



Research Article

# Comparative Pathogenic Potential of Avian Influenza A/H9N2 Viruses Isolated from Commercial, Backyard and Fancy Birds

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## Authors' Contributions

TY and MYT conceived and designed the experiment. MFS and MA did experimental work. MFS and AA performed statistical analysis. MFS and AR wrote the manuscript. SY did bioinformatic analysis.

## Keywords

Antigenic variants, H9N2, Histopathology, Pathogenicity, Serobiochemical

**Abstract** | Poultry industry is confronting numerous viral infections particularly low pathogenic avian influenza virus (LPAIV) H9N2, which can bring exceptional abatement in trade of poultry products in endemic countries such as Pakistan. The current study was conducted to evaluate the infectious and antigenic potential of three different AIV (A/H9N2) variants isolated from commercial, backyard poultry and fancy birds (partridge). Commercial broiler birds were infected experimentally at the dose of  $10^6$  EID<sub>50</sub> by using these three isolates. Results revealed that onset of clinical signs was started at 2<sup>nd</sup> day post infection (DPI) followed by severity in redness and swelling of eyes, sneezing and lacrimation at 3<sup>rd</sup> DPI that lasts up to 5<sup>th</sup> DPI. Cross reactivity of antiserum response to H9N2 was compared among reference isolates when challenged in broilers, Partridge origin anti-H9N2 antibodies showed varied hemagglutination inhibition results ( $4\log_2$  to  $15\log_2$ ) when cross reacted with different antigens, suggesting H9N2 viruses are circulating with different antigenic characteristics. Sero-biochemical analysis of blood collected at 7<sup>th</sup> DPI revealed an increase in aspartate amino transaminase (246-308 U/L), alanine amino transferase (31-58 U/L) and blood urea nitrogen (11.21-22.58 mg/dl).

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## Introduction

Avian Influenza Virus (AIV) belongs to the family *Orthomyxoviridae* consists of three different groups (A, B, C) classified on the basis of nucleocapsid (NP) and matrix (M) proteins. Spreading of AIV H5N1 is the most significant problem facing by poultry industry in the world but beside that there is also underestimated challenge, which is the spread of AIV subtype H9N2 in Asia (Gharaibeh, 2008). In South Asian countries H9N2

was found circulating with varying pathogenic characteristics (Nili and Asasi, 2002). Similarly, in Pakistan, it was reported earlier that H9N2 subtype of AIV produce marked pathogenicity with high mortality rate in chickens (Naeem *et al.*, 1999) but now H9N2 virus has low pathogenic nature with low mortality rate (Sarwar *et al.*, 2013). So, the infection with H9N2 does not bring about high mortality in poultry but rather it can possibly do only in case of immune-compromised birds subsequent of vaccine stress or any environment effect (Bano *et al.*, 2003).

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The H9N2 pathogenicity is variable and it also depends upon the presence of other pathogens circulating

in the field (Banet-Noach *et al.*, 2007). As H9N2 has vast range of avian hosts including terrestrial poultry birds worldwide, so there is chance of continuous evolution of virus as the virus circulate among different hosts and thus it has become severe panzootic threat in Eurasia during last two decades (Choi *et al.*, 2004). It has also been clear from the previous study that change in biological diversity of H9N2 plays a critical role in potentiating the antigenic potential of virus and thus it can cause severe pandemics in future (Choi *et al.*, 2004). H9N2 virus is under continuously evolution process to better adapt in multiple host which led to change in pathogenic potential in chicken (Naeem *et al.*, 1999; Nili and Asasi, 2002; Gharaibeh, 2008). Paucity of data regarding pathogenic potential in different avian species neglected for H9N2 vaccination, however, kept in close contact to human. Therefore, current study was planned to investigate the pathogenicity and tissue tropism of H9N2 virus isolated from different hosts and localities.

## Materials and Methods

### Viruses

Three Avian Influenza, affirmed H9N2 viruses, isolated from different hosts were secured from Influenza laboratory, Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan. The hemagglutinin (HA) and neuraminidase (NA) gene sequences of these three H9N2 viruses are available under accession numbers: MG720814 (A/Chicken/Pakistan/660BYP/2015/H9N2), MG720815 (A/Chicken/Pakistan/871CF/2015/H9N2) and MG720816 (A/Partridge/Pakistan/260/2015/H9N2) for HA gene. While the accession numbers for the sequences of NA gene are: MF280168 (A/Chicken/Pakistan/660BYP/2015/H9N2), MF280169 (A/Chicken/Pakistan/871CF/2015/H9N2) and MF280170 (A/Partridge/Pakistan/260/2015/H9N2) (Ali *et al.*, 2017).

### Experimental infection

After achieving the approval letter from the institution having IERB number 645/Bact on October 2015, the present challenge study was conducted. To investigate the infection potential of aforementioned LP AIV isolates, a challenge or experimental infection in commercial poultry was conducted during November 2015 to June 2016. For this purpose, n=40, day old chicks were acquired from a commercial hatchery and raised under controlled environmental conditions in chicken isolators. The feed and water were supplemented to birds ad-libitum during the experiment period. Prior to two days challenge, all birds were screened out for any subclinical infection of AIV H9N2, Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV). For the screening of these viruses, tracheal swabs were collected from the birds

and polymerase chain reaction (PCR) was performed via earlier reported primers (Rashid *et al.*, 2009) after extracting RNA using QIAmp® viral RNA mini kit (cat: 52906). After confirming the depletion of maternal antibodies against H9N2 by using hemagglutination inhibition (HI) test (Alexander and Chettle, 1977) at age of 5 week, birds were divided into different four groups (A, B, C and D) having 10 birds each in a group and placed in different chicken isolators. The group A, B, and C were challenged with A/Chicken/Pakistan/660BYP/2015/H9N2, A/Chicken/Pakistan/871CF/2015/H9N2 and A/Partridge/Pakistan/260/2015/H9N2, respectively via oronasal route with the calculated dose of  $10^6$  EID<sub>50</sub>. The calculation of EID<sub>50</sub> of all three studied isolates was done according to the earlier reported method (Reed and Muench, 1938). The D group was kept as control and sterile phosphate buffer saline (PBS) was given via oronasal route.

### Clinical signs, histopathological examination

After infection, all birds were inspected daily for any clinical signs. At 5<sup>th</sup> and 9<sup>th</sup> DPI, n=3 random birds were slaughtered from each experimental group and all the gross lesions were recorded. Tissues samples including trachea, lungs, kidney and liver were fixed in 10% neutral buffered formalin and were processed in standard techniques for fixation, dehydration, clearing, embedding, sectioning and staining as described by Drury and Wallington (Drury and Wallington, 1967).

### Serological analysis

Blood samples were collected at 0, 3, 7 and 14<sup>th</sup> DPI from the trial birds to collect serum. Immune response (titer) of serum collected from each trial group was assessed by HI test utilizing 4 hemagglutinin units (4HAU) of that particular virus with whom challenge group was infected. Along with, the cross-HI of all studied H9N2 strains was performed against specific antisera collected from recovered birds. HI test of sera was performed by making 2 fold serial dilution in 96 well plate using 4HAU of studied H9N2 strains with 1% chicken RBC's (Alexander and Chettle, 1977).

### Serum chemistry analysis

Serum aspartate amino transaminase (AST), Serum alanine amino transferase (ALT) and Blood urea nitrogen (BUN) of collected serum samples of trial birds were determined by Reitman and Frankel method (Reitman and Frankel, 1957).

### Statistical analysis

The obtained data was analyzed using GraphPad Prism version 6.01 (Swift, 1997). To know any significant link of enzyme status within and between different groups at different time interval, two way-ANOVA and multiple comparison were employed. The differences between enzyme levels of different challenged groups were

separated by Tukey's test. P values < 0.5 were considered statistically significant.

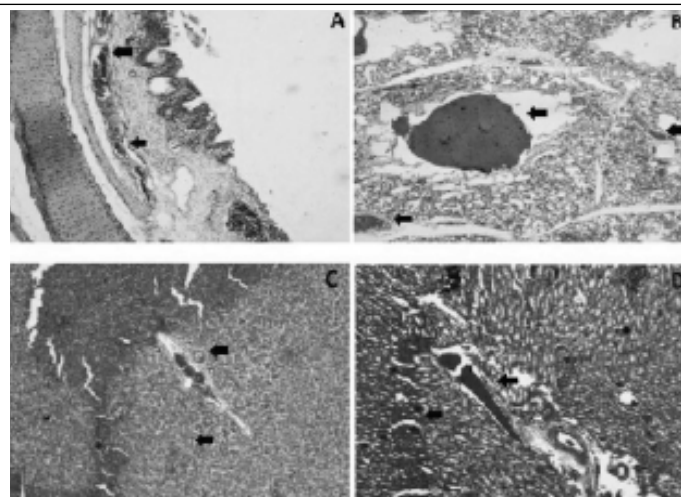
## Results

Results revealed no mortality in all groups when challenged with A/H9N2 isolates. However, most of the effected birds revealed slight depression, low feed and water intake along with mild clinical signs like lethargic, sneezing, gasping, facial swelling, nasal discharge, redness of eyes and lacrimation on 2<sup>nd</sup> day post infection (DPI). These clinical signs were observed up to 5<sup>th</sup> DPI with gradually decrease in severity. Diarrhea was only observed in group C and birds were slightly depressed with ruffled feathers till 9<sup>th</sup> DPI. The most recurrent gross lesions in infected birds were congestion of lungs and trachea along with swollen kidney in all challenged groups. There were no apparent gross lesions observed in any other organs of infected birds. Apparently, birds were started to recover naturally on 6<sup>th</sup> DPI with signs and symptoms disappeared till 10<sup>th</sup> DPI. No clinical signs were observed in control group.

Histopathological examination of tracheal tissue showed inflammation and necrosis. Sloughing of epithelial lining of trachea with lymphocytic infiltration was also observed in trachea. Lungs showed congestion and perivascular hemorrhages with neutrophilic infiltration. There was congestion of portal vein in liver with aggregation of leukocytes and macrophages. Hepatocytic swelling and necrosis was also observed in liver. Severe congestion, glomerulus inflammation, vacuolar degeneration in tubular epithelium along with leukocytic infiltration and peri-glomerular fibrosis was observed in kidney. However, neutrophilic infiltration in all tested organs were observed higher at 9<sup>th</sup> DPI when compared with 5<sup>th</sup> DPI in all challenged groups (Figure 1).

Serological investigations revealed that antisera against H9N2 of partridge host (group C) produced varying cross reactivity with backyard (group A; A/Chicken/Pakistan/660BYP/2015/H9N2) and commercial (group B; A/Chicken/Pakistan/871CF/2015/H9N2) poultry origin H9N2 virus (Table 1).

No amino acid mutation was observed at receptor binding residues 191, 198, 234-235 (H9 numbering) of the three studied isolates. Furthermore, motif KSSR/G at HA cleavage site confirmed the low pathogenic nature of all three isolates. Off note, NA stalk length showed mutation at residue I62T (N2 numbering) in partridge origin isolate (group C; A/Partridge/Pakistan/260/2015/H9N2). However, no mutation was observed in rest of the NA key residues of stalk length (38-39, 46-50, and 63-64), heamadsorbing site (372, 402-403) and heamadsorbing loop (313, 368, 370, 381).



**Figure 1: Histopathology of broiler chicken at 9th day p.i. infected with H9N2 virus (H&E 100X). (A) Trachea of broiler chicken shows the infiltration of neutrophils and sloughing off epithelium. (B) Lung of broiler chicken shows the congestion, infiltration of neutrophils and engorgement of protein. (C) Liver of broiler bird show the infiltration of inflammatory cells and inflammation of hepatocytes. (D) Kidney of broiler chicken shows the infiltration of neutrophils and hemorrhages in glomerulus.**

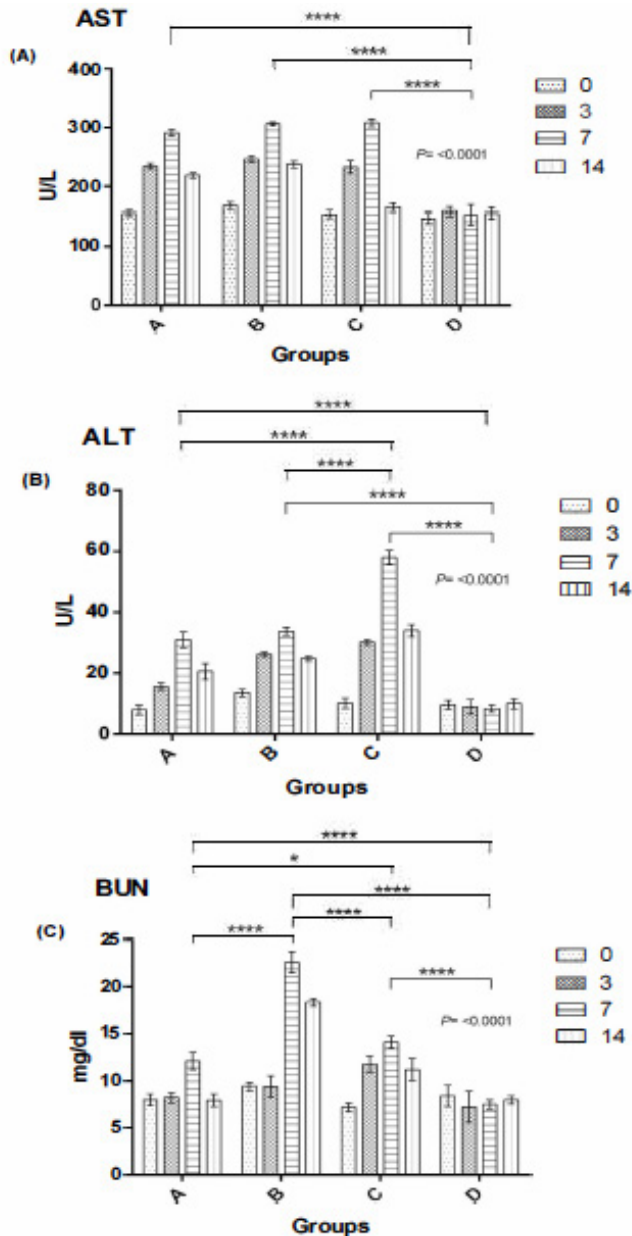
Steady increase in liver enzymes (AST, ALT) and kidney function test (BUN) was recorded till post infection to 7<sup>th</sup> DPI and then sudden fall was observed at 14<sup>th</sup> DPI. Maximum level of AST (246-308 U/L), ALT (31-58 U/L) and BUN (11.21-22.58 mg/dl) in all challenged groups was seen at 7<sup>th</sup> DPI (Table 2). Significant ( $p < 0.001$ ) increase in liver enzymes (AST, ALT) and BUN was recorded between experimental groups (Figure 2). The level of ALT increased with high significant in all challenged groups as compared to the control group at 7<sup>th</sup> DPI. Similarly, the level of ALT also increased with high significance ( $p < 0.001$ ) in all challenged groups but level of ALT was higher in the group C ( $58 \pm 2.34$  U/L) than other challenged groups A ( $31 \pm 2.54$  U/L) and B ( $33.8 \pm 1.30$  U/L) at 7<sup>th</sup> DPI. The level of BUN was also significantly ( $p < 0.001$ ) increased in all challenged groups but the level was a bit higher in group B ( $22.58 \pm 1.13$  mg/dl) than other challenged groups A ( $12.11 \pm 0.91$  mg/dl) and C ( $14.1 \pm 0.60$  mg/dl) at 7<sup>th</sup> DPI. The liver enzyme (ALT, AST) and BUN was dropping to normal level at 14<sup>th</sup> DPI.

## Discussion

Current study revealed varying clinical signs in chickens challenged with H9N2 viruses of different host origin, may suggest circulation of different H9N2 variants in birds in Pakistan. Although all birds recovered naturally but indicate that fancy birds neglected with H9N2 vaccine may provide opportunity for genetic mingling and genesis of novel influenza viruses. The clinical infection and

**Table 1: Cross reactivity of raised hyperimmune sera against different isolates of H9N2 virus.**

Isolates	HA Activity (log <sub>2</sub> )	HI of hyperimmune sera (log <sub>2</sub> )			
		Antiserum Group A	Antiserum Group B	Antiserum Group C	Antiserum Control
Group A (A/Chicken/Pakistan/660BYP/2015(H9N2))	11	10	16	13	1
Group B (A/Chicken/Pakistan/871CF/2015(H9N2))	7	9	12	13	2
Group C (A/Partridge/Pakistan/260/2015(H9N2))	9	4	15	14	2

**Figure 2: The level of sero-biochemical analytes at different days in different groups.**

histo-pathological evidences also confirmed that circulating H9N2 viruses are of low pathogenic in nature and clustered in sub-lineage B2 under G1 lineage of Middle East group (Ali *et al.*, 2017). In Pakistan, frequent outbreaks of H9N2 have been observed from last two decades which shows endemic circulation in the country. Birds of group A and B challenged with H9N2 virus showed clinical presentation similar to previous studies conducted in Pakistan in 2009 (Subtain *et al.*, 2011) and December 2019 | Volume 34 | Issue 2 | Page 146

2015 (Aslam *et al.*, 2015) which suggests that our H9N2 viruses isolated from commercial and backyard poultry has same pathogenic potential with the isolates of H9N2 used by Aslam *et al.* (2015) and Subtain *et al.* (2011). The same clinical findings were also observed in experimentally infected chicken in other countries like Egypt in 2018 (Awadin *et al.*, 2018). This may be due to the reason that H9N2 isolate used in Egyptian study was of G1 lineage circulating in Middle East is closely related to the H9N2 isolates used in our study as described in our previous study (Ali *et al.*, 2017). While clinical sign like diarrhea was only observed in group C which was infected with the H9N2 virus of Partridge origin but the other clinical signs of respiratory system were same as observed in previous studies (Subtain *et al.*, 2011; Aslam *et al.*, 2015). In our study it is clearly observed that the A/H9N2 viruses isolated from the field can produce clinical infection which is contradicted to the results of a previous study reported in India (Dash *et al.*, 2015) in which the H9N2 virus isolated from field outbreak does not produce clinical infection in the experimental birds. This contradiction might be due to the change in antigenic nature of used isolates in the study. Present study showed severity of lesions highest at 9<sup>th</sup> day post infection in group C, however, in other studies it was recorded till 6<sup>th</sup> day post infection (Subtain *et al.*, 2011). Our results suggest that circulating H9N2 viruses has the higher pathogenic potential and is under strong selection pressure.

H9N2 isolates used under current study produced higher HA titer (8–11 log<sub>2</sub>) than reported previously (7–9 log<sub>2</sub>) in Pakistan in year 2011 (Subtain *et al.*, 2011). This contradiction is assumed on basis of antigenic variation in H9 virus isolates with passage of time (Kim *et al.*, 2006). Varying response of cross reactivity of antisera with the different field A/H9N2 isolates (n=3) suggests circulation of H9N2 subtype with different immunogenic properties at the same time in different localities and hosts in poultry of Pakistan. Current results also highlighted the importance of systematic surveillance and further investigation of pathogenic characteristics of endemically circulating H9N2 virus, which may help to select best H9N2 virus as a vaccine candidate.

No mutation was recorded in HA gene at functionally important key residues; however, one mutation was observed in NA stalk length in partridge origin H9N2

virus, might be associated with host adaptation and increased virulence (Iqbal *et al.*, 2009). Results confirmed that NA gene might be under strong selection pressure (Ali *et al.*, 2017). Off note, mutation was only observed in group C, also exhibited severity of clinical signs along with higher level of varying cross reactivity titer to other chicken and backyard poultry isolate.

**Table 2: Serobiochemical parameters of birds infected with different isolates of H9N2.**

Experimental Birds	Day PI	AST (U/L)	ALT (U/L)	BUN (mg/dl)
Group A	0	155.8±4.32	8±1.58	7.98±0.61
	3	233.8±5.11	15.6±1.40	8.22±0.52
	7	291±5.09 <sup>a</sup>	31±2.54 <sup>bc</sup>	12.11±0.91 <sup>ef</sup>
	14	218.6±4.39	20.6±2.70	7.94±0.66
Group B	0	167.4±5.27	13.6±1.40	9.42±0.42
	3	246±5.29	26.2±0.83	9.4±1.14
	7	306.2±3.03 <sup>a</sup>	33.8±1.30 <sup>bd</sup>	22.58±1.13 <sup>efg</sup>
	14	237±5.74	24.8±0.83	18.34±0.40
Group C	0	154±5.83	10.2±1.64	7.2±0.45
	3	232.6±10.66	30.2±0.83	11.8±0.84
	7	308±6.44 <sup>a</sup>	58±2.34 <sup>bcd</sup>	14.1±0.60 <sup>efg</sup>
	14	164.8±6.61	34±2	11.21±1.15
Control	0	147.±8.98	9.6±1.51	8.4±1.14
	3	158.2±7.49	9±2.44	7.23±1.65
	7	152.8±16.25 <sup>a</sup>	8.4±1.14 <sup>b</sup>	7.5±0.47 <sup>c</sup>
	14	155.4±9.37	10±1.58	7.98±0.43

PI: Post Infection; AST: Aspartate amino transaminase, U/L: unit per liter; ALT: Alanine amino transferase; BUN: Blood urea nitrogen, mg/dl: milligram per deci liter; a,b,c,d,e,f,g: Different letters within the same column indicate significant differences among levels according to Tukey's test ( $p \leq 0.05$ )

Increased level of ALT and AST suggest that pathogenic involvement of liver in H9N2 and this was supported by gross lesion and histopathological examination of liver in infected birds. As liver is considered to be the major source of plasma protein like ALT and AST, so damage to this organ leads to change in levels of liver enzymes in blood serum (Egbal *et al.*, 2007). Results of sero-biochemical analytes revealed that studied H9N2 viruses has also strong affiliation to liver and kidney as reported by other researchers (Subatain *et al.*, 2011; Aslam *et al.*, 2015). The higher level of ALT in group C also supports the clinical sign like diarrhea which was only observed in this group. This suggests that H9N2 virus of partridge origin has strong affiliation to liver and viruses isolated from different hosts may have different pathogenic potential which has also been reported previously (Cong *et al.*, 2007; Yu *et al.*, 2008).

## Conclusion

Our results indicate circulation of multiple H9N2 variants with varying pathogenic potential in different avian species. Observed pathogenic potential and mutation in molecular marker of fancy bird isolate suggest urgent need to devise strategies because of close interaction to human. Therefore, future investigations related to genomic variations due to influence of host adaptation of virus or immune pressure of irrational usage of field vaccine are warrant.

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### Conflict of interest statement

The authors declare no conflict of interest.

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