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Original Article

A perspective of the prevalent H9N2 virus with a special focus on molecular and pathological aspects in commercial broiler chicken in Punjab, Pakistan

Perspectiva do vírus H9N2 prevalente com foco especial em aspectos moleculares e patológicos em frangos de corte comerciais em Punjab, Paquistão

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Abstract

Frequent outbreaks of avian influenza H9N2 virus in Pakistan revealed that this subtype has become endemic in the poultry industry and, besides economic losses, poses a threat to public health. The present study describes the molecular characterization and pathological alterations in naturally infected broiler chickens with the current H9N2 field strain and their phylogenomic dynamics. In this study, tissue samples (trachea, lung, kidney and intestine) from 100 commercial chicken flocks were collected from July 2018 to August 2019. Samples were subjected to molecular detection, phylogeny and subsequent pathological examination. The complete length of the HA gene was successfully amplified in five samples. Nucleotide sequencing revealed positive samples placed in a clade belonging to the B2 sub-lineage of the G1 genotype and categorized as LPAIV based on the amino acid sequence of the HA gene at the cleavage site (PAKSSR/G). Genetic analysis of the haemagglutinin (HA) gene revealed nt: 80.5%-99.5%; aa: 83.8%-98.9% homology to H9N2 strains reported previously from Pakistan, neighbouring countries, and (A/Quail/Hong Kong/G1/97). Gross lesions include a slight airsacculitis, mild hemorrhages, diffuse congestion and purulent exudate in tracheal mucosa, fibrinonecrotic cast in the trachea lumen and mild pulmonary congestion. Histopathological alterations include sloughing of epithelial cells and infiltration of inflammatory cells in the trachea, mononuclear cells (MNCs) infiltration, pulmonary congestion and exudate in the lumen of parabronchi, peritubular congestion in the kidneys with degeneration of tubular epithelial cells and degenerative changes in the intestinal villi epithelial cells and goblet cell hyperplasia. Immunohistochemistry analysis confirmed the presence of AIVH9N2 antigen in the trachea, lungs, kidney and intestine. Electron microscopy revealed ultrastructural changes in the trachea, including degenerated cilia, mitochondrial swelling and enlarged endoplasmic reticulum. Based on all essential analysis, the present study revealed the distribution of the H9N2 virus of G1 genotype in Punjab, Pakistan, with mild to moderate pathogenicity.

Keywords: avian influenza, polymerase chain reaction, phylogenetic analysis, electron microscopy, histopathology, immunohistochemistry.

Resumo

Surtos frequentes do vírus da gripe aviária H9N2 no Paquistão revelaram que esse subtipo se tornou endêmico na avicultura e, além das perdas econômicas, representa uma ameaça à saúde pública. O presente estudo descreve a caracterização molecular e as alterações patológicas em frangos de corte naturalmente infectados com a atual cepa H9N2 e sua dinâmica filogenômica. Neste estudo, amostras de tecidos (traqueia, pulmões, rim e intestino) de 100 lotes comerciais de frangos foram coletadas de julho de 2018 a agosto de 2019. As amostras foram submetidas à detecção molecular, filogenia e posterior exame patológico. O comprimento completo do gene HA foi amplificado com sucesso em cinco amostras. O sequenciamento de nucleotídeos revelou amostras positivas colocadas em um clado pertencente à sublinhagem B2 do genótipo G1 e categorizado como LPAIV com base na sequência de aminoácidos do gene da hemaglutinina (HA) no local de clivagem (PAKSSR/G). A análise genética do gene da HA revelou: nt = 80,5%-99,5%; aa = 83,8%-98,9% de homologia com cepas de H9N2 relatadas anteriormente no Paquistão e em países vizinhos (A/Quail/Hong Kong/G1/97). As lesões macroscópicas incluíram aerossaculite leve, hemorragias leves, congestão difusa e exsudato purulento na mucosa traqueal, cilindro fibrinonecrótico no lúmen da traqueia e congestão pulmonar leve. As alterações histopatológicas incluíram descamação de células epiteliais, infiltração de células inflamatórias na traqueia, infiltração de células mononucleares (MNCs), congestão pulmonar e exsudato no lúmen dos parabrônquios, congestão peritubular nos rins com degeneração das células epiteliais tubulares,

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alterações degenerativas nas células epiteliais das vilosidades intestinais e hiperplasia de células caliciformes. A análise imunoistoquímica confirmou a presença do antígeno AIVH9N2 na traqueia, nos pulmões, no rim e no intestino. A microscopia eletrônica revelou alterações ultraestruturais na traqueia, incluindo cílios degenerados, inchaço mitocondrial e retículo endoplasmático aumentado. Com base em todas as análises, o presente estudo revelou a distribuição do vírus H9N2 do genótipo G1 em Punjab, Paquistão, com patogenicidade de leve a moderada.

Palavras-chave: gripe aviária, reação em cadeia da polimerase, análise filogenética, microscopia eletrônica, histopatologia, imunoistoquímica.

1. Introduction

Avian influenza (AI) is a zoonotic viral disease caused by the avian influenza virus (AIV), a single-stranded RNA virus, member of the family *Orthomyxoviridae* and belongs to the genus Influenza A. The poultry sector suffers significant financial losses worldwide due to its rapid transmission among the flocks (Ashraf et al., 2017; Hasni et al., 2021). Antigenic diversity of surface glycoproteins classified AIVs into eighteen haemagglutinin (HA) subtypes (H1 to H18) and eleven neuraminidase (NA) subtypes (N1 to N11), with the majority of potential combinations of all subtypes isolated from avian species (Bouwstra et al., 2017).

Based on the amino acid sequence spanning the cleavage site of the HA protein, AIVs are phenotypically distinguished into low-pathogenic avian influenza virus (LPAIV) and high-pathogenic avian influenza virus (HPAIV) (Sultan et al., 2017). LPAIVs have mono or dibasic cleavage sites at their HA cleavage site, allowing only proteases (trypsin-like) to cleave them in the respiratory and digestive systems and are characterized by low pathogenicity as measured by the intravenous pathogenicity index (Kumosani et al., 2017). On the other hand, HPAIVs have polybasic cleavage sites that allow them to replicate in various organs, including vascular endothelium and perivascular endothelial cells resulting in high pathogenicity in chickens (Ali et al., 2021).

Avian influenza H9N2 (AIH9N2) viruses have maintained a prevalent epidemiological disease profile since their first identification in Wisconsin, the United States, in 1966 from turkeys (Xu et al., 2018). The H9N2 viruses have become prevalent in poultry in Pakistan and evolved through reassortment with HPAIVs (H5N1 and H7N3), forming novel genotypes of H9N2 (Munir et al., 2013; Shahzad et al., 2020). Birds show mild to severe respiratory symptoms, drop in egg production, immunosuppression and very high mortality, as high as nearly 100% (Yunhua et al., 2020). More severe clinical signs observed in the field than laboratory-induced infection is due to co-infection of other respiratory pathogens and immunosuppression (Purohit et al., 2020). Predisposing factors like infectious bursal disease, coccidiosis, *Mycoplasma gallisepticum* and Marek's disease aggravate the H9N2 magnitude in the field (Sha et al., 2020).

Irrespective of extensive vaccination strategies, H9N2 has been prevalent in Pakistan and neighbouring countries for two decades. Frequent mutation (antigenic shift) has upraised alarms about the possibility of H9N2 virus evolution (Sehrawat et al., 2021). Also, continuous field circulation of AIVH9N2 with some evolution has been a significant constraint causing high morbidity and mortality in the poultry sector (Shahzad et al., 2020). Therefore, the present study was aimed to identify field prevailing H9N2 strains to elucidate the potential pandemic risk and associated pathological features by monitoring gross and ultrastructural changes, particularly in the respiratory tract.

2. Materials and methods

2.1. Sample collection

Tissue samples (trachea, lung, kidney and intestine) from 100 commercial chicken flocks (5 birds/flock) were collected from July 2018 to August 2019. These birds were presented at poultry disease diagnostic centres in Lahore, Pakistan, namely, University Diagnostic Laboratory & Necropsy Section of University of Veterinary and Animal Sciences, Lahore, Pakistan and GP Laboratory of Big Bird (Pvt) Limited Lahore, Pakistan.

2.2. Inclusion criteria for selection of birds for isolation of AIVH9N2

Chickens with a history of mild to moderate respiratory problems, drop in egg production, and other clinical signs such as depression, sneezing, coughing, dyspnea, gasping, anorexia and decreased water intake were selected. Tissue samples with characteristic *AIVH9N2* lesions i.e., mild hemorrhages and accumulation of mucus/exudate in the trachea, were selected for virus isolation and pathological studies (Seifi et al., 2010).

2.3. Genomic extraction of AIVH9N2

Virus isolation was carried out according to the procedures described by Kariithi et al., (2020). Pooled tissue homogenate representing one flock was aseptically inoculated into 9-day old embryonated eggs through the chorioallantoic route. Hemagglutination test was performed on harvested allantoic fluid and identified as H9 subtype by hemagglutination inhibition (HI) assay using specific antisera against H9 subtype and Newcastle disease virus (NDV). RNA was extracted from allantoic fluids using the TRIZol® Reagent, followed by reverse transcription using RevertAidTM First Strand cDNA (Thermo Scientific™) kit.

2.4. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Complete haemagglutinin (HA) gene was amplified by using already reported specific two sets of primers; F-5'-GCAAAAGCAGGGGAATTTCT -3'; R-5'-GTGTACTGTTTAAGCCACCT -3 and F-5'- ATGGTATGGACACGTTCTCT -3'; R-5'-ACAAGGGTGTTTTTGCTAACT -3' with expected amplicon size of 918bp and 948bp, respectively (Ali et al., 2021). A reaction mixture of 25 µL containing 2 µL template cDNA,

1.25 µL (10pmol each primer), 12.5 µL of 2X master mix (Thermo Scientific, United States) and 8 µL of nuclease-free water was prepared and set to amplification conditions; preheating (94°C for 2 min) followed by 35 cycles of denaturation (95°C for 10 sec), annealing (53°C for 30 sec) and extension (72°C for 45 sec) with a final extension (72°C for 5 min). PCR products were visualized in a gel documentation system after gel electrophoresis.

2.5. Sequencing and phylogenetic analysis

Purified PCR products were subjected to the Sanger Sequencing method to get the raw nucleotide sequences. The sequences were aligned through Clustal W using BioEdit software. The phylogenetic relationship was determined by incorporating the study isolates and other strains sequences retrieved from the NCBI database (Bahari et al., 2015). The evolutionary tree was generated with the neighbour-joining method using MEGA 6 software. The homology (%) of nucleotides (nt) and amino acids (aa) was determined with DNASTAR Lasergene software (Sultan et al., 2022).

2.6. Histopathological examinations

Formalin-fixed tissue samples (trachea, lung, kidney and intestine) of PCR positive cases were processed and stained with hematoxylin and eosin dye (H&E) for microscopic examination (Anjum et al., 2020).

2.7. Immunohistochemistry

Tissue sections (4-6 µm) were processed and stained using the 3rd Gen IHC detection kit (SuperPicture™ Invitrogen, USA) for immunohistochemical detection of antigens. Briefly, tissue sections were de-paraffinized, rehydrated, antigen retrieved with quenching solutions, rinsed with PBS, followed by incubation with a primary monoclonal antibody against nucleoprotein of type A influenza virus subtype H9N2 (60 min) and horseradish peroxidase (HRP) polymer conjugate (10 min), and stained with diaminobenzidine (DAB) chromogen solution and evaluated under a fluorescent microscope (Nadeem et al., 2021).

2.8. Electron microscopy

Trachea tissue blocks (1 mm) from freshly dead birds were treated with 2% glutaraldehyde, absorbed on grids (carbon-coated), and washed with deionized water. Sections were stained with 2% sodium phosphotungstate (pH 6.5) and evaluated in a transmission electron microscope at a magnification of 5,000- 25,000X at National Institute for Biotechnology and Genetics Engineering (NIBGE), Faisalabad, Pakistan (Bouwstra et al., 2017).

3. Results and Discussion

3.1. AIVH9N2 isolation and phylogenetic analysis

AIVH9N2 was successfully isolated from five samples (Figure 1a, 1b). Due to high similarity, these sequences were submitted to the GenBank database with the following accession numbers; MW287132 and MZ340551. These two isolates were placed in a single sister clade containing Pakistan-originated Influenza A H9N2 virus and grouped into the Quail/Hong KongG1/97 lineage. Further, India, Bangladesh, Iran, Hong Kong, Middle East Territories and Pakistan originated H9N2 virus formed a monophyletic group known as G1-Lineage (Figure 2). G1 lineage has two sub-lineages (B1 and B2). Previously identified isolates from Pakistan, India, Bangladesh, and the Middle East are present in B1 sub-lineage. However, the current study isolates were clustered with the B2 sub lineage.

The H9N2 viruses can be separated into two groups: American and Eurasian. These two primary lineages can identify additional clusters with a complicated evolutionary history (Xu et al., 2018). Most American strains are reported in Chile, Peru, Argentina and Brazil (Trovão et al., 2020; Mathieu et al., 2021). In contrast, Eurasian viruses are separated into three sub-lineages; G1-

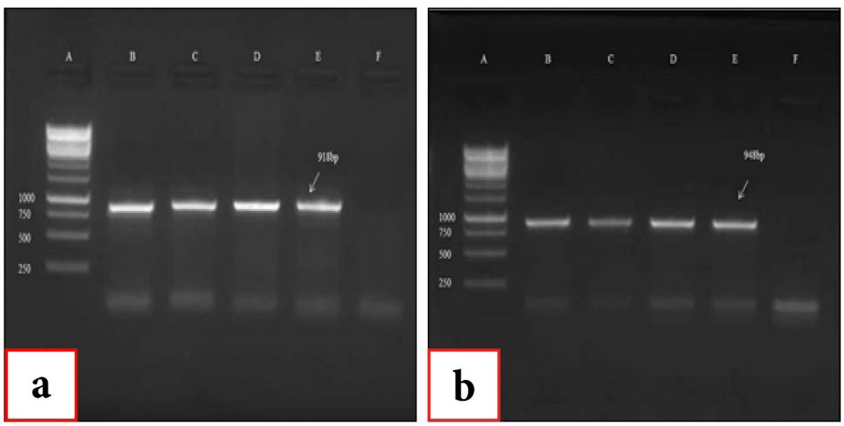

Figure 1. Agarose gel with PCR amplified product of HA gene of present study isolates. (a) Lane A: DNA Ladder: 1 KB; Lane B: Positive control; Lane C, D and E: Positive samples for H9N2 (HA1 gene fragment) with amplicon size 918bp; Lane F: Negative control; (b) Lane A: DNA Ladder: 1 KB; Lane B: Positive control; Lane C, D and E: Positive samples for H9N2 (HA2 gene fragment) with amplicon size 948bp; Lane F: Negative control.

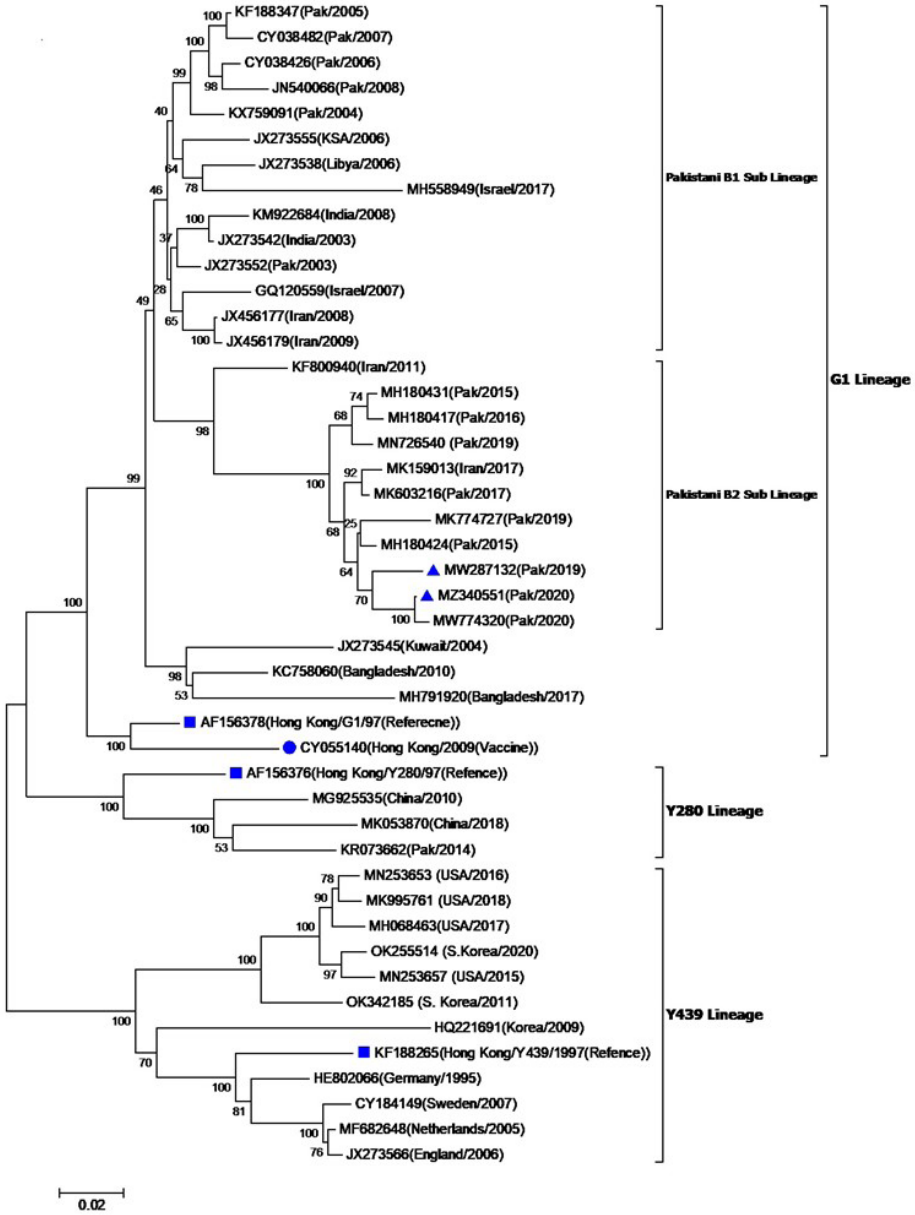

Figure 2. HA gene based phylogenetic analysis of H9N2 using the Neighbour-joining method with 1,000 bootstrap replicates with the help of Tamura Nei model (Tamura et al., 2013) through MEGA 6 software. Blue triangles revealed under study isolates. Blue squares showing reference strains and blue circle showing vaccinal strain.

h9.4, BJ94-h9.3 and Y439-h9.2, mostly found in commercial poultry (Peacock et al., 2019). Viruses belonging to the Y439 sub-lineage were first discovered in chickens and ducks in South Korea and Hong Kong, with subsequent sporadic detections in wild and domestic birds in other countries (Abolnik et al., 2016; Awuni et al., 2019). Also, H9N2 viruses isolated from wild birds in Europe belong to the Y439 sub-lineage (Alexander, 2007). In China and Southeast Asia, H9N2 viruses of the BJ94-h9.3 sub-lineage (Y280 or G9) are usually found in poultry (Okamatsu et al., 2017).

Geographically, the most common strains among poultry are G1-h9.4. These strains have slowly moved from Southeast Asian countries to the Middle East and North and Sub-Saharan Africa (Davidson et al., 2014). The G1 lineage can be further separated into Eastern (G1-h9.4.1) and Western (G1-h9.4.2) sub-lineages based on genetic similarity and geographical diversity (Dong et al., 2011).

The G1-h9.4.1 strains are widely associated with small poultry like quail and guinea fowls and are found in China, Vietnam, and Cambodia. In contrast, G1-h9.4.2 strains are

endemic in chickens in Pakistan, India, Afghanistan, Egypt and the Middle East (Hosseini et al., 2017). Most viruses detected in South Asian poultry are 'Western' viruses, with a few Y439 sporadically infecting poultry from wild birds. The G1 'Western' sub-lineage circulating in these regions evolved from the genetic reassortment of co-circulating HPAIV H7N3 and LPAIV H9N2 viruses which overwhelmed other local clades (Iqbal et al., 2009).

3.2. Homology matrix of study isolated H9N2 viruses

Nucleotide and amino acid similarity matrix based on HA gene of current isolates have nt: 80.5%-99.5%; aa: 83.8%-98.9% homology to H9N2 strains previously reported from Pakistan, isolates of neighbouring countries (China, Iran, India and Bangladesh), G1-lineage ancestor isolate (A/Quail/Hong Kong/G1/97) and turkey Wisconsin 1966 isolate (Figure 3).

3.3. Pathogenicity determination of isolated H9N2 viruses

The haemagglutinin receptor-binding site motif determines the host range. Cleavage site in HA gene is considered as an indicator of pathogenicity. The glutamine (Q) at position 234 results in binding to avian receptors, whereas leucine (L) at the same location results in attachment with human receptors (Butt et al., 2010). The current study isolate showed the presence of L at position 234 of HA amino acids and highlighted its zoonotic potential. The present study isolates were identified as low pathogenic AIV based on the amino acid sequence PAKSSR/G at the proteolytic cleavage site in the HA gene (Figure 4).

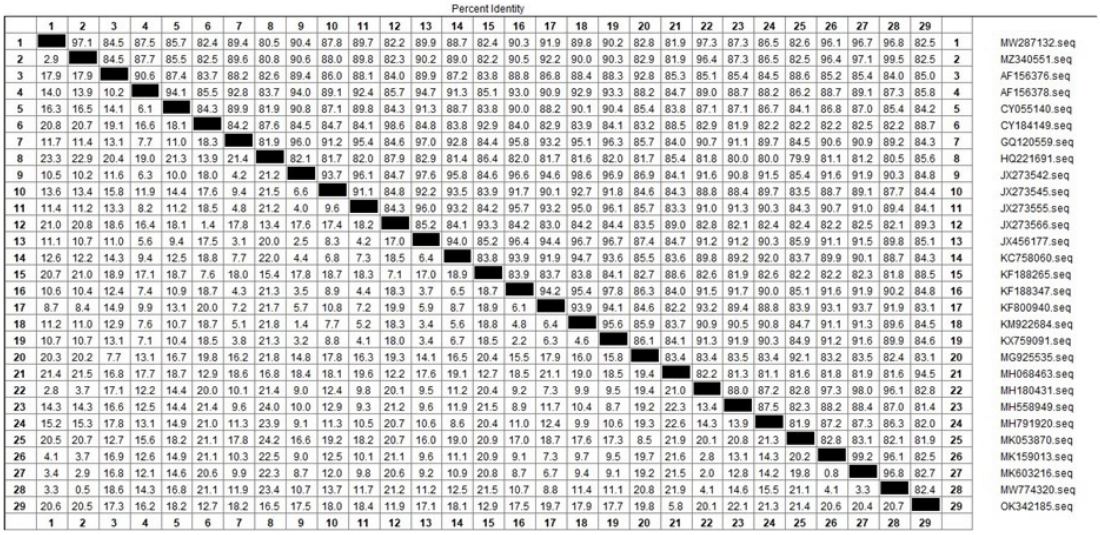

Figure 3. Nucleotide homology percentage of study isolate with other H9N2 strains reported in different countries worldwide.

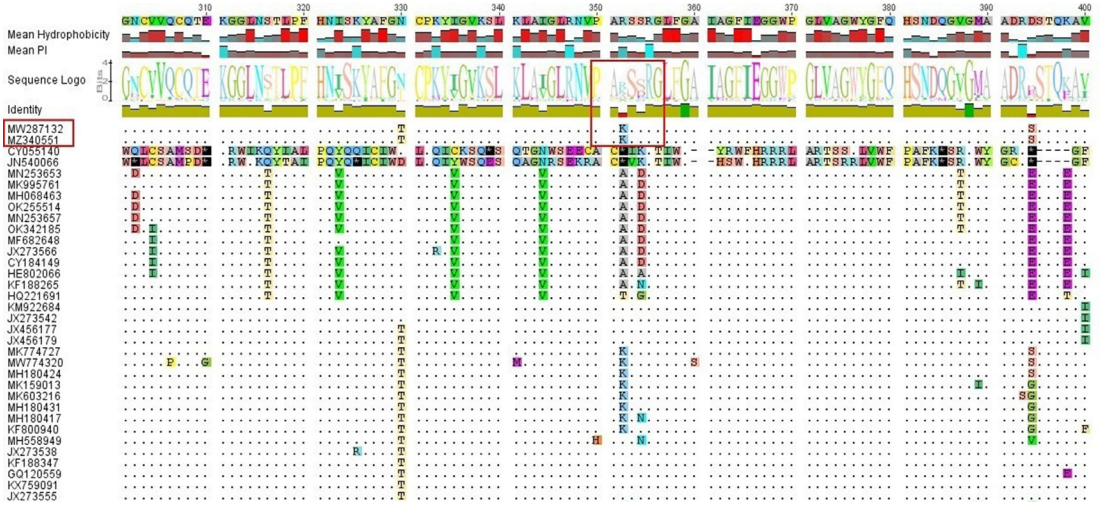

Figure 4. The amino acid cleavage sequence of HA protein cleavage site from the present study isolates showing low-pathogenic H9N2 in red marked areas.

3.4. Gross lesions

Necropsy findings revealed a slight airsacculitis, mild hemorrhages (Figure 5a), diffuse congestion and purulent exudate in tracheal mucosa (Figure 5b), fibrinonecrotic cast in the trachea lumen (Figure 5c) and mild pulmonary congestion (Figure 5d). Some gross lesions were persistent, like slight airsacculitis, mild hemorrhages, and diffuse congestion. In contrast, severe lesions, including purulent exudate in tracheal mucosa and fibrinonecrotic cast in the trachea lumen, were observed in a few cases. The pathological lesions identified in this study mimicked the same pattern as described by Nili and Asasi (2002), Arafat et al. (2020).

3.5. Histopathological examinations

Microphotographs of normal trachea, lungs, kidney and intestine showed no microscopic lesions (Figure 6a, 6c, 6e, 6g). The prominent microscopic changes include sloughing of epithelial cells and infiltration of inflammatory cells in the trachea (Figure 6b). Also, mononuclear cells (MNCs) infiltration, pulmonary congestion and exudate in the lumen of parabronchi were noticed (Figure 6d). Peritubular congestion in the kidneys with degeneration of tubular epithelial cells was also detected (Figure 6f). Degeneration in the intestinal villi epithelial cells along with mild infiltration of inflammatory cells and goblet cell hyperplasia were observed (Figure 6h). The lesions observed in trachea, lungs, kidneys and intestine were found in agreement with the findings of Aslam et al. (2015), Awadin et al. (2018) and Purohit et al. (2020).

3.6. Immunohistochemistry (IHC)

In the present study, *AIVH9N2* antigen was detected in the trachea (Figure 7a), lungs (Figure 7b), kidney (Figure 7c) and intestine (Figure 7d) of infected birds, which is in line with the findings of Aslam et al. (2015), Arafat et al. (2018). Different methods can be used to detect the localization of viruses like H9N2 in tissue specimens, and IHC is one of them. This method is more accurate, rapid and has diagnostic importance than other molecular techniques used for the same purpose.

3.7. Electron microscopy

The ultrastructural changes in the trachea include degenerated cilia, distortion of membranes with changes in the internal structure, including mitochondrial swelling and enlarged endoplasmic reticulum (Figure 8a, 8b). Similar findings were observed in H9N2 infected birds as reported by Nili and Asasi (2002), Qi et al. (2016).

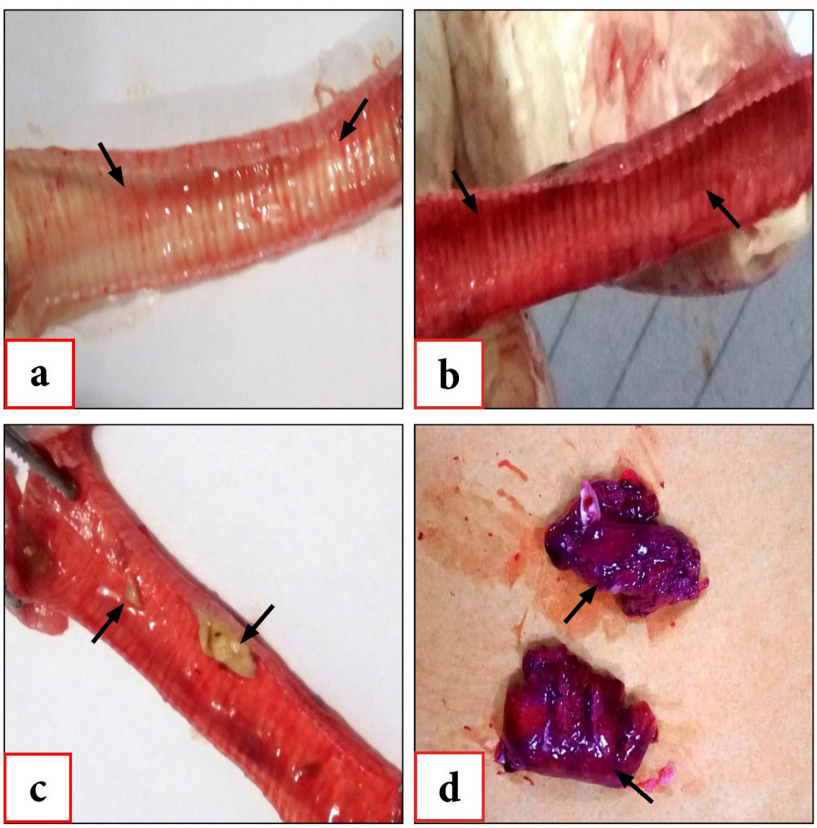

Figure 5. Gross lesions of H9N2 infected chickens. (a) Mild hemorrhages in trachea; (b) Diffuse congestion in tracheal mucosa; (c) Fibrinonecrotic cast in the tracheal lumen; (d) Mild pulmonary congestion.

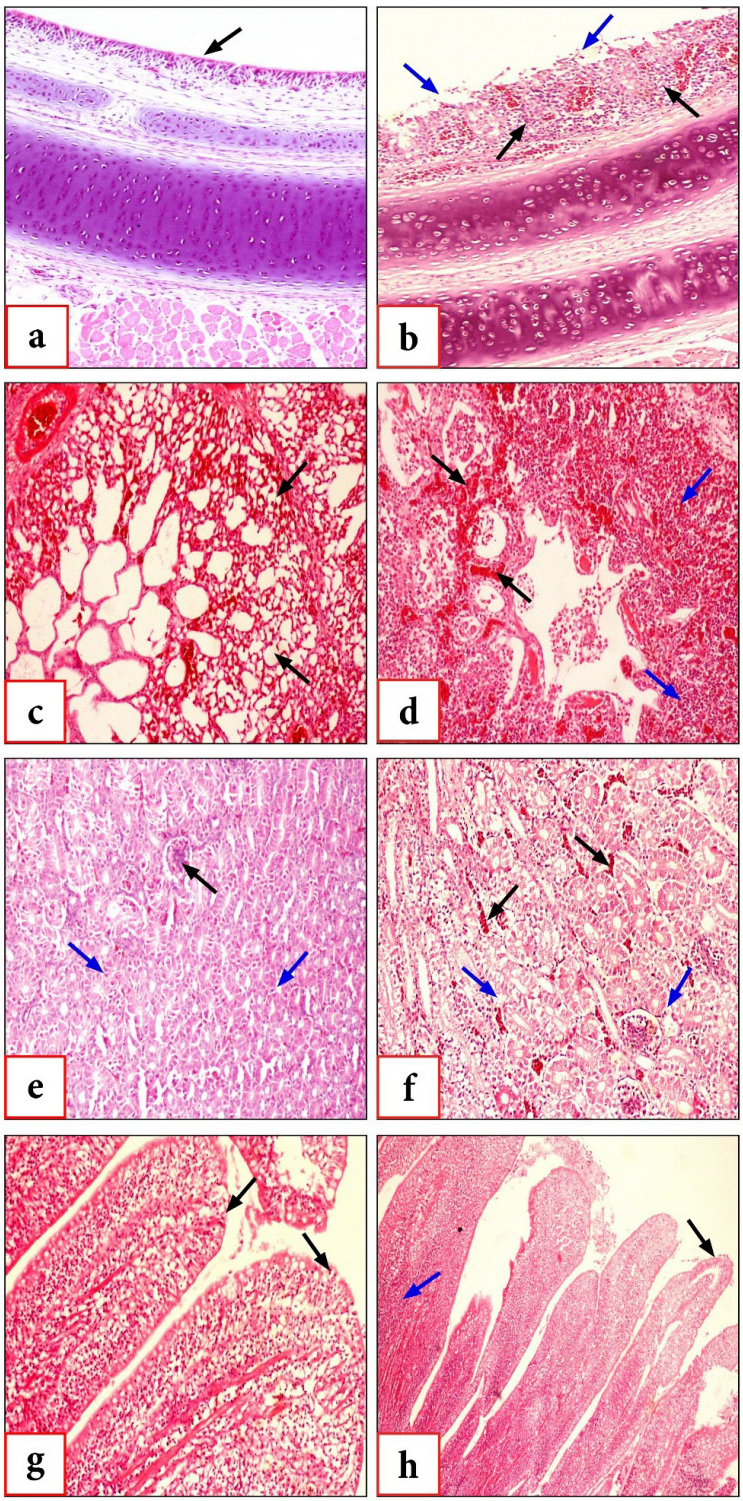

Figure 6. Photomicrographs of histopathological alterations in H9N2 infected chickens (H&E staining, 10X). (a) Trachea with intact pseudostratified ciliated columnar epithelium (black arrow); (b) Sloughing of ciliated epithelium (blue arrow) and inflammatory cell infiltration in mucosa of trachea (black arrow); (c) Normal lungs with intact parabronchi (black arrow); (d). Pulmonary congestion (black arrow) and inflammatory cells aggregation in the lumen of parabronchi (blue arrow); (e) Normal glomerulus (black arrow) and renal tubules (blue arrow); (f) interstitial renal hemorrhages (black arrow) and degeneration of renal tubular epithelial cells (blue arrow); (g) Normal architecture of intestinal villi (black arrow); (h) Necrosis of columnar intestinal epithelial cells (black arrow) and inflammatory cells accumulation in lamina propria of mucosa (blue arrow).

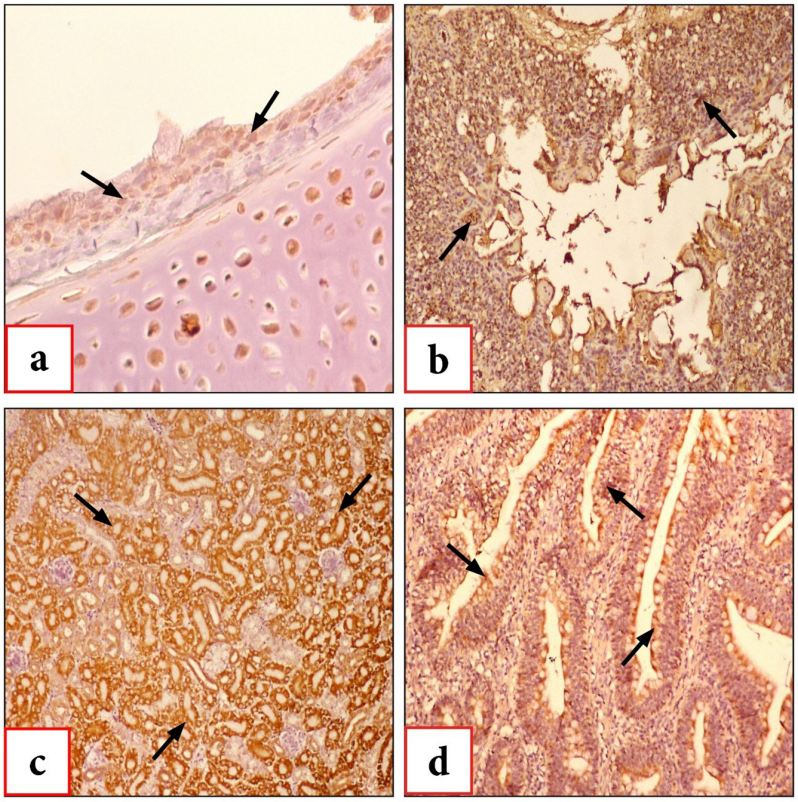

Figure 7. Photomicrographs of H9N2 infected chickens (IHC, 10X). Immunolabelling of H9N2 antigens within; (a) Tracheal epithelium; (b) Pulmonary bronchioles; (c) Renal tubular epithelial cells; (d) Intestinal villi epithelial cells.

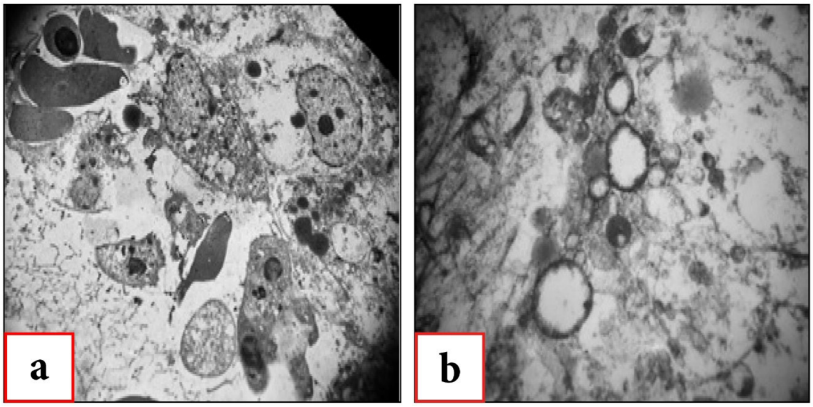

Figure 8. Electron photomicrograph of trachea of H9N2 infected chickens (Magnification, 25000X). (a) Loss of cilia; (b) Distorted mitochondria.

4. Conclusions

The present study investigated the circulating H9N2 virus of (B2 sub lineage; G1-lineage) in commercial poultry flocks with mild to moderate pathological changes in the trachea, lungs, kidney, and intestine. Current isolates carried nucleotide change including Q226L substitution in HA gene that contributes to increased ability to transmit into mammalian hosts. Our findings suggest potential threats of H9N2 to the poultry industry in the country with zoonotic risk. Further studies should be designed to evaluate transmission potential due to reassortment and characterization to better understand zoonotic public health risk.

Acknowledgements

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