

Study on Coinfection of *Mycoplasma gallisepticum* and Low Pathogenic Avian Influenza Virus H9 in Broilers

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Abstract

Avian influenza viruses (AIVs) belong to the family *Orthomyxoviridae* which consists of three genera known as *Influenza virus -A, -B* and *-C*. The influenza viruses, which can cause infections in avian species belong to genus *influenza virus A* which can produce two different types of diseases on the basis of their virulence and pathogenicity. Thus are classified as a highly pathogenic avian influenza virus (HPAIV) and a low pathogenic avian influenza virus (LPAIV). Recent outbreaks of LPAIV H₉N₂ in Asian countries such as in Pakistan and China revealed that this subtype has become endemic in poultry population of these countries. Meager literature is available about its pathogenicity. This study was made to ascertain the pathogenesis of H₉ subtype AIVs in broilers along with most prevalent respiratory pathogen *Mycoplasma gallisepticum* under field conditions. In first part of study, challenge organisms (H₉ virus and *Mycoplasma gallisepticum*) were isolated from field samples and experimental inoculum was prepared. In second part, pathogenesis of H₉ virus in association with *M. gallisepticum* was carried out under controlled environmental conditions. The virus prevailing in Pakistan was found nonpathogenic as only slight diarrhoea and depression in few birds was seen while clinical signs and lesions produced grossly and microscopically were more severe in form of severe conjunctivitis and facial edema and respiratory involvement with hemorrhagic and pneumonic lungs and degenerative and lytic necrosis in kidneys in birds infected co-infected with H₉ virus and *Mycoplasma gallisepticum* as compared to other groups which were infected with single pathogens. Immunohistochemical detection of viral antigen revealed that replication of virus was more severe in birds infected with H₉ virus and *Mycoplasma gallisepticum* as compared to other groups. In conclusion, the virus subtype H₉ circulating in Pakistan is low pathogenic in nature with affinity for respiratory system and urinary system while coinfection with *Mycoplasma gallisepticum* is the most important factor enhancing the pathogenicity of H₉ virus under field conditions.

Keywords: H₉N₂ virus; Pathogenicity; Avian influenza virus; *Mycoplasma gallisepticum*; Coinfection

Introduction

Influenza virus A can infect domesticated poultry birds and other mammals including man, whereas wild migratory and water birds serve as the natural reservoir hosts. In these species, virus causes little or no disease but co-exists in almost perfect balance [1,2]. However in avian species and other poultry birds, it can cause a disease which ranges from a mild, asymptomatic to an acute and fatal condition.

Influenza virus actually belongs to orthomyxoviridae family which was firstly discovered in 1955 [3]. The viruses are classified into types A, B and C on the bases of antigenic difference of the NP and M1 protein [4]. Further subtyping is based on the antigenicity of two transmembrane glycoproteins on the surface of virus which are named hemagglutinin (H or HA) and the neuraminidase (N on NA). These viruses are currently separated into sixteen H (H1- H16) and nine N (N1- N9) antigenic subtypes [5,6].

H₉N₂ viruses have the ability to cause severe respiratory distress accompanied by high morbidity and mortality and a marked reduction in egg production paradoxically under field conditions, despite being completely nonpathogenic in experimental conditions [7]. The frequent heavy losses incurred and increased mortality in infected flocks has raised serious concerns for the poultry industry in many countries. Severity of infection not only depends upon the virulence of subtype, but factors such as age, species susceptibility, environmental and management conditions and concurrent infection with secondary pathogens may also be the responsible for increased morbidity and mortality in chickens [8,9]. Among these factors, secondary respiratory pathogens either bacteria or viruses or both along with LPAIV exacerbated the replication of these viruses in host tissues ensuing in

manifestation of severe disease, although the virus subtype isolated in these cases still produced little or no disease under experimentally infected domesticated birds [10,11]. One of the most important pathogen such as *Mycoplasma gallisepticum*, when co-infected with H₉ virus, resulted in the high mortality which may reach up to 10-60% [12-14].

This experimental study was designed to investigate pathogenic potential of H9 virus infection in the presence of *Mycoplasma gallisepticum* infection on the bases of clinical signs, gross pathology, histopathology and immuno-histochemical staining.

Materials and Methods

Virus isolation was done by inoculating H₉ virus, isolated from field samples in 10 days embryonated eggs according to the protocol adopted by OIE (2005). All the work was performed by using class 2 biosafety cabinets. The chorio-allantoic fluid (CAF) was harvested and viral stock for a challenge was prepared by 1:10 dilution of fresh CAF having 4 HA unit with sterile PBS and 0.2 ml / bird was injected

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intravenously through brachial vein in each bird [15]. Isolation of *Mycoplasma gallisepticum* (MG) [16] was carried from the tracheal swabs, which were cultured at 37°C in Frey's broth for 4 days, sub-cultured onto Frey's agar for another 4 days and observed under low power magnification. One suspected colony with a fried egg colonial morphology was tested against MG specific chicken antibodies and presence of the organism was confirmed. The challenge inoculum was collected from the Frey's broth by centrifugation at 15557 xg for 15 minutes. The isolate was reconstituted in 30 ml of sterile saline and the Haemagglutination test was performed against 0.1% of chicken red blood cell suspension [17]. The isolate was further diluted with normal saline to reach 2 HA units and 0.5 ml inoculum was administered through intra-tracheal route in the experimental birds. Eighty day old broiler chicks were obtained from Big Bird hatchery, Raiwind Road, Lahore, Pakistan and reared in the Experimental Rooms, Department of Pathology, University of Veterinary & Animal Sciences Lahore, during the month of March-April 2009, under standard housing conditions. The birds were fed commercially prepared feed and water ad libitum and were vaccinated against Newcastle disease and Infectious bursal disease. All the chickens were tested negative for antibodies to avian influenza virus H₉ at the age of 30 day, randomly divided into four groups designated as A, B, C and D, and were housed in separate rooms (Table 1). The chickens in group A were inoculated with sterile phosphate-buffered saline (PBS) and were kept as unchallenged control. In group B, chickens were challenged through intravenous (I/V) route by using brachial vein with a volume of 0.2 ml of H₉ virus inoculum at 31st day of age. The chickens in group C were inoculated with 0.5ml of *Mycoplasma gallisepticum* inoculum through intra-tracheal at 25th day of age. The chickens in group D were given infection with *Mycoplasma gallisepticum* on 25th day of age while co-infected with H₉ virus on 31st day of age. The birds in all groups were monitored daily for 14 days (till the end of experiment) for their general conditions, clinical signs of disease and mortality and all observations were recorded. On 5th, 9th and 14th day post-challenge, three birds from each group were randomly selected and slaughtered, all the gross lesions were recorded and organs such as trachea, lungs, liver and kidney were collected for histopathology and virus isolation. Similarly necropsy was conducted of the birds which died during the experimental period.

Results

Avian influenza A virus subtype H₉ was successfully isolated from

Treatment	Inoculation day	Groups			
		A Control	B H ₉	C MG	D H ₉ +MG
PBS	31 st	+	-	-	-
H ₉ virus	31 st	-	+	-	+
MG	25 th	-	-	+	+

+ Inoculated
- Not inoculated

Table 1: Experimental design.

Groups	Depression	Ruffled feathers	Diarrhoea	Respiratory involvement	Nasal discharge	Fascial odema	Mortality
A (Control)	-	-	-	-	-	-	-
B (H ₉ Infected)	++	-	+	-	-	-	-
C (MG Infected)	-	-	-	++	+	-	1/20
D (H ₉ +MG Infected)	++	++	-	+++	+++	++	5/20

- Negative
+ Mild condition
++ Moderate condition
+++ Severe condition

Table 2: Clinical signs recorded in experimental groups.

field isolates and confirmed by using HI test. Chorio-allantoic fluid (CAF) collected from inoculated embryonated eggs were first tested by HA test for the presence of virus. Calculated 4 HA unite was 1: 64. HI test was conducted by using 4 HA unite against H₉ specific reference antisera. The titer obtained ranged from 1:64 to 1:128 indicating that CAF contains subtype H₉ virus which was used as inoculum in the experimental infection. Under low magnification MG positive growth plates showed colonies with characteristic fried-egg like appearance with an opaque central zone deeply grown and a translucent peripheral zone on the surface. In group A all the birds were normal while in group B, between 2-7 days post infection (PI), most of the birds were depressed slightly with low intake of feed. Among these 4 birds developed diarrhea, one bird showed facial swelling while 3 birds were more depressed on 5th day PI. All the birds were recovered from depression after 7 days PI while diarrhea persisted up to 12th day. The birds in group C were suffering with respiratory signs but diarrhea was not evident. On 7th day PI, 3 birds were showing lacrimation and conjunctivitis accompanied by mucoid nasal discharge in next two days. The pronounced respiratory signs were coughing, sneezing and gasping. Moist rales were also observed in 2 birds. One bird was found dead on 9th day PI. In group D, birds showed more pronounced clinical signs in the form of respiratory involvement. The clinical signs evident at day 3 PI were depression, crouching, huddling, ruffled feathers and respiratory signs including sneezing, gasping and nasal discharge. These signs were more pronounced on 5th to 7th day in 6 out of 20 birds while 3 birds showed conjunctivitis, lacrimation and facial swelling on 7th day PI. Two birds were found dead on 6th day PI while three more birds were found dead on 8th, 12th and 14th day of the experiment. All the clinical signs are shown in Table 2. In group A, all the visceral organs were normal with no abnormal gross changes. In group B, trachea, lungs and liver were normal in most of the birds. Only slight hyperemia and congestion was observed in trachea and lungs in two birds each which were slaughtered on 5th day and 9th day PI while kidneys were swollen in most of the birds. The frequency of changes was 40 % in kidneys while only 10% in trachea and lungs. The birds in group C showed congestion and presence of mucoid or catarrhal exudate and in trachea and lungs. Lungs revealed more patches of variable sizes of mild congestion and petechial hemorrhages. The air sacs were cloudy in appearance. In group D, main postmortem findings were accumulation of fibrinous casts in tracheal bifurcation which was extended upto secondary bronchi of lungs. The trachea and lungs were severely congested and edematous, air sacs were opaque and thickened, liver was enlarged and kidneys were swollen with the deposition of urates and areas of necrosis seen. All the gross lesions are shown in Table 3. No histopathological changes were observed in group A while in group B slight changes were observed in respiratory system, which were declination, congestion and infiltration of leukocytes in trachea. Lungs showed the infiltration of leukocytes as well as congestion on 5th day which were more severe on 9th day PI. A significant change observed was swelling of glomeruli and presence of inflammatory cells in tubular region in kidneys. These changes were noted with increasing intensity on 9th and 14th day PI.

Groups	Congestion			Hypertrophy				Fibrillation				Exudate/ odema				Necrotic foci				
	T	L	LI	K	T	L	LI	K	T	L	LI	K	T	L	LI	K	T	L	LI	
A (Control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B (H9 Virus Infection)	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
C (MG Infected)	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
D (H9+MG Infected)	+	+	-	+	-	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-

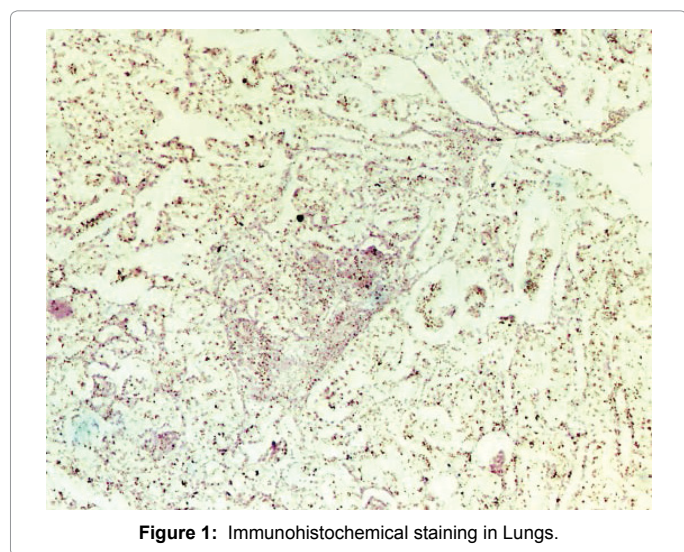
- Absent,
 + Present,
 T Trachea,
 L Lungs,
 LI Liver,
 K Kidney

Table 3: Gross pathological lesions in experimental groups.

Groups	Congestion/ haemorrhage				Leukocytic infiltration				Odema/swelling				Necrosis/degeneration/ Sloughing				
	T	L	LI	K	T	L	LI	K	T	L	LI	K	T	L	LI	K	
A Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B H9	+	+	-	++	+	+	-	++	+	-	-	++	+	-	-	-	
C MG	++	+	-	-	++	++	-	-	+	+	-	-	+	-	-	-	
D MG+H ₉	++	+++	+	+++	++	+++	+	+	+	+++	-	++	-	++	+	++	

- Absent
 + Mild condition
 + + Moderate condition,
 + + + Severe condition,
 T Trachea
 L Lungs
 LI Liver
 K Kidney

Table 4: Histopathological lesions in experimental groups.



However no significant changes were observed in liver except the presence of leukocytic infiltration. In group C, main changes were hyperemia, congestion and swelling and degeneration of goblet cells in trachea. Lungs were also hyperemic and congested with accumulation of mucoid exudate in alveoli. Hyperplasia of alveolar wall was noted due to infiltration of mononuclear inflammatory cells and giant cells. In group D, prominent changes were tracheal and pulmonary hyperemia, congestion and mononuclear cells infiltration on 5th day PI. Goblet cells were degenerated in trachea, in some cases emphysema and presence of casious exudate was seen in alveoli on 9th and 14th day PI. In kidneys congestion, non-supportive focal interstitial nephritis and foci of hemorrhages and lytic necrosis, swelling of glomeruli with urate deposition was observed. Liver sinus ides were congested and granular

degeneration was observed in some cases. All the changes are shown in Table 4. The collected unstained paraffin-embedded sections were prepared from all tissue samples collected and immune-histochemical (IHC) staining was performed to disclose whether the pathological changes were induced by H₉ virus or not. Viral antigen was not evident in tissues of chickens in group A and C while it was identified in kidneys and lungs tissues of infected birds in group B and D. Positive immunohistochemical staining was obvious as dark brown deposits in the nuclei of pulmonary epithelial cells and within nuclei or cytoplasm of necrotic renal tubular epithelium in kidneys (Figures 1 and 2). It was found that viral detection was 70% in birds in group D while in group B it was detected in 40% of tissues.

Discussion

H₉N₂ viruses have been responsible for several outbreaks in domestic ducks, chickens and turkeys in Germany during 1998 and 2004 [18,19], in chickens in Italy in 1994 and 1996 [20], in ostriches in South Africa in 1995 [21], in turkeys in USA during 1995-1996 [22], in chickens in Korea in 1996 [23], and in Pakistan in 1998 [24]. Importantly infection rate is being increasing noticeably in Iran, Pakistan and United Arab Emirates [7,12,24-29]. The published data revealed that the scientists and researchers throughout the world did not pay any attention to LPAIV especially to H9N2 subtype, that is why its infection, mainly in domesticated chickens, has become endemic associated with high rates of outbreaks and elevated pathogenicity across large geographical areas throughout the world since the mid 1990s [24,30,31].

The pathogenicity of low pathogenic avian influenza viruses varies with the viral strain, the species and age of host. Incubation period ranges from 3-7 days and may be up to 21 days [32]. Low pathogenic virus infection may remain almost asymptomatic and in general, may cause distinct as ruffled feathers, a slight and transient decline in egg production in layers or some reduction in weight gain in broilers [33]. But under some conditions more severe pathology was reported in chickens in the form of mild respiratory symptoms like mild tracheal rales, coughing and sneezing [34,35], and diarrhea under some conditions was observed by Davison et al. [36]. Most of the workers did not report mortality due to H9N2 infection [26], but during some studies mortality was reported as 30% mortality was reported in layers [37,38] while in Iran 5% mortality was seen in field conditions in broilers [39].

So far, there has not been any understandable justification for a huge discrepancy in the morbidity and mortality rates between field conditions and experimental induction of the infection in a controlled environment. Many researchers worked to sort out the lack of association between pathogenicity of H9N2 subtype under these two different conditions. Similarly this study was intended to observe the role of *Mycoplasma gallisepticum* infection enhancing the replication of H9 virus and exacerbating pathogenicity in experimental conditions.

In this study H9 virus was isolated from flocks which were suffering from respiratory signs resulting in mild mortality which was up to 5 to 10 % under field conditions. The isolation of *Mycoplasma gallisepticum* was conducted through culturing the tracheal swabs on PPLO broth, the growth of the organism appeared with typical colony characteristics of fried-egg like appearance with an opaque central zone deeply grown and a translucent peripheral zone on the surface of broth.

The overt clinical signs were not much evident in chickens of group B which were infected with H₉N₂ virus, only diarrhoea and slight depression was recorded in few birds while rests of the birds were found normal. The findings of author are similar with the findings of the Davison et al. [36] who similarly reported diarrhea in some conditions in experimental birds. But our findings are different to some extent with the findings of Mutinelli et al. [40] and Anon [35], who reported mild respiratory symptoms like mild tracheal rales, coughing and sneezing in broiler chickens. This could be due to difference in the subtype of virus used, strain of the birds and different climatic conditions from those of author's during experimental study.

Birds in group D which was co infected with H₉ virus and *Mycoplasma gallisepticum*, showed severe clinical signs in the form of severe conjunctivitis and facial edema and respiratory involvement. Gross and histopathological lesions in these groups were severe

congestion, swelling and degenerative and lytic necrosis in kidneys and lungs were severely congested, hemorrhagic and pneumonic as compared to the birds of group B. Inflammation and congestion of kidney, urate deposits and tubule-interstitial nephritis were noted in our study and these findings were according to the results of Vasfi Marandi and Bozorgmehri-Fard et al. [41], Hablolvarid et al. [42]. Our results are agreed with the findings of Nili and Asasi [28] Barbour et al. [43] and Pazani J. et al. [14]. Similar results were obtained by Toroghi and Momayez [44] and Pourbakhsh et al. [45]. Similar results were reported by Banani et al. and Nili and Asasi [13] who demonstrated that concurrent infections with Infectious Bronchitis virus and secondary bacterial infection such as *Ornithobacterium rhinotracheal*, and *M. gallisepticum* may be more important enhancers of the, signs than the other factors in H9N2 infection in chickens. The possible reason for the enhanced pathogenicity could be the release of proteases enzymes by the replication of bacteria such as *Mycoplasma gallisepticum*. These enzymes might recognize a monobasic cleavage signal at HA of influenza virus which plays an important role in the pathogenicity of the virus. Tashiro et al. [46] documented that the protease of *S. aureus* activated the HA of the influenza virus, allowing multiple cycles of virus replication in the lungs of mice. On the bases of this observation it is thought that co-infection with *M. gallisepticum* may confer a similar effect on H9N2 virus replication in chickens. An alternative explanation for the exacerbation of the pathogenicity of H9N2 influenza virus infection is that the stress of bacterial infection affects the immune system of chickens. Cytotoxic T cells play an important role to prevent the viral infection but under stressful condition these cell become deficient and cannot confer protection against viruses. Thus it can be concluded that this pathogen provided favorable conditions for the increased pathogenicity of H9 virus which resulted in the form of severe morbidity and mortality.

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