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SHORT COMMUNICATION

Comparative Molecular Characterization and Pathogenicity of H9N2 Avian Influenza Virus in Commercial Poultry Flocks in Pakistan

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ABSTRACT

The current study was designed to investigate the molecular characterization and pathological potential of field isolated H9N2 low pathogenic avian influenza virus (LPAIV). Trachea, lungs, kidney, and spleen tissue samples of 413 chickens with respiratory signs were collected from one hundred poultry flocks, processed for RT-PCR, phylogenetic analysis, and histopathological examinations. A PCR product of 918 bp corresponding to a fragment of the HA gene was detected in 14 samples. After nucleotide sequencing and alignment, the positive samples were grouped into two isolates. Field isolates of the current study have 95.02-98.85% nucleotide and 90.04-98.21% amino acid homology to previously reported H9N2 strains from Pakistan. Microscopic examination revealed degenerative changes, necrosis, congestion, hemorrhages, and mononuclear cell infiltration in the trachea, lungs, kidney, and spleen. Based on the molecular analysis, the results depicted that studied isolates shared 95.02-98.85% nucleotide identity, and 90.04-98.21% amino acid similarity with other Pakistan originated H9N2 LPAIV and referenced A/Quail/Hong Kong/G1/97 isolates, hence, clustered in G1-lineage. Moreover, H9N2 LPAIV also induced gross and microscopic alternations in the studied organs. Further investigations on potential transmission risks, pathotyping, and causes of vaccine failures are required.

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INTRODUCTION

H9N2 is a low pathogenic avian influenza virus (LPAIV) that belongs to the *Orthomyxoviridae* family. It is an enveloped RNA virus having eighteen hemagglutinin (HA) and 11 neuraminidase antigenically distinctive subtypes (Shahzad *et al.*, 2020). It infects a variety of domestic and wild birds and imposes an enormous health risk with the potential to cause recurring catastrophic pandemics. Likewise, H9N2 strains have a tremendous economic impact, including a substantial decline in egg production, immunity, and incremented mortality rate (Sultan *et al.*, 2017).

Since its first outbreak reported in 1998 in Pakistan, avian influenza outbreaks are periodically published in Pakistan caused by H9N2 strains resorted with highly pathogenic variants of H5N1 and H7N3 (Sultan *et al.*, 2017). Recently, H9N2 LPAIV was reported in 5.7% of 905 flocks of commercial and backyard poultry in Pakistan (Umar *et al.*, 2019). Molecular diagnostic techniques like polymerase chain reaction (PCR) shows superior sensitivity compared to that of conventional assays such as ELISA and are now becoming acceptable as new gold standards. The prime objective of the current study was to isolate H9N2 LPAIV strains from clinically infected birds with respiratory symptoms, study the genetic diversity and their associated pathologies. Due to the risk of gene mutation and the emergence of new strains, analyzing the HA gene sequences was also part of this study.

MATERIALS AND METHODS

Sample collections: Trachea, lungs, kidney, and spleen tissue samples were collected from 413 birds exhibiting

respiratory illness brought from one hundred different poultry farms from September 2018 to August 2019. Gross pathological lesions of three to five birds were recorded from each flock presented at University Diagnostic Laboratory, University of Veterinary and Animal Sciences Lahore, and GP Laboratory (Pvt) Lahore. Tissue samples were divided into two parts, i.e., one part for reverse transcriptase PCR (RT-PCR) and the other for histopathological examinations.

Genome extraction and RT-PCR: Tracheal and lung tissues of each flock were pooled and triturated in sterile phosphate buffer saline (PBS) admixed with antibiotic streptomycin (1000U/ml), penicillin (1000U/ml) and antifungal amphotericin B (20µg/ml) solutions. Tissue homogenates were centrifuged at 5000x*g*, and the supernatant was collected and inoculated (0.2 ml) in a 9-day old chicken embryo. After 72h, the embryos were chilled at 4°C to harvest the allantoic fluid, which was examined for haemagglutination test to screen out the non-agglutinating viruses. Positive samples were subjected to RNA extraction, cDNA synthesis, and RT-PCR**.** Already reported F-5'- GCAAAAGCAGGGGA ATTTCT -3' and R-5'- GTGTACTGTTTAAGCCACCT -3 primers were used (Ali *et al*., 2018), targeting the haemagglutinin (HA) gene with an amplicon size of 918bp. A reaction mixture of 20µl [1µl template cDNA, 1µl of each primer (10pmol), 10µl of 2X master mix and 7µl of nuclease-free water] was used to perform the RT-PCR with the following conditions; initial denaturation 94°C for 2 min, subsequently 40 cycles of (95°C for 10 sec, 53°C for 30 sec, 72°C for 45 sec) and ended with a final extension at 72°C for 5 min (Kausar *et al*., 2018). The amplified products were sent to ABI Scientific Pvt. Ltd. for HA gene sequencing.

Phylogenetic analysis: The oligonucleotide sequences were aligned through ClustalW using BioEdit software. The phylogenetic tree was constructed and inferred by the Maximum likelihood-method with statistical analysis based on 1,000 bootstrap replicates performed on MEGA-X software. Nucleotide and amino acid percentage identity was compared with the MegAlign program (DNASTAR, Lasergene®). Nucleotide sequences (n=88) of H9N2 LPAIV were retrieved from the NCBI-GenBank database to conduct phylogenetic analysis.

Accession numbers: The partial gene sequences of the present study were submitted to the NCBI-GenBank database under accession numbers MN726540 (A/chicken/Pakistan/PATH-I/2019) and MN726541 (A/chicken/Pakistan/PATH-II/2019).

Necropsy findings and histopathological examinations: Gross pathological lesions were noted after postmortem of suspected birds, and tissues were fixed in 10% neutral buffered formalin for histopathological analysis (Purohit *et al*., 2020).

RESULTS AND DISCUSSION

A PCR product of 918 bp corresponding to a fragment of the HA gene was detected in 14 samples out of 100 flocks samples. After nucleotide sequencing and alignment, the positive samples were grouped into two isolates; MN726540 and MN726541. Phylogenetic and antigenic analyses indicated that H9N2 LPAIV is classified into G1 lineage, Y280 lineage, and the Y439/Korean lineage associated with specific genetic changes suggesting their increased pandemic potential (Butt *et al*., 2010). The phylogenetic analysis revealed that both study isolates were homologous to H9N2 strains previously reported from Pakistan and clustered with H9N2 strains in G1-lineage (Fig. 1). In Pakistan, the first outbreak of H9N2 LPAIV was reported in 1998 which was genetically close to the G1-lineage virus circulating in Hong Kong during 1997 and subsequently reported in Asia, Middle East and Africa (Lee *et al*., 2016; Kariithi *et al*., 2020). Nucleotide similarity matrix based on HA gene indicated that both sequences have high similarities with HA sequences of H9N2 strains reported in neighboring countries, i.e., India, Iran, and Bangladesh.

There was some degree of variation among study isolates, previously reported Pakistani isolates, and the referenced strain A/Quail/Hong Kong/G1/97. Our isolates shared 95.02-98.85% nucleotide identity, and 90.04-98.21% amino acid similarity with Pakistani originated H9N2 LPAIV reported earlier. In comparison, both isolates shared 94.64% (nucleotide) and 90.04% (amino acid) similarities with A/Quail/Hong Kong/G1/97 strain (Table 1).

On necropsy, a slight airsacculitis, congestion, and petechial haemorrhages in tracheal mucosa, mildly congested lungs, and swollen kidneys were grossly seen in H9N2 LPAIV infected birds which are in concurrence with the conclusions of Eladl *et al*. (2019) and Kariithi *et al*. (2020). Histopathological examinations revealed sloughing of epithelial cells in the trachea, dilated mucus glands with thinned epithelium, lymphoplasmacytic aggregations and necrosis of individual mucosal cells. In the lungs, mononuclear cells (MNCs) infiltration, necrosis of parabronchi walls, pulmonary congestion, and engorgement of red blood cells in capillary beds were noticed. In kidneys, heterophilic tubulointerstitial nephritis and hypercellularity of glomeruli were evident. In the spleen, reticuloendothelial hyperplasia and necrotic splenocytes were observed (Fig. 2). The microscopic lesions in the trachea, lungs, kidney, and spleen were found in agreement with the reports of Awadin *et al*. (2018) and Purohit *et al*. (2020).

Conclusions: The present study demonstrates the molecular characterizations and pathological lesions induced by H9N2 LPAIV in poultry flocks. Nucleotide and amino acid identity matrix represented that Pakistan originated H9N2 LPAIV strains are genetically similar to A/Quail/Hong Kong/G1/97 isolates and therefore placed in a single monophyletic group i.e., G1-lineage. These viruses tend to cause mild to moderate gross and microscopic changes in the trachea, lungs, kidney, and spleen. The findings from this study emphasize the potential threats of H9N2 LPAIV to the poultry industry globally as well as in Pakistan. Future studies should be aimed at evaluating potential transmission risks, pathotyping, and causes of vaccine failures in poultry flock.

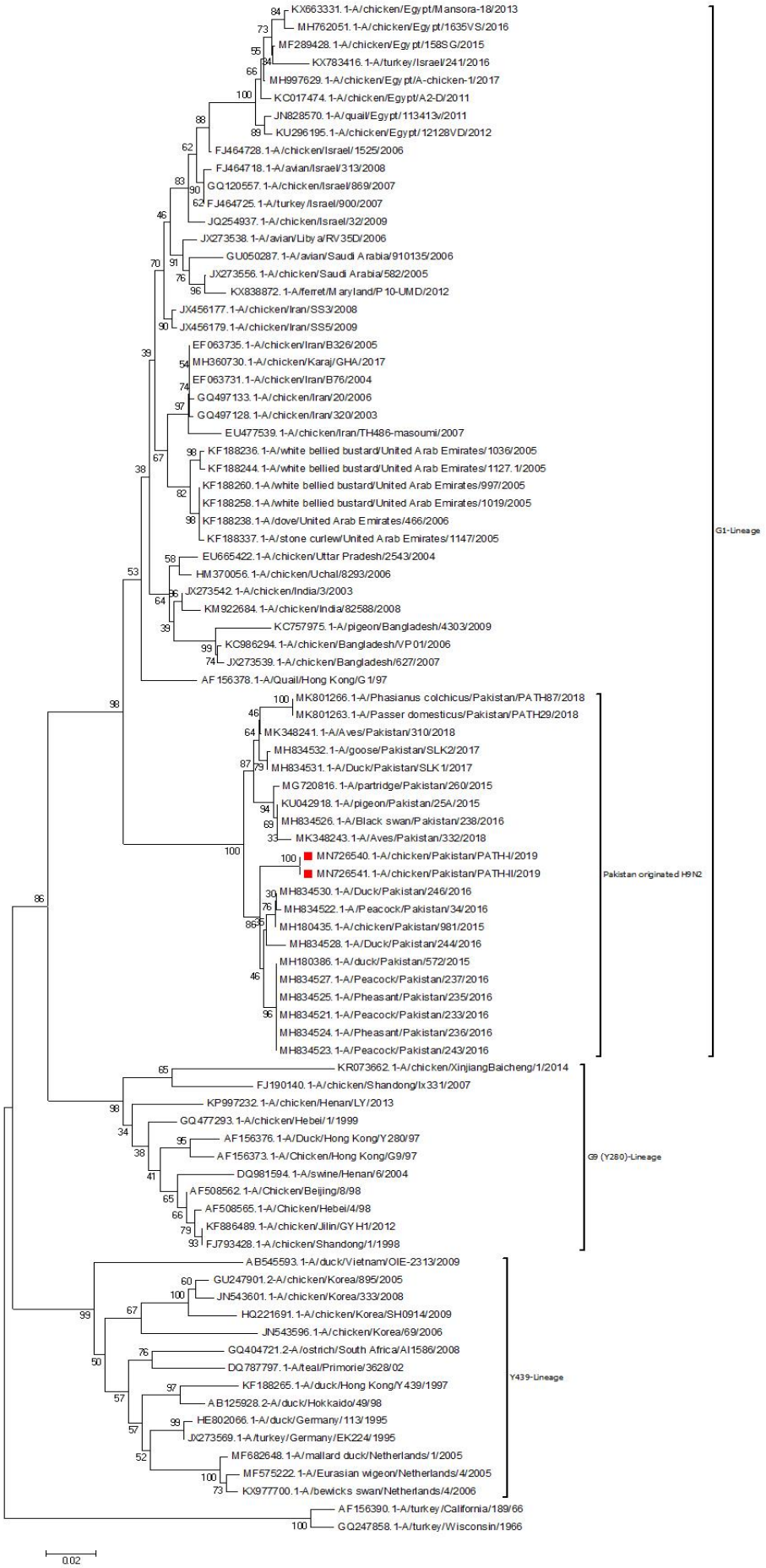

Fig. 1: Phylogenetic tree analysis of HA gene of studied H9N2 LPAIV isolates and other sequences retrieved from NCBI-GenBank database. Pakistan originated isolates of this study are indicated by solid red rectangular.

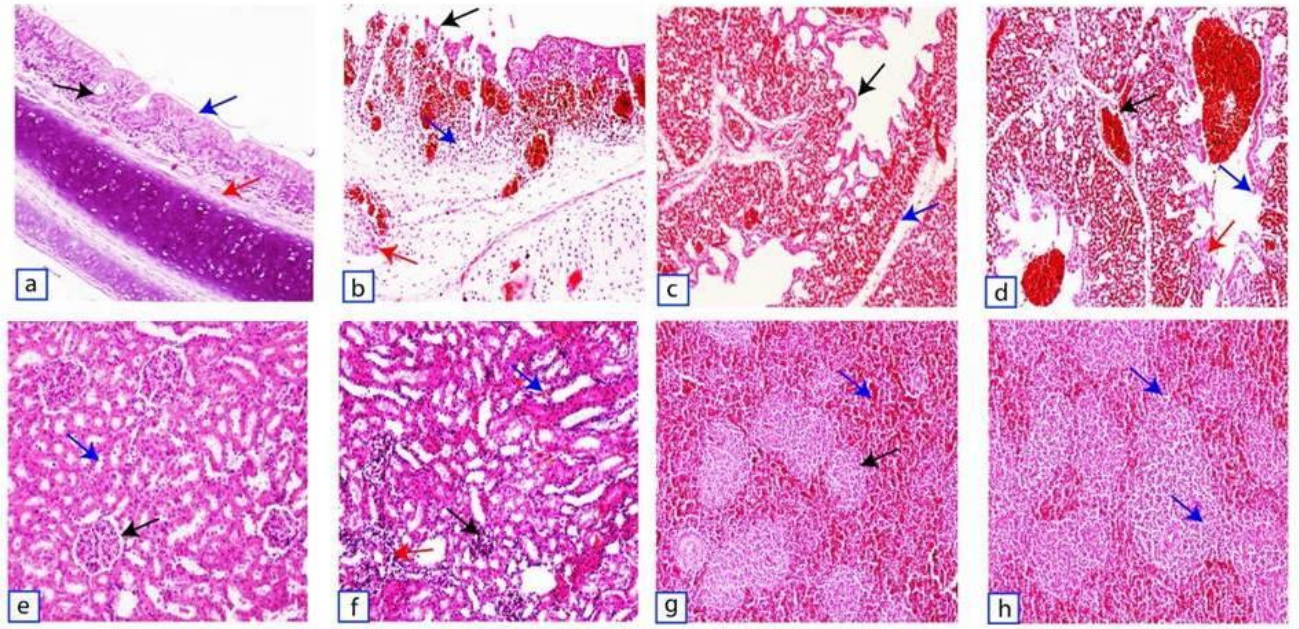

Fig. 2: Photomicrographs of subjected organs (hematoxylin and eosin staining; 10X) ***a:*** *Normal trachea with intact pseudostratified ciliated columnar epithelium (blue arrow), mucous gland (black arrow) and hyaline cartilage* (red arrow). **b:** *Sloughing of the epithelium (black arrow), necrotic cells (blue arrow) and* lymphoplasmacytic infiltration (red arrow) in the trachea of H9N2 LPAIV infected birds. **c.** Normal lungs with intact parabronchi *(blue arrow) and intact simple cuboidal epithelium with atrium and infundibulum (black arrow).* ***d.*** Congestion *(black arrow), distorted epithelium (blue arrow) and mononuclear cells (*MNCs) infiltration (red arrow) in the lungs of H9N2 LPAIV infected birds. **e.** Normal kidney with intact glomerulus *(black arrow)* and renal tubules *(blue arrow)*. **f.** Glomerular hypercellularity *(black arrow),* tubulointerstitial nephritis *(red arrow)* and interstitial haemorrhages (blue arrow) in the kidney of H9N2 LPAIV infected birds. **g.** Normal spleen with intact white pulp *(black arrow)* and red pulp *(blue arrow)* **h:** Necrotic splenocytes in white pulp *(blue arrow)* of spleen of H9N2 LPAIV infected birds.

Table 1: Analysis of homology between viruses of the current study, previously reported Pakistani isolates, and Quail/Hong Kong/G1/97 strain

Other isolates	Similarity (%)		Sites of amino acid substitute	Total
	Nucleotides	Amino acids		
MH834522.1-A/Peacock/Pakistan/34/2016	98.85	96.55	(I90M, I156V, D230E)	3
MK348241.1-A/Aves/Pakistan/310/2018	98.85	96.17	(I90M, I156V, D230E)	3
MH834527.1-A/Peacock/Pakistan/237/2016	98.47	98.21	(S55N, I90M, I156V, D230E)	4
MH834530.1-A/Duck/Pakistan/246/2016	98.47	97.96	(I90M, I156V, D230E, V232I)	4
MH180435.1-A/Chicken/Pakistan/981/2015	98.47	97.96	(I90M, I156V, D230E, L255F)	4
MH834528.1-A/Duck/Pakistan/244/2016	98.47	97.96	(L47M, I90M, I156V, D230E)	4
MH834525.1-A/Pheasant/Pakistan/235/2016	98.47	97.83	(S55N, I90M, I156V, D230E)	4
KU042918.1-A/Pigeon/Pakistan/25A/2015	98.47	97.06	(I90M, G95D, I156V, D230E)	4
MH834521.1-A/Peacock/Pakistan/233/2016	98.47	96.17	(S55N, I90M, I156V, D230E)	4
MG720816.1-A/Partridge/Pakistan/260/2015	98.47	95.91	(I90M, G95D, I156V, D230E)	4
MK348243.1-A/Aves/Pakistan/332/2018	98.47	95.53	(I90M, G95D, I156V, D230E)	4
MH834526.1-A/Black swan/Pakistan/238/2016	98.47	95.53	(I90M, G95D, I156V, D230E)	4
MH180386.1-A/Duck/Pakistan/572/2015	98.08	98.21	(S55N, I90M, I156V, D230E, A256V)	5
MH834524.1-A/Pheasant/Pakistan/236/2016	98.08	98.21	(S55N, I90M, I156V, D230E, A256V)	5
MK801266.1-A/Phasianuscolchicus/Pakistan/ PATH87/2018	98.08	98.08	(I90M, I156V, D230E, G238N, T239N)	5
MK801263.1-A/Passer domesticus/Pakistan/ PATH29/2018	98.08	98.08	(I90M, I156V, D230E, G238N, T239N)	5
MH834523.1-A/Peacock/Pakistan/243/2016	98.08	98.08	(S55N, I90M, I156V, D230E, A256V)	5
MH834532.1-A/Goose/Pakistan/SLK2/2017	98.08	96.55	(I36V, I90M, S127N, I156V, D230E)	5
MH834531.1-A/Duck/Pakistan/SLK1/2017	97.7	96.3	(I36V, I90M, S127N, I156V, S222A, D230E)	6
JX273552.1-A/Chicken/Pakistan/47/2003	96.17	91.70	(K53R, G95D, V101I, I112V, R117K, I129V, I156V, T219R, D230E, V259F)	10
KX759089.1-A/Chicken/Sihala/NARC-04N-087/2004	96.17	91.06	(K53R, I90M, G95D, I112V, R117K, I129V, I156V, T219R, D230E, V259F)	10
JX273553.1-A/Chicken/G-Karachi/2003	95.79	90.68	(K53R, I90M, G95D, V101I, I112V, R117K, I129V, I156V, T219R, D230E, V259F)	11
KF188325.1-A/Chicken/Pakistan/NARC-1617/2005	95.79	90.68	(K53R, I90M, G95D, I112V, R117K, I129V, I156V, T219R, Q227L, D230E, V259F)	11
CY038426.1-A/Chicken/Pakistan/UDL-01/2006	95.40	90.80	(A52D, K53R, I90M, G95D, I112V, R117K, I129V, I156V, M187V, T219R, D230E, V259F)	12
AF508556.1-A/Chicken/Pakistan/5/99	95.02	90.04	(I36V, G37R, K53R, I90M, G95D, V101I, I112V, R117K, I129V, I156V, T219R, D230E, V259F)	13
AF156378.1-A/Quail/Hong Kong/G1/97	94.64	90.04	(I36V, G37R, I45V, A52D, I90M, G95D, V101I, I112V, R117K, I129V, I156V, T219R, D230E, V259F)	14

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Authors contribution: MA, MUR and SM conceived and designed the study. MA, AA, IH and SU executed the experiments. MA, AA, MSY, AM and SU prepared the manuscript. MUR, AA and HUR reviewed the manuscript. MUR evaluated and supervised the study. All authors have read and approved the manuscript.

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