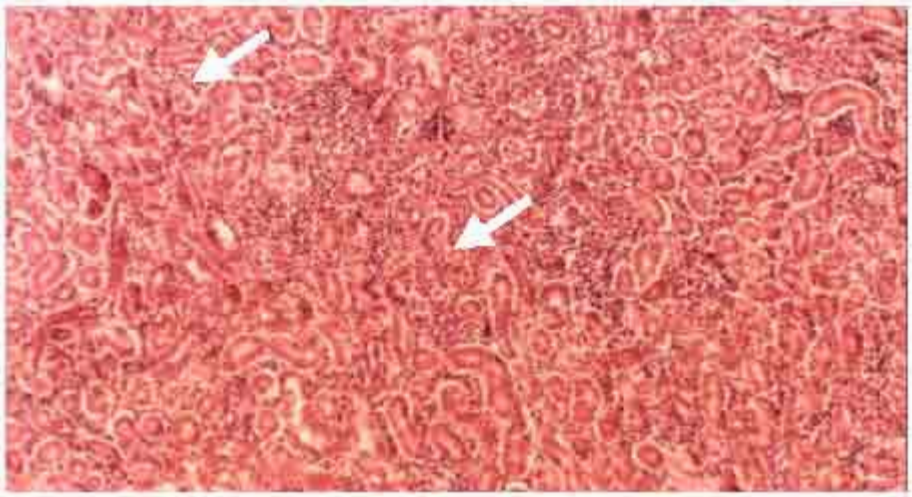

Fig. 4: Kidney of 37-day-old Mg+ chicken was inoculated with a H9N2 subtype via IV rout, died 7th PID. Nonsuppurative tubulointerstitial (arrows) nephritis is seen (H and E, × 100).

of H9N2 subtype of AIV in experimental and field conditions has been investigated by many researchers. It has been declared that the factors such as management, concurrent bacterial or viral diseases, immunosuppression agents, age and strain of chicken, are the main reasons of the pathogenicity variation of H9N2 isolates (Aamir *et al.*, 2007; Capua and Alexander, 2004; Guo *et al.*, 2000; Toroghi and Momayez, 2006). Bano *et al.* (2003) indicated that H9N2 subtype of AIV as a nonpathogenic virus can cause a severe infection in field condition in presence of opportunist secondary pathogens. They also showed that in chemically bursectomised chickens, H9N2 subtype can cause high mortality. Banani *et al.* (2002) and Nili and Asasi (2003) suggested that concurrent infections with Infectious Bronchitis and secondary bacterial infection such as *Ornithobacterium rhinotracheal*, *E. coli* and *M. gallisepticum* may be more important enhancers of the signs than the other factors in H9N2 infection in chickens. Proteolytic cleavage of the influenza virus HA glycoprotein by cellular proteases is a prerequisite for virus infectivity, spread, tissue tropism and its pathogenicity. Some bacterial enzymes recognize a monobasic cleavage signal at HA of the mammalian and the nHPAI viruses. Amino acid sequence analysis of HA gene of cleavage site of A/Chicken/Tehran/ZMT-173/99 subtype is crucial to demonstrate any probable change in the pathotype of virus (Callan *et al.*, 1997; Rott *et al.*, 1995).

During the influenza outbreak of Italy in 1999-2000,

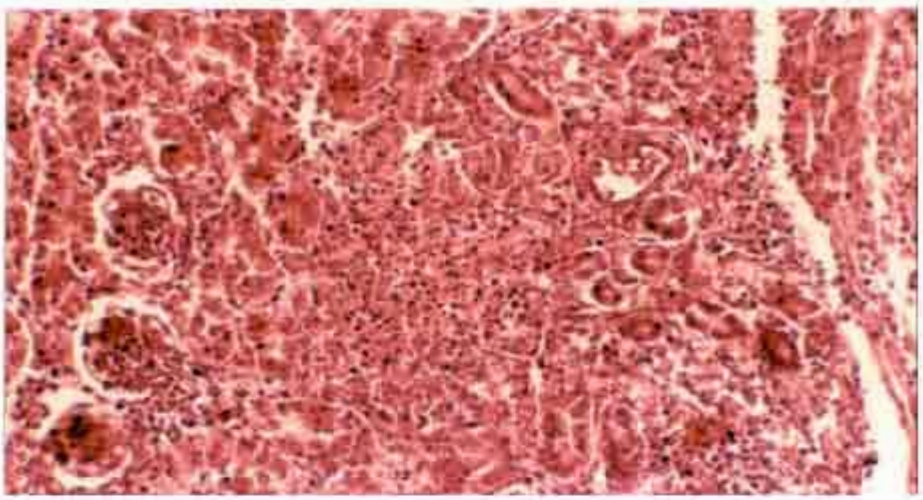

Fig. 5: Kidney of 37-day-old Mg+ chicken that was inoculated with a H9N2 subtype via IV rout died 7th PID. Focal necrosis with infiltration of mononuclear and granulocytic inflammatory cells is seen. Necrotic debris is present centrally (H and E, × 200).

Fig. 6: Bursa of fabricious of 35-day-old Mg+ chicken was inoculated with a H9N2 subtype via IV&ON routs, died 5th PID. Mild to moderate lymphoid depletion, atrophy of lymphoid follicles, and corrugation of epithelium are seen (H and E, × 40).

which occurred over two time periods of LPAI outbreak followed by a HPAI outbreak, there was reported variable mortality ranging from 5% to 90%, depending on the age of the affected birds and the presence of secondary infections such as *Pasteurella multocida*, *O. rhinotracheal* and Mycoplasmas (Zanella *et al.*, 2001). Kim *et al.* (2006) showed that a Korean avian H9N2 influenza virus was able to cause 30% mortality and the reduction of egg production. Guo et al. (2000) reported that a Chinese H9N2 virus strain caused 80% mortality rates in 12-weeks old layer chickens, despite its failure to meet the criteria for a HPAI virus. The discrepancies observed in the pathogenicity of H9N2 strain between this experimental infection and those reported by Toroghi and Momayez (2006), Vasfi Marandi and Bozorgmehri-Fard (2002) and Pourbakhsh *et al.* (2000) may be due to strain of chickens, health status of commercial chickens, the routs of virus inoculation as well as strain of H9N2 viruses. In this trial, 10% mortality occurred in Mg+ experimental subgroups, suggesting that Mg infection may be an enhancer of the pathogenicity of the H9N2 strain and may caused increased mortality in Mg + broiler flocks.

The rout of inoculation has direct effects on the resulted illness in experimental trials. Mild respiratory changes observed in IV, IT and ON inoculated SPF chickens with

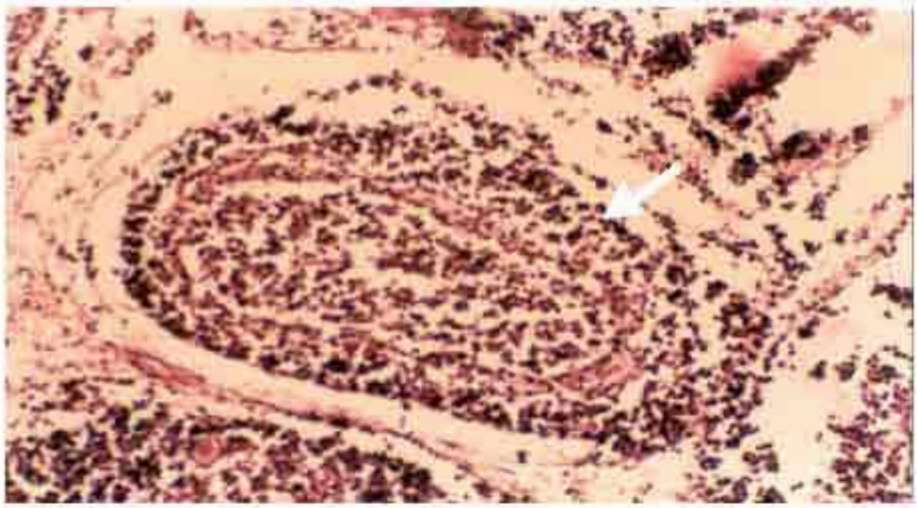

Fig. 7: Bursa of fabricious of 35-day-old Mg+ chicken, was inoculated with a H9N2 subtype via IV&ON routs, died 5th PID. Moderate lymphoid depletion with prominence of reticular epithelium between cortex and medulla in a lymphoid follicle (arrow) and severe interfollicular edema are present (H and E, × 200).

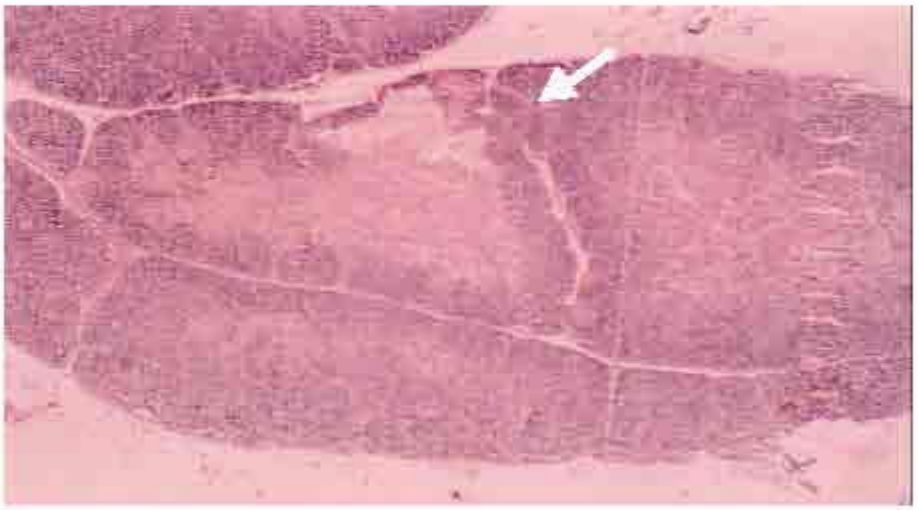

Fig. 8: Thymus of Mg+, 35-day-old chick was inoculated with a H9N2 subtype via IV&ON routs, died 5th PID. There is severe congestion and focal necrosis (arrow) and sparse lymphocytic population in the cortex (H and E, × 50).

a H9N2 subtype strain, indicated the effect of rout of inoculation on induced disease (Hablolvarid *et al.*, 2004 and 2003). In this trial, in order to study the pathogenicity of A/Chicken/Tehran/ZMT-173/99 (H9N2) subtype, we firstly designed a pilot study and inoculated Mg+ chickens by IV rout alone, but formation of cast in tracheal bifurcation were not observed. Therefore, a novel experimental model including IVPI evaluation by using Mg+ chickens by IV along with ON routs was adopted. Severe congestion and presence of exudative casts in tracheal bifurcation were important respiratory lesions. Tracheal casts were only observed in a chicken inoculated via both IV and ON routs and died at 5 PID because of asphyxia which has not been previously shown in experimental infections both in SPF and commercial chickens. In histopathologic examinations, infiltration of inflammatory cells in the wall of bronchi and lungs confirmed involvement of respiratory system. This observation suggests that simultaneous inoculation of chicken play an important role in formation of tracheal casts. Previous studies described with LP H9N2 subtype by the IT (intra tracheal) or IN (intra nasal) routs, commonly resulted in virus replication lesions, both within the trachea, lung and kidneys of chickens (Hablolvarid *et al.*, 2004)

In present study, the general congestion and observable

lesions in most of the visceral organs were apparent in macroscopic and microscopic examinations and confirmed H9N2 viremic nature, in both dead chickens. This was predictable because of IV inoculation and real viremia. The general lesions especially in visceral organs in different studies with IV inoculation confirmed a viremic period in chickens (Hablolvarid *et al.,* 2004 and 2003; Halverson *et al.,* 1980; Swayne and Halverson, 2003). Vasfi Marandi and Bozorgmehri-Fard *et al.* (2003) indicated that congestion of kidney and urate deposits are observable signs which were seen in this study, too. The renal lesions which are mainly interstitial nephritis or tubulointerstitial nephritis were frequently reported in outbreaks of HPAI and n-HP AI (Hablolvarid *et al.,* 2003; Halverson *et al.,* 1980; Swayne and Halverson, 2003). According to the renal lesions (tubular necrosis and nephritis) reported by Swayne and Slemons (1998) and detection of the virus from these sites, it can be suggested that kidneys are a suitable place for replication of AIV. However, since 1969, Swayne and many other researchers have indicated the nephrotropism nature and nephropathogenicity of AIV (Hablolvarid *et al.,* 2003; Swayne and Alexander, 1994; Swayne and Slemons, 1990). As the observations of Vasfi Marandi and Bozorgmehri-Fard (2002) in during various outbreaks of H9N2 subtype with high mortality in the broiler flocks of Tehran province in 1998, the rate of virus isolation from swollen kidneys has been significantly increased. It seems that the high mortality reported in some of these flocks related to the invasion of AIV to the kidneys and resulted renal failure. Regarding the lesions observed in necropsy and histopathologic examinations in the present study, kidney lesions such as severe inflammation and focal lytic necrosis were the most predominant histological lesions. It is suggested that renal lesions are principal cause of mortality, so, early death caused by internal imbalance due to renal failure, may be the main reason of absence of respiratory sings such as pneumonia and fibrinous casts in tracheal bifurcation.

Necrotic depletion of lymphoid centers in turkeys infected with Turkey/Ont/7732/66 (H5-H9) influenza virus had been described (Resende, 1980). Resent studies indicates that Turkey/Ont/7732/66 also produces severe lymphoid necrosis in experimentally inoculated chickens, this necrosis was evident in lymphoid cells present in spleen, thymus, bursa, intestinal tract and lung. Birds that die from LPAI have lymphocyte depletion and necrosis or apoptosis of lymphocytes in the cloacal bursa, thymus and other areas with lymphocyte accumulations. In experimental studies in mallard Ducks, LPAI virus infections suppressed T cell function (Swayne and Halverson, 2003). Immunosuppression of chickens due to H9N2 infection as well as bacterial co-infection such as *M. gallisepticum* and *E. coli* might increase the pathogenicity of H9N2 infection (Callan *et al.,* 1997; Rott *et al.,* 1995).

In the present study, in chickens inoculated IV or IV along with ON routs, presence of congestion, edema and apparent hemorrhages and lymphocytes depletion in thymus, seemingly indicates it's involvement during influenza disease. Presence of the same signs in bursa of fabricious along with the lesions in thymus can indicate the involvement of immune system and immunosuppressive effect of H9N2 subtype to chicken, but of course, further studies are needed to show such effect of H9N2 subtype on immune system of chickens.

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