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Full Length Article

Avian Influenza Subtype H9N2 Isolated from Various Districts of Punjab Pakistan during 2019–2020

Kinza Khan[1,3*], Tahir Yaqub[1], Muhammad Zubair Shabbir[1] and Asim Aslam[2]
[1]*Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[2]*Department of Pathology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[3]*Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan*
[*]For Correspondence: 2018-phd-1040@uvas.edu.pk
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Abstract

Avian influenza subtype H9N2 is endemic in Pakistan. The virus being low pathogenic has a low mortality rate but high morbidity rate with huge economic losses in terms of low production. In present study, a total of 500 samples were collected from commercial and backyard poultry of Faisalabad, Toba Tek Singh, Multan, and Bahawalpur districts from September 2019 to February 2020. A total of 39 samples were found to be positive for H9N2 from Faisalabad, Toba Tek Singh, Multan and Bahawalpur which showed an overall prevalence of 7.8%. The samples were further sequenced for HA and NA complete genes and phylogenetic analysis was performed. Results showed that new isolates have close association with recent previous isolates of 2015–2016. The sequence analysis showed that field virus and local vaccine virus has a maximum homology of 98.5 and 99.59% based on HA and NA genes respectively with the field isolates. While imported vaccine viruses have homology with field isolates at a maximum of 89.95 and 90.58% based on HA and NA genes respectively. This shows that vaccines prepared from local field isolates are more successful than the imported vaccines to control H9N2 in the field. © 2021 Friends Science Publishers

Keywords: H9N2; Low pathogenic; Prevalence; Phylogenetic analysis; Homology

Introduction

Avian Influenza viruses belong to Type A Influenza viruses and have a genome composed of RNA comprised of eight segments (Killian 2008). Each segment is responsible to encode one or two specific proteins either structural or non-structural (Fiala *et al.* 2018). Segment 4 is responsible to encode for Hemagglutinin (HA) protein and segment 6 is responsible to encode for Neuraminidase (NA) protein (Bouvier and Palese 2008). Up till now, 18 HA and 11 NA subtypes of Influenza A types have been discovered (Tong *et al.* 2013). Both of these proteins are the surface glycoproteins of influenza viruses and thus responsible for virus entry and exit from the host cell (Jin *et al.* 2014). NA mainly breaks the mucin in the respiratory tract which is a protective secretion of respiratory tract and clears most of the bacterial and viral entry in the respiratory tract. Thus NA assists the virus to approach the sialic acid receptors on the host cell surface (Butt *et al.* 2010). Secondly, NA helps the virus to fuse with the host cell membrane and thirdly, its enzymatic activity cleaves the sialic acid from the sialo glycoconjugates at the time of viral exit from the cell. So it also helps in spread of viral infection to the new cells (Gubareva *et al.* 2000). HA protein mainly interacts with the terminal sialic acid on the host cell surface glycoconjugate and helps in viral adsorption (Tønnessen *et al.* 2013). Host immune responses are also generated against these proteins of the virus to control the infection (Kosik and Yewdell 2019). Low Pathogenic Avian Influenza (LPAI) viruses cause a significant level of morbidity but occasionally high mortality leading to high economic losses worldwide in the poultry (Foster 2018). Among these viruses, H9N2 subtype is of special concern regarding Pakistan (Ahad *et al.* 2013). This subtype was first discovered in 1966 from turkeys in Wisconsin (Dong *et al.* 2011). In Pakistan, it is endemic and was first reported in 1998 (Naeem *et al.* 1999). Since then, it has been continuously circulating and evolving among the poultry population (Cameron *et al.* 2000). A recent comprehensive study on broiler birds showed 92% prevalence of H9N2 in Pakistan (Kausar *et al.* 2018). The poultry sector plays an important role to meet the protein requirement of the nation (Hussain *et al.* 2015). Even after good management practices and vaccination strategies, every year losses due to various diseases are beard by poultry sector (Hafez and Attia 2020). Avian influenza also plays its role in progress inhibition of this sector (Gado *et al.* 2017). Various commercial farmers use imported as well as local vaccines to minimize this threat, yet this problem is

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faced by them each year (Irshad *et al.* 2018). The current study was designed to isolate recent field variants of H9N2 and their comparison was done with previous isolates as well as some common vaccines being used in the field against H9N2 in Pakistan.

Materials and Methods

Sampling and transportation

Total 500 oral swabs and organ samples (trachea) samples were collected from commercial and backyard farms of different localities of Faisalabad, Toba Tek Singh, Multan, and Bahawalpur districts. The samples were then transferred to the lab dipped in BHI (Brain Heart Infusion broth) as a transport medium added with antibiotic (Gentamycin at 0.5 mg/mL of BHI) in icebox.

Virus cultivation and harvesting

The samples were then cultivated on 9–11 days old chicken embryonated eggs through chorio-allantoic sac (CAS) route (Senne 1989) and allantoic fluid (AF) was harvested after 48 h of incubation (Khalili *et al.* 2013).

Haemagglutination test (HA Test) and haemagglutination inhibition (HI) assay

The AF was checked for hemagglutination activity through HA test (Maff 1984). The positive samples were then checked for HI test using specific antiserum against subtype H9N2 of avian influenza (Villegas and Purchase 1998).

Molecular characterization and sequencing

Out of 21 positive samples, four representative samples of four different districts were then processed for RNA extraction using QIAmp viral RNA extraction mini kit using the manufacturer's instructions. From this RNA, complementary DNA was synthesized using Revert Aid (Thermo Scientific®) First Strand cDNA Synthesis Kit using the manufacturer's instructions. Complete HA and NA genes were amplified using specific reported primers for H9 and N2 genes following (Ali *et al.* 2017). Sequencing was performed using specific primers which were used for PCR by Sangers' sequencing method.

Phylogenetic and sequence analysis

The phylogenetic tree was generated neighbor-joining method using 1000 Bootstrap values in MEGA-X by using CLUSTAL W algorithm (Kumar *et al.* 2018). Sequences analysis of recent and previous isolates of Pakistan and vaccinal strains currently being used in the field was performed for percentage identity and divergence (Gado *et al.* 2017).

Results

Prevalence

A total of 500 samples were collected from various localities of District Faisalabad (n=105), Toba Tek Singh (n=113), Multan (n=122) and Bahawalpur (n=160). Out of 500 samples, 65 were found positive for HA test and 39 were found positive for H9 by using the specific anti-serum. The overall prevalence of H9N2 was found to be 7.8% in these areas (Table 1). Prevalence of each district was 8.57 (13/105), 8.84 (21/113), 6.55 (16/122) and 7.5% (15/107).

Sequence analysis

The HA and NA nucleotide sequences of four new isolates (one representative of each district) were submitted to GenBank with accession numbers mentioned in Table 2.

Phylogenetic analysis

Phylogenetic analysis of HA and NA genes (Figs. 1, 2 and 3) of new isolates in comparison with isolates of 2015–2016 and previous Pakistani isolates showed that the recent isolates have more resemblance with the isolates of 2015–2016. The study isolates form a distinct clade with 2015–2016 isolates in G1 lineage from those which were isolated before 2010. Moreover, the local vaccine virus (A/chicken/Pakistan/vac/2018) falls in the same clade and as those of recent isolates. While the imported vaccine viruses are being currently used in the field in Pakistan A/chicken/Guangdong/SS/94, A/chicken/Egypt/114940v/NLQP/2011, A/chicken/Iran/Av1221/1998 fall in different clade than those of recent isolates.

Discussion

H9N2 is most prevalent influenza virus among terrestrial poultry throughout Eurasia (Dong *et al.* 2011). The virus is endemic in Pakistan and outbreaks are continuously being reported among the poultry every year in Punjab province of Pakistan (Shaukat *et al.* 2016). In current study the overall prevalence in various districts of Punjab was found to be 7.8%. Another recent study showed a comparable prevalence of 6.7% in commercial and 2.7% in backyard poultry of Pakistan (Ali *et al.* 2017). A study in Quetta district of Balochistan province showed the seroprevalence of 14.03% H9N2 in broiler birds (Arif *et al.* 2015). Another country wide study showed 92% prevalence of H9N2 in broiler birds (Kausar *et al.* 2018) while a study of Faisalabad district showed a prevalence of 60% in commercial layers (Akhter *et al.* 2017). These all show that H9N2 has a higher prevalence in commercial poultry as compared to backyard poultry. On the basis of phylogenetic studies, there are mainly three lineages H9N2 viruses

Table 1: Study isolates with their accession numbers for HA and NA genes on GenBank

S. No.	Isolates of H9N2	District	Type of Poultry	HA accession No.	NA accession No.
1	A/Chicken/Pakistan/041CP/2020	Faisalabad	Commercial	MW767044	MW786686
2	A/Chicken/Pakistan/061CP/2020	Multan	Commercial	MW774320	MW786666
3	A/Chicken/Pakistan/062BYP/2020	Bahawalpur	Backyard	MW769799	MW786665
4	A/Chicken/Pakistan/046CP/2019	Toba Tek Sing	Commercial	MW767039	MW786664

Nucleotide sequences analysis for percentage identity and nucleotide diversity among the study isolates, previous recent isolates and vaccine viruses showed a maximum identity of field isolates with the local vaccine virus and minimum identity with imported vaccine viruses. This shows that study isolates have 98.5 % homology with local vaccine virus as compared to imported vaccine viruses (89.95%) based on HA gene (Table 2) while based on NA gene, 99.59% homology with local vaccine virus as compared to imported vaccine viruses (90.58%) as mentioned in Table 3. Moreover, the nucleotide divergence showed that NA gene is more conserved among the field isolates as compared to HA gene

Table 2: Nucleotide diversity & percentage identity between field isolates and vaccine viruses based on HA gene

Sr. No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	**A/chicken/Pakistan/982/2016**		2.4	2.7	1.7	2.7	5.9	0.8	0.7	0.5	3.1	2.8	4.3	2.9	16.6	11.9	13.4	3.2
2	**A/chicken/Pakistan/994/2015**	97.69		0.3	2.4	0.3	5.9	2.0	2.2	2.2	4.6	4.2	4.8	4.0	16.2	11.9	13.4	4.8
3	**A/chicken/Pakistan/844/2016**	97.46	99.65		2.7	0.0	6.1	2.3	2.5	2.5	4.8	4.5	5.1	4.3	16.5	11.9	13.7	5.1
4	**A/chicken/Pakistan/845/2016**	98.38	97.69	97.46		2.7	5.8	1.3	1.5	1.5	3.8	3.6	3.9	3.6	16.5	11.6	13.0	3.9
5	**A/chicken/Pakistan/835/2016**	97.46	99.65	100	97.46		6.1	2.3	2.5	2.5	4.8	4.5	5.1	4.3	16.5	11.9	13.7	5.1
6	**A/chicken/Pakistan/540CF/2015**	94.65	94.7	94.47	94.76	94.47		5.4	5.7	5.7	7.6	7.3	8.4	7.6	15.1	11.3	12.1	7.8
7	**A/chicken/Pakistan/740CF/2015**	99.19	98.04	97.81	98.73	97.81	95.11		0.7	0.6	3.0	2.7	3.9	2.7	16.0	11.4	13.0	3.2
8	**A/chicken/Pakistan/1108CF/2016**	99.25	97.87	97.64	98.56	97.64	94.82	99.25		0.5	2.9	2.6	4.2	2.7	16.8	11.6	13.4	3.0
9	**A/chicken/Pakistan/401BYP/2015**	99.48	97.87	97.64	98.56	97.64	94.82	99.36	99.42		2.9	2.6	4.0	2.7	16.3	11.5	13.1	3.0
10	**A/Chicken/Pakistan/046CP/2019**	97.06	95.8	95.57	96.49	95.57	93.27	97.18	97.23	97.23		1.3	5.9	2.3	17.6	13.3	14.5	1.5
11	**A/Chicken/Pakistan/041CP/2020**	97.35	96.08	95.85	96.66	95.85	93.55	97.46	97.52	97.52	98.67		5.5	2.0	18.4	13.7	15.1	3.0
12	**A/Chicken/Pakistan/062BYP/2020**	96.03	95.57	95.34	96.37	95.34	92.69	96.37	96.08	96.26	94.7	94.99		6.1	18.9	14.1	15.8	5.9
13	**A/Chicken/Pakistan/061CP/2020**	97.29	96.26	96.03	96.6	96.03	93.32	97.41	97.46	97.46	97.75	98.04	94.47		18.6	13.1	15.1	4.0
14	**A/Chicken/Guangdong/SS/94**	86.83	87.11	86.88	86.88	86.88	87.8	87.23	86.71	87.06	86.19	85.73	85.39	85.62		14.3	7.7	18.1
15	**A/chicken/Egypt/114940v/2011**	86.83	86.83	86.83	87.06	86.83	87.29	87.17	86.88	87.11	85.85	85.56	85.27	85.96	85.16		9.4	13.5
16	**A/chicken/Iran/av1221/1998**	88.95	89.01	88.78	89.24	88.78	89.93	89.24	88.95	89.18	88.21	87.86	87.34	87.8	93.21	88.67		15.0
17	**A/chicken/Pakistan/vac/2018**	96.95	95.57	95.34	96.37	95.34	93.15	96.95	97.12	97.12	98.5	97.18	94.7	96.26	85.91	85.68	87.92	

% Identity

% Nucleotide Diversity

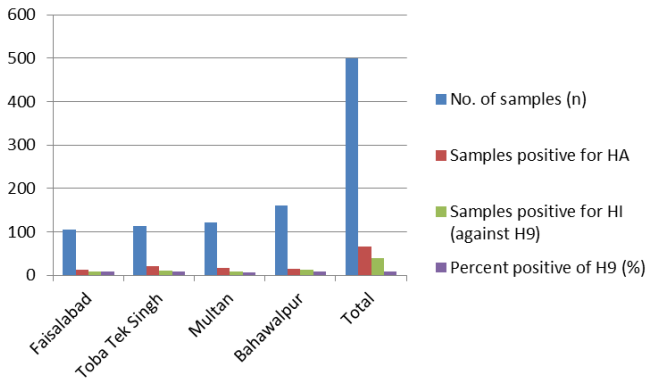

Fig. 1: Prevalence of H9N2 in various districts of Punjab, Pakistan

including Y280 like (A/Duck/Hong Kong/Y280/97), G1 like (A/Quail/Hong Kong/G1/97) and Korean like (A/Chicken/Korea/38349-p96323/96) (Hosseini *et al.* 2017). The Pakistani isolates of H9N2 are found to be linked with G1 like lineage (Ali *et al.* 2019). Study isolates in this research, also fall in G1 lineage and form a distinct clade as compared to previous isolates before 2010 which shows that the virus has evolved much during the recent decade from 2011–2020. Sequence analysis for percentage identity and nucleotide diversity on the basis of HA and NA genes showed that study isolates from commercial and backyard poultry are not much variant and have a maximum diversity of 7.3 and 7.4% on the basis of HA and NA genes respectively. The local vaccine isolate is much more identical to the study isolates and previous isolates of Pakistan. A study from Sharkia Governorate of Egypt also showed similar results (Gado *et al.* 2017) and indicated that due to wide host range, H9N2 viruses (Dong *et al.* 2011) rapidly evolved with time, and there is a need for continuous monitoring to upgrade the vaccinal seed for immunization of birds.

Conclusion

Current study shows that H9N2 is continuously circulating and evolving in Pakistan. So there is a need to upgrade the vaccine virus on regular basis to combat this problem.

Table 3: Nucleotide diversity & percentage identity between field isolates and vaccine viruses based on NA gene

Sr. No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	**A/chicken/Pakistan/835/2016**		0.0	1.0	0.8	0.4	6.0	0.6	1.4	1.4	1.8	2.4	1.5	1.5	13.2	10.0	10.7	1.9
2	**A/chicken/Pakistan/844/2016**	100		1.0	0.8	0.4	6.0	0.6	1.4	1.4	1.8	2.4	1.5	1.5	13.2	10.0	10.7	1.9
3	**A/chicken/Pakistan/845/2016**	98.97	98.97		0.9	1.2	6.3	0.8	0.6	1.6	2.6	3.2	2.2	2.2	13.5	10.8	10.9	2.7
4	**A/chicken/Pakistan/982/2016**	99.18	99.18	99.11		1.0	6.0	0.3	1.2	1.2	2.2	2.8	2.0	2.0	13.6	10.8	11.2	2.4
5	**A/chicken/Pakistan/994/2015**	99.59	99.59	98.84	99.04		6.3	0.7	1.5	1.5	1.9	2.5	1.7	1.7	13.4	10.2	10.8	2.1
6	**A/chicken/Pakistan/540CF/2015**	94.61	94.61	94.4	94.61	94.33		5.8	6.8	6.6	7.1	7.4	6.8	6.8	13.5	10.9	10.8	7.2
7	**A/chicken/Pakistan/740CF/2015**	99.38	99.38	99.18	99.65	99.24	94.81		1.2	1.0	2.0	2.6	1.8	1.8	13.1	10.5	10.8	2.1
8	**A/chicken/Pakistan/1108CF/2016**	98.63	98.63	99.38	98.77	98.49	93.92	98.84		2.0	3.0	3.6	2.7	2.7	13.5	11.1	11.1	3.1
9	**A/chicken/Pakistan/401BYP/2015**	98.63	98.63	98.43	98.77	98.49	94.13	98.97	98.09		2.8	3.4	2.6	2.6	13.6	11.3	11.6	3.0
10	**A/Chicken/Pakistan/046CP/2019**	98.29	98.29	97.54	97.88	98.15	93.72	98.09	97.2	97.33		1.1	0.7	0.7	13.4	11.2	11.8	0.4
11	**A/Chicken/Pakistan/062BYP/2020**	97.74	97.74	96.99	97.33	97.61	93.45	97.54	96.65	96.79	98.9		1.9	1.9	14.3	11.6	12.4	0.7
12	**A/Chicken/Pakistan/061CP/2020**	98.49	98.49	97.88	98.09	98.36	93.92	98.29	97.4	97.54	99.24	98.15		0.0	13.5	10.7	11.3	1.2
13	**A/Chicken/Pakistan/041CP/2020**	98.49	98.49	97.88	98.09	98.36	93.92	98.29	97.4	97.54	99.24	98.15	100		13.5	10.7	11.3	1.2
14	**A/chicken/Guangdong/SS/94**	84.78	84.78	84.58	84.51	84.65	84.58	84.85	84.58	84.51	84.65	84.1	84.58	84.58		10.4	5.6	13.5
15	**A/chicken/Egypt/114940v/2011**	90.58	90.58	90.04	90.04	90.45	89.97	90.24	89.76	89.63	89.69	89.42	90.1	90.1	87.58		6.9	11.3
16	**A/chicken/Iran/av1221/1998**	90.38	90.38	90.17	89.97	90.24	90.31	90.31	90.04	89.69	89.56	89.15	89.9	89.9	91.13	92.36		11.9
17	**A/chicken/Pakistan/vac/2018**	98.15	98.15	97.4	97.74	98.02	93.65	97.95	97.06	97.2	99.59	99.31	98.84	98.84	84.58	89.63	89.49	

% Identity

% Nucleotide Diversity

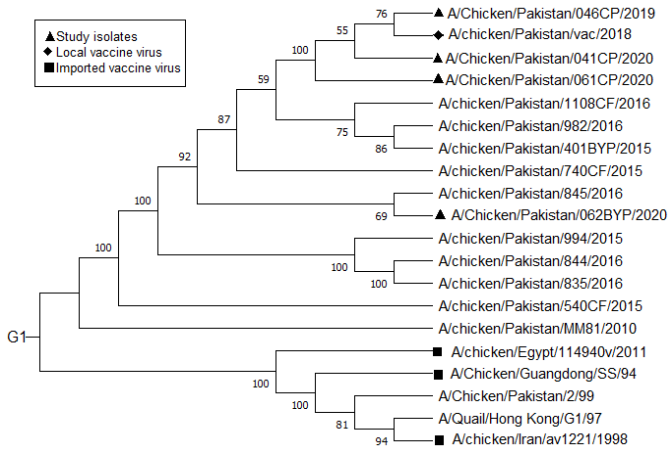

Fig. 2: Phylogenetic tree (based on HA gene) showing recent isolates of H9N2 viruses in Pakistan compared with the previous isolates and the vaccine viruses

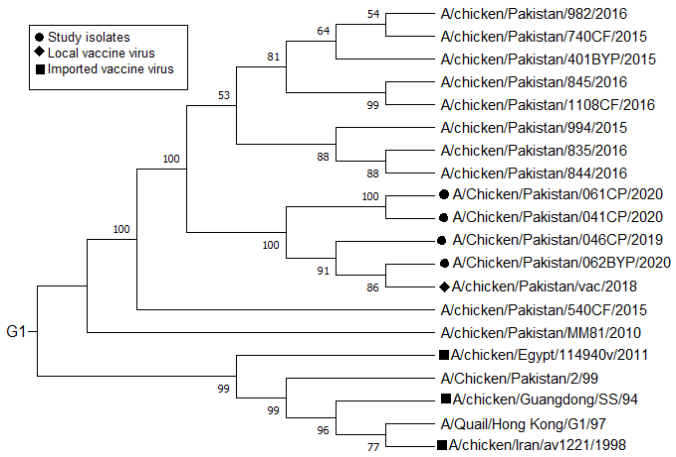

Fig. 3: Phylogenetic tree (based on NA gene) showing recent isolates of H9N2 viruses in Pakistan compared with the previous isolates and the vaccine viruses

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Author Contribution

This article is a part of Ph. D research work of KK of this manuscript while the KK, TY and MZS have contributed in making the research plan and conducting the research work. All the authors contributed equally in write up of this work and approval of final copy.

Conflict of Intrests

Authors declear no confilict of intrest

Data Availibilty

Data will be availbal on demand

Ethics Approval

During this study, oral samples were taken from the field from suspected birds and virus isolates were further studied through sequencing and bioinformatics tools. No trial on birds was done such as disease induction or immune protection analysis. So, there is no need of such approval.

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