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Research Article

SEROPREVALENCE OF H5, H7, and H9 INFLUENZA VIRUSES IN BROILERS PREVAILING IN DIFFERENT ECOLOGICAL ZONES OF KHYBER PAKHTUNKHWA, PAKISTAN

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ABSTRACT:

Background: Avian influenza poses a significant risk to communities as it impacts birds and results in a high mortality rate and global economic losses of up to 89%. This study aimed to assess the seroprevalence of the H5, H7 and H9 strain of AIV between the years2017 to 2020 in Khyber Pakhtunkhwa, Pakistan.

Methods: 650 blood samples from broiler birds were collected from five (Dera Ismail Khan, Tank, Abbottabad, Mansehra, and Peshawar) different ecological zones of Khyber Pakhtunkhwa, Pakistan. Initial screening was done using the HA and HI tests. H9 samples were further confirmed using PCR.

Results: Overall seroprevalence of avian influenza virus subtypes H5, H7, and H9 was 22.43%, 5.384%, and 25.23%, respectively. Maximum seroprevalence was reported in DIK district, followed by Abbottabad, Peshawar, Mansehra, and Tank. A phylogenic study of H9 samples depicted H9 strains clustering closer to previously reported Pakistan-originated strains. Overall, the AIV H9N2 strain under study showed 10-12% genomic distance to strains of B1 sub-lineages reported from Pakistan, India, and Iran. Similarly, a 12.9–17% genomic distance was observed between viruses of the G1_Mideast_Group A lineage, followed by 19–22.5% between viruses of the Y9280-like lineage and 24-28% between viruses of the Y439/97-like lineage.

Conclusion: The current research region had an epidemic of avian influenza. Therefore, it is crucial to isolate infected birds in order to prevent and control the further spread of the disease.

KEY WORDS

Avian influenza Seroprevalence, Human health, PCR, Pakistan

ARTICLE HIGHLIGHTS

1. The current study estimates the seroprevalence of avian influenza virus subtypes H5, H7 and H9 in five different ecological zone of KPK Pakistan.
2. H9 was more prevalent as compared to H5 and H7.

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3. A genomic distance ranging from 12.9% to 17% was identified between viruses belonging to the G1_Mideast_Group A lineage.
4. This was followed by a distance of 19% to 22.5% between viruses of the Y9280-like lineage, and a distance of 24% to 28% between viruses of the Y439/97-like lineage.

1 | INTRODUCTION

The poultry sector is an important part of agriculture industry having a pivotal role in socio economic development of Pakistan. This industry not only helps to fulfill the food requirements of the country but also creates several employment opportunities. The Northern and Southern region of Khyber Pakhtunkhwa has more poultry forms because of suitable environment throughout the year. Avian influenza of high pathogenic (HP) nature was first time reported in Pakistan in 1995[1-2]. Since then, the outbreak has been repeatedly reported in several locations across the country from several areas of poultry farming. Based on the importance of poultry industry, the protection from disease is very important to be considered. An invasion of the AIV from wild birds to poultry may occur and then may transferred to domestic birds that may cause almost nothing or may cause minor clinical signs. At present avian influenza A virus subtypes H5 and H7 considered high pathogenic, while subtypeH9N2 is low pathogenic prevailing having capability to spread and maintain through the natural sources[3-5]. The natural reservoirs for AIVs were observed in wild birds, domestic birds and animals that are in contact with waterfowl. Pigs act as a intermediate host that were found to transmit the virus to others. In certain Southeast Asian countries, a native waterfowl was found to harbor H5N1 avian influenza virus for many years[6-10]. The H9N2 virus has garnered significant attention due to its swift dissemination among indigenous avian species. This virus, which has minimal pathogenicity, is able to survive in chicks and is transmitted to unaffected birds through the fecal-oral route, even though it causes severe clinical symptoms. The avian influenza virus subtype H9N2 induces severe respiratory disease in chickens with impaired immune systems. The phenomenon leads to a rise in premature chick death and a significant decrease in egg output in hens, resulting in financial detriment[7]. In Japan, studies have shown that cargo agents were spreading H5N1 and H9N2 while spreading was also associated with bird and pet disease[11-12]. The worldwide Avian Influenza viruses outbreaks caused acute respiratory tract infection causing significant disease[13]. The signs were designated by Bird flu that caused great economic loss[14]. Avian influenza virus outbreaks are the cause of diseases and deaths worldwide, including humans, domestic birds and mammals. In recent years, some strain of AI has spread the fence of nature to humans, causing severe medical symptoms and mortality[13]. The objective of this study was to evaluate the prevalence of the H5, H7, and H9 subtypes of Avian Influenza Virus (AIV) in Khyber Pakhtunkhwa, Pakistan, from 2017 to 2020.

2 | MATERIAL AND METHODS

The selected area included five districts of Khyber Pakhtunkhwa including Peshawar, Mansehra, Abbottabad, Tank and Dera Ismail Khan. Random collection of blood samples was done from 650 birds during winter and summer season. This was accomplished by using a disposable sterile syringe to collect blood samples from the wing veins of commercial broilers and placing them in vacutainer tubes that had been treated with EDTA. For preventing the rupture of the suspended erythrocytes, the contents of the vacutainers tube were mixed using a gentle tapering technique. For serum separation, the samples were subsequently transported to the laboratory of the Department of Biological Sciences at Gomal University, D.I. Khan, where they were kept in a cool environment. After that, the serum samples were kept in a low temperature freezer at a temperature of -20 degrees Celsius for later use. All of the blood samples were subjected to quantitative analysis using the recognized antigens H5, H7, and H9 (control positive), which were received from the Poultry Research Institute (PRI) in Rawalpindi, Pakistan. The Haemagglutination Inhibition (HI) test was used to determine the prevalence of Avian Influenza throughout the samples. Genomic RNA extraction was done for H9 samples using commercially available genome extraction kit (QIAmp Viral RNA Kit, Qiagen, USA) while amplification of genome, the cDNA synthesis kit was used to synthesize the cDNA from the extracted RNA (Thermo scientific Reert Aid First Strand Kit).

For genome amplification, previously reported primers[15] targeting HA gene (forward; 5′-AGC AAA AGC AGG GGA AYW WC-3′) and (reverse: 5′-CCA TAC CAT GGG GCA ATT AG-3′) were used. The HA gene was subjected to be amplified using PCR protocol as previously described yielding a product of approximately 808bp[15]. PCR product amplicons were purified by using Wizard SV Gel and PCR Clean-Up System. Following the manufacturer's guideline (Promega, Co. Madison, WI, USA). Primers used for amplification with the help of Big Dye Terminator method and detected in ABI PRISM Genetic Analyzer 3130x1 version were used for the sequencing

of the HA genome (Applied Biosystems, Foster City, CA, USA).The Gene Bank database was used for comparison of obtained sequences with the help of BLAST tool at NCBI(https://blast.ncbi.nlm.nih.gov/Blast.cgi). The HA genome sequences representing so-far reported distinct AIV H9N2 strains were retrieved from the public database. Assembled sequence of each isolate was aligned with strains representing distinct AIV H9N2 strains (GenBank) using Clustal W methods in BioEdit version 5.0.6 (Hall 1999) for subsequent HA gene-based phylogenetic analysis. To know about the phylogenetic cluster of the present study isolates, HA gene sequences of isolates were compared recently reported distinct AIV H9N2 strains throughout the world with the help of distance-based neighbour-joining (1000 replication bootstrap values) method in MEGA version 6.0 software (http://www.ncbi.nlm.nih.gov/). To reveal evolutionary dynamic, all positions containing gaps and/or missing codon were eliminated with codon positions as 1st, 2nd, 3rd and non-coding.

3 | RESULTS

Out of 650 samples, the largest antibody titer was 1:4 and the lowest was 1:32, with no samples having a titer more than 32. In the current study, researchers looked at the overall sero-prevalence of avian influenza (H5, H7 and H9) in five ecologically distinct districts of Khyber Pakhtunkhwa, Pakistan was alarming.

Table 1 Seroprevalence of avian influenza viruses from five different ecological zones of KPK Pakistan

Districts	Strain	No. of samples Observed	Positive	Prevalence (%)
Dera Ismail Khan	H5N1	130	43	33.08%
	H7N3	130	2	1.54%
	H9N2	130	45	34.62%
Tank	H5N1	130	35	26.92%
	H7N3	130	0	0%
	H9N2	130	35	26.92%
Abbottabad	H5N1	130	47	36.15%
	H7N3	130	15	11.54%
	H9N2	130	51	39.23%
Mansehra	H5N1	130	22	16.92%
	H7N3	130	13	10%
	H9N2	130	38	29.23%
Peshawar	H5N1	130	28	21.54%
	H7N3	130	5	3.84%
	H9N2	130	30	23.07%

The analysis of the seroprevalence of the avian influenza A virus subtypes H5, H7, and H9 was presented in study table 1. The results showed that compared to H5 (22.43%) and H7 (5.38%), the H9 (25.23%) subtypes had a higher prevalence.

Table 2 Haemagglutination inhibition titers were recorded for the avian influenza strains.

Specific Antisera		A (H7) Antigen	B (H9) Antigen	C(H5) Antigen
Group A (H7)	R1	1: 16	1: 32	1: 32
	R2	1: 64	1: 32	1: 32
	R3	1: 32	1: 32	1: 64
	R4	1: 64	1: 64	1: 64
	R5	1: 64	1: 64	1: 32
	GMT	**39.4**	**39.4**	**24.3**
Group B (H9)	R1	1: 32	1: 32	1: 64
	R2	1: 32	1: 32	1: 64
	R3	1: 32	1: 64	1: 16
	R4	1: 64	1: 64	1: 32

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	R5	1: 64	1: 64	1: 32
	GMT	**39.4**	**48.5**	**36.8**
Group C	R1	1: 64	1: 16	1: 64
(H5)	R2	1: 16	1: 32	1: 64
	R3	1: 64	1: 64	1: 64
	R4	1: 64	1: 64	1: 32
	R5	1: 32	1: 32	1: 128
	GMT	**39.4**	**36.8**	**78.3**
Group D	R1	-	-	-
(Control group)	R2	-	-	-
	R3	-	-	-
	R4	-	-	-
	R5	-	-	-
	GMT	-	-	-

Group A H7 (rabt) serum exhibited a minimum heterogeneous antibody titer of 1:16 against H5 and H9, whereas the maximum titer detected was 1:64. This was the case for both H5 and H9. According to Table 2, the highest heterologous HI titers of Group B rabbit is serum against H9 isolate antigen ranged from 1:16 to 1:64. On the other hand, the highest heterologous HI titers of Group C rabbit is serum against H5 isolate antigen ranged from 1:16 to 1:64 across the two groups. The PCR gel bands (808bp) indicate the presence of the HA gene, which is used to identify the avian influenza virus H9N2 in samples obtained from sick hens (**Figure 1**).

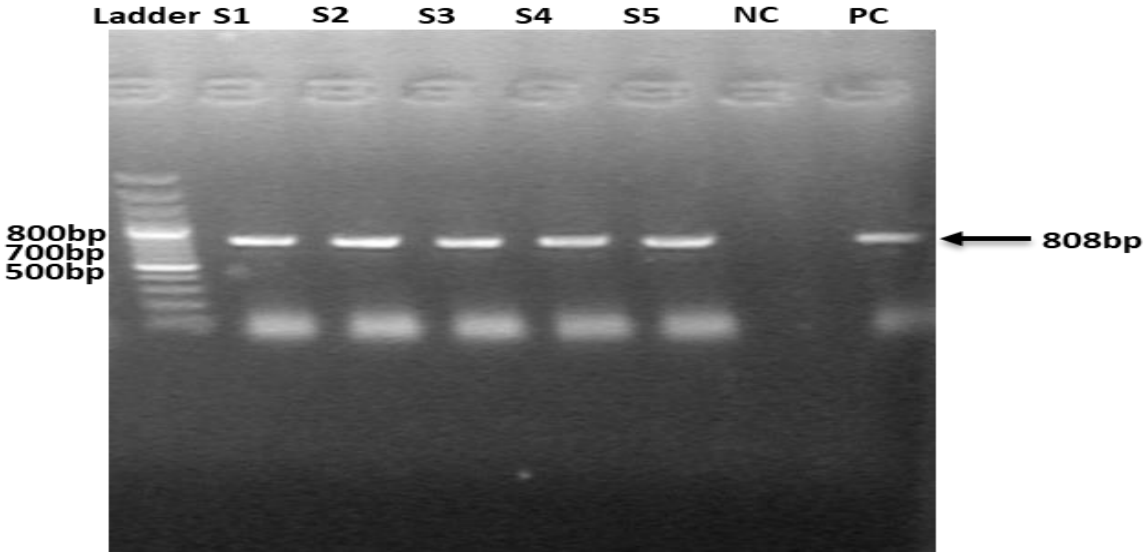

Figure 1 PCR gel bands (808bp) showing the presence of HA gene used for the identification of avian influenza virus H9N2 in samples collected from morbid chickens. **Note:** Ladder of 100bp was used. S1-S5: Samples 1-5, NC: Negative control, PC: Positive control

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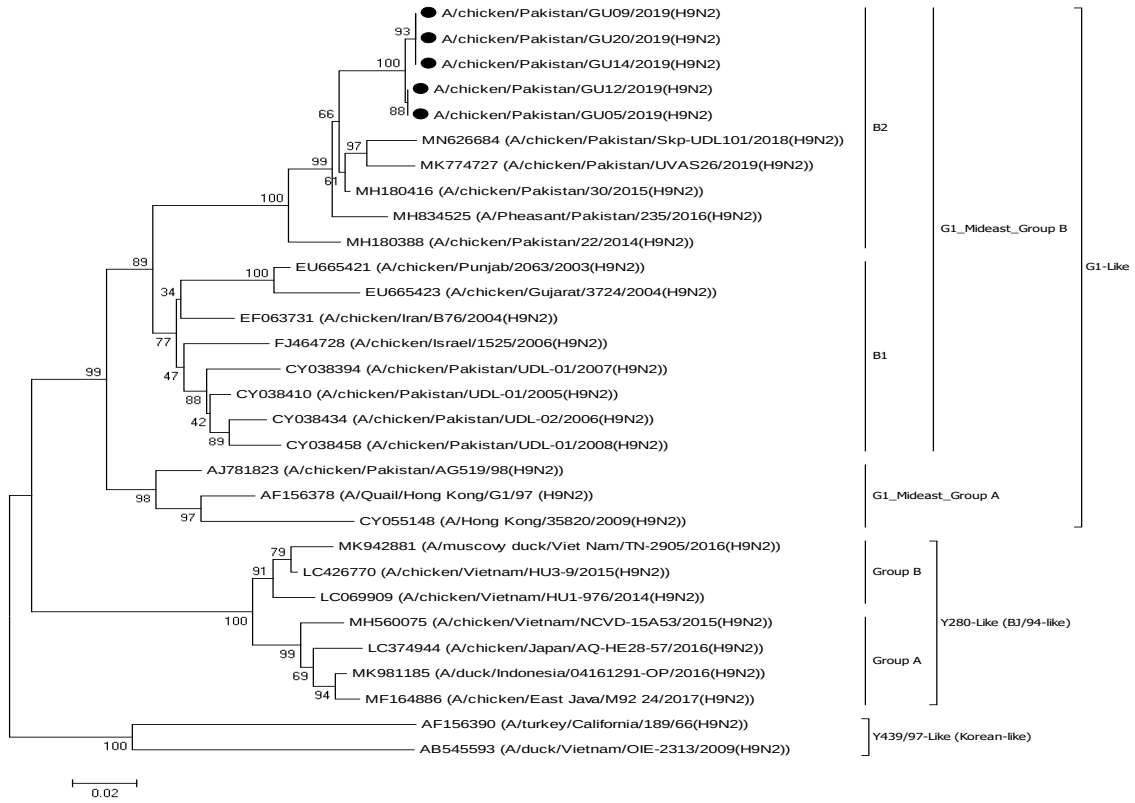

Fig 2 Genomic and Phylogenetic analysis of H9N2virus with Mega XI program (neighbor joining).

Owing to the presence of amino acid sequence (PAKSSR/G) at HA proteolytic cleavage site, all the H9N2 strains tested were designated as low pathogenic avian influenza viruses (LPAIVs). Based on HA gene, the phylogenetic analysis clustered all under study AIV H9N2 strains with those viruses that belonged to sub-lineage B2 within G1_Mideast_Group B viruses. All AIV H9N2 strains clustered closer to previously reported Pakistan-originated strains reported during the year 2015-2019. Under study AIV H9N2 strains showed 98.9-97.2% nucleotide homology compared to AIV H9N2 strains reported from chickens in Pakistan during 2015 (MH180416). Whereas, 95.8-95.1% nucleotide homology was observed between under study strains and those strains reported from chickens in Pakistan during 2018-2019. Overall, under study AIV H9N2 strains showed 10-12% genomic distance to strains of B1 sub-lineages reported from Pakistan, India and Iran. Similarly, a 12.9-17% genomic distance was observed between viruses of G1_Mideast_Group A lineage followed by 19-22.5% between viruses of Y9280-like lineage and 24-28% between viruses of Y439/97-like lineage (**Figure 2**).

4 | DISCUSSION

Avian influenza is a highly contagious and virulent illness that affects both animals and humans. The samples were collected from five distinct districts of Khyber Pakhtunkhwa, Pakistan, each characterized by various ecological conditions. The present study was designed to estimate the occurrence of avian influenza viruses (H9, H5 and H7) in broiler chickens. The study focused on examining three significant subtypes of AI (H5, H7, and H9) in broilers throughout five key districts of Khyber Pakhtunkhwa, due to their potential for causing a pandemic. High sero-prevalence of both H5 and H9 was observed in serum samples of broilers. The observed high seroprevalence was anticipated due to the documented circulation of these virus types in various areas of chicken production in Pakistan since 1998[1]. Similar findings have also been discovered in adjacent nations to Pakistan, namely Iran[17]. The study found that the prevalence of H9 antibodies in serum samples from broilers was higher compared to the prevalence of H5 and H7 antibodies. This aligns with the findings of the prior study conducted by[16].

This study aimed to evaluate the seroprevalence of various strains of Avian Influenza virus in broiler chickens. The high sero-prevalence of influenza A (H9N2) identified in this investigation was not surprising, given that this strain of influenza has been widespread in poultry in this region since 1998[16]. The results were in line with the findings of comparable studies conducted in Pakistan[16] and Iran, a nearby nation, where a similar seroprevalence rate of 23-87% was found[17]. However, a separate study conducted in China found a significantly lower seroprevalence rate (ranging from 2.9% to 11.1%) of A (H9N2) among different categories of poultry specialists[18]. This can be attributed to the contrasting poultry production and marketing systems in the respective countries. The level of direct interaction with poultry or poultry products may differ depending on the style of poultry husbandry and live bird markets in each country. A study conducted in Nepal on the awareness and behaviors of poultry workers about Influenza revealed comparable findings. The study stated that 30% of the poultry professionals utilized gloves, while 27% used face masks. Neighboring nations in this region, such as India, China, and Egypt, have also reported serological evidence of human infection with A (H9N2) viruses[19-20]. The sero-prevalence in different groups of poultry professionals varied between 1.2% and 17% in these investigations. However, our investigation found that 30.61% of individuals have antibodies against the A (H9N2) virus. The elevated seroprevalence in Pakistan could be ascribed to the enhanced avian-to-human transmission capability of the avian influenza virus, resulting from genetic changes and reassortments. The greater sero-prevalence observed may be attributed to the regular use of vaccination against Influenza A in chicken populations in Pakistan. This practice may lead to the undetected survival of the disease, enabling its silent transmission to other populations. The study has limitations due to its cross-sectional design, which only spanned six months, perhaps resulting in the omission of seasonal patterns.

In present study, AIV H9N2 was dectected from chicken birds showing typical clinical presentation of avian influenza infection including sneezing, nervous signs (torticollis and paralysis), coughing and darrhoea. It is not unusual because previous reports have confirmed the AIV infection in broilers[22], layer[23, 21], backyard poultry[15] and wild birds[24-26] in Pakistan. All studied AIV H9N2 sequences were clustered into sub-lineage B2 within G1_Mideast_Group B. Our findings agree with observation of previous study who also observed similar clustering pattern for this particular subtype of avian influenza[15]. Additionally, AIV H9N2 viruses of sub-lineage B1, sub-lineage B2 and sub-lineage A have previously been reported from Pakistan indicating the circulation of multiple AIV strains[27-28,29]. Such evidences highlight the virus propensity to be evolved with the passage of time and therefore an emergence of novel strains in the future[30-31]. AIV H9N2 viruses emergence may lead to mismatching of field used vaccine[26-28] that seubsequent may create hardles in disease control particularly in disease-endemic countries such as Pakistan where frequenc occurrence of disease is not uncommon.

5 | CONCULUSION

The study emphasizes the occurrence of three (H9, H5 and H7) distinct strains of avian influenza in Khyber Pakhtunkhwa, Pakistan. The current research validates the evidence that the H9 subtype exhibits a significantly higher prevalence compared to other subtypes, posing a substantial threat to the poultry population in the studied area. Implementing rigorous biosecurity protocols and widespread vaccination efforts will effectively alleviate the prevalence and impact of the disease**.**

Conflict of Interests

All authors declare no potential conflict of interest.

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