SERO-SURVEILLANCE OF AVIAN INFLUENZA SUBTYPES H5, H7, AND H9 IN BACKYARD POULTRY

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Abstract. Avian influenza viruses (Bird flu) cause sickness and mortality in backyard and commercial poultry. Poultry is one of the most successful segments of the livestock business, with a 700-billion-rupee investment. Pakistan is the world's 11th producer but since last few decades' avian influenza virus outbreaks have increased globally including Pakistan. The current study was conducted to determine Sero-surveillance of avian influenza subtypes H5, H7 and H9 in backyard poultry of Abbottabad region. Total 400 random samples were collected from Abbottabad, Havelian, Lora and Lower Tanawal for serological analysis at Veterinary Research and Disease Investigation Center Abbottabad (VR&DIC). Out of 400 blood and tissue samples, 60.85% were found positive while 39.15% were negative. The positive cases for H5 remained 73.16%, H7: 9.39% and H9: 80.10% as confirmed by serological testing and RT-PCR. The highest prevalence was observed in Golden Fayoumi breed of birds. The highest prevalence of H5 was in Abbottabad (9.25%) in Golden Fayoumi (15.32%). 20 weeks old birds were more affected by H5 (16.66%). H7 was at highest prevalence in Lora (27.27%) and lowest in Abbottabad (10.09%) compared to Havelian (22.85%) and Lower Tanawal (10.20%) respectively. More effected breed of H7 was Fancy (21.62%). The highest prevalence of H7 was observed in birds in age group of 20 to 60 weeks (27.23%). The highest prevalence of H9 was observed in Abbottabad (76.15%) in Golden Fayoumi birds (76.64%) followed by Havelian (75.24%), Lora (69.32%) and Lower Tanawal (72.45%) were less prevalent than Abbottabad. The highest prevalence age was 20 to 60 weeks for H9 (96.17%). Also, co-infection of H5 and H9 was reported during current study. Avian influenza can be controlled by proper and in time vaccination of birds. In addition, it is advisable to avoid congested environment to prevent spread of virus from one bird to another. Moreover, to prevent avian influenza one must avoid contaminated food and water to the birds and awareness of farmer and bird owners about vaccination and cleanliness. The current study strongly suggested that continuous surveillance is needed to reduce AIVs infection.

Keywords: *pathogenic strains, backyard chicken, chicken breeds, highly pathogenic AIV, glycoproteins*

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Introduction

Avian influenza is a viral infection caused by negative sense single stranded RNA virus that belongs to family *Orthomyxoviridae*. Seven genera includes in family Orthomyxoviridae, in which influenza A, B, C and D causes influenza virus in vertebrates whereas, influenza A virus infect wild and domestic birds (Chen et al., 2014). Avian Influenza is classified on basis of surface glycoproteins which are Hemagglutinin and Neuraminidase. There are 18 subtypes of hemagglutinin (H1-H18) and 11 subtypes of neuraminidase (N1-N11) identified (Ciminski and Schwemmle, 2019). The first case of avian influenza was reported in 1997 in Hong Kong that led to illness in 18 people (Lai et al., 2016). Avian influenza became a pandemic by crossing two main barriers, Animal –human transmission barrier and Human- human transmission barrier (Tong et al., 2013).

One of known highly pathogenic avian influenza virus (HPAI), called Fowl plague, first reported in 1878, affected chickens in Italy (Ma, 2022). The causative agent from isolated chicken was described in 1902 as influenza virus. Another outbreak was reported in European countries and later on all over world (Dhingra et al., 2018). Later in 1941, hemagglutination reactions and further assays were introduced (Danilenko et al., 2021). Highly pathogenic strains of subtype H5 were revealed in Scotland (Lee et al., 2020). In avian species, avian influenza virus leads to an assortment of syndromes from asymptomatic to upper respiratory tract disease, results in loss of egg production and other lethal systemic disease (Yeo and Gan, 2021)**.** Some factors to control severity of infection includes, ability of virus to cause a disease, stress and immunity level of host, some supplementary bacterial infection (Rohaim et al., 2021).

Influenza virus is spherical, having spikes on surface and its genome consists of 8 RNA fragments which encodes 10 proteins. It has nucleoproteins (NP), matrix proteins (M2), and two antigenic surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA) having various subtypes (Kumari et al., 2023). These glycoproteins are capable to evoke immune response in different subtypes. On the basis of pathogenicity of these glycoproteins, influenza A virus is grouped into Eighteen hemagglutinin and eleven neuraminidase subtypes (Kosik and Yewdell, 2019). Two different spikes having length of approximately 16 nm encodes Hemagglutinin and on surface of virion Neuraminidase molecules are present. The hemagglutinin is trimeric rod shaped and embedded in envelop whereas, NA is tetramer and mushroom shaped (Wolff and Veit, 2021). Short sequence of amino acid harbors two glycoproteins to envelop.

The most thriving segment of livestock is poultry industry. The current investment of poultry industry is more than Rs. 700 billion. Over the course of last few years, this industry has grown and increased to an amazing rate of 8 to 10% per year. Pakistan is now 11th largest poultry producer in the world (Rehan et al., 2019). Poultry meat account for 34 percent (1,518 thousand tons) of the country's overall meat production (4,478 thousand tons). Poultry meat output increased by 12.8%, while egg production increased by 5.6 percent (19.0 billion no.) in 2020-21 compared to the previous year's (Hoda et al., 2021). The total estimation of population of Back Yard Poultry was 88.49 million, while organic eggs was recorded as 17.93 million and organic meat production was 0.54 million tons (Hasni et al., 2021). One of the most serious of these threats is avian influenza. AIV was first reported in Pakistan during 1995 when highly pathogenic outbreak occurred in Hazara region killing two million birds and destroyed broiler breed in region (Dubal et al., 2014).

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Avian influenza is highly prevalent in Pakistan and there is a need of effective surveillance to control spread in backyard poultry. For the last few years Avian influenza subtypes H5, H7, H9 are continuously being reported in backyard poultry (Ahmed et al., 2021). In January 2018 outbreak of avian influenza has been reported in Islamabad, Pakistan to World Organization of Animal Health (OIE), effected species were ducks, chicken, swans. Symptoms seen in these species included nasal discharge, respiratory problem, diarrhea and sudden death. The dead birds were then submitted to National Reference Laboratory of Poultry Disease which were (NRLPD) tested positive for Influenza A virus H5N8 (OIE WI-O, 2008).

Keeping in view the above facts, it is concluded that due to versatile nature of Avian Influenza-A Viruses and its dispersed outbreaks, it cannot be ignored and its continuous surveillance in Back Yard poultry is required to prevent its outbreaks and losses not only to commercial poultry but also to human population. Therefore, the current study is designed for surveillance of AIV in Back yard poultry to determine the prevalence of Avian Influenza, its strains and molecular characterization of subtypes in Abbottabad region.

Materials and methods

Study was conducted at Veterinary Research and Disease Investigation Center (VR&DIC) in Abbottabad district during 2022.

Sample collection

Blood samples were collected from different birds that were selected, in Gel Vacutainers. Total 400 random samples were collected in 3 cc sterile syringe and placed in gel vacutainers for serological analysis from different Tehsils of District Abbottabad viz; Abbottabad, Havelian, Lora and Lower Tanawal. The samples were shifted to Virology Lab at Veterinary Research and Disease Investigation Center (VR&DIC) Abbottabad in cold chain for further Lab analysis.

Sample analysis

Blood samples were subjected to centrifugation at 3000 rpm for 3-4 min and serum (supernatant) was separated in Eppendorf tubes.

Preparation of 1% RBCs suspension

1% red blood cells (RBCs) stock was prepared and then working solution of 5 ml (200 µl RBCs + 4800 µl PBS) was prepared from stock (Alkhorayef, 2021).

Haemagglutination (HA) and inhibition (HI) tests

HA and HI were performed using standard protocols.

Differentiation of subtypes using PCR

RNA extraction

After HI, positive samples were preceded for extraction and PCR. For extraction purpose GeneAll Biotechnology kit (Korea-based manufacturer of nucleic acid extraction/purification manual kit) used to determine subtypes of avian influenza virus.

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Procedure:

- First 5 ml of serum sample was centrifuge for 5 min at 4000 rpm. Take another tube and add 1 ml of RiboEx.
- 100 µl of sample was taken and added to tube having RiboEx. Vortex sample for 5 s and incubate for 5 min at room temperature.
- 200 µl of chloroform was added to sample tube, again vortex for 5 s and incubate at room temperature for 5 min.
- Eppendorf tube having mixture was centrifuge for 15 min at 12000 rpm and attain 3 portions, from which 350 µl of white portion was separated to another Eppendorf tube.
- Then 350 µl of RB-1 buffer was added to tube and mixed with hands, add all solution of 700 µl to spin column tube along with collection tube and centrifuge at 10000 rpm for 30 s.
- Solution from collection tube was discarded, add 500 µl of SW-1 buffer in spin column tube and centrifuge for 30 s at 10000 rpm. Again, solution from collective tube was discarded.
- 500 µl of RNW buffer was added in tube and centrifuge for 10000 rpm for 30 s. Collection tube was discarded along with pellet and run empty spin column tube in centrifuge for 10000 rpm for 1 min.
- After that RNase free water of 100 µl was added to tube and incubate for 2 min at room temperature. Centrifuge tube for 1 min at 10000 rpm and after that spin column tube was discarded and solution from collection tube added to another Eppendorf tube.

Complementary DNA synthesis

For synthesis of complementary DNA thermo Scientific Revert Aid First Strand cDNA Kit was used. A microtube was taken and 2 µl of reaction buffer, 1 µl of reverse transcriptase, 0.5 µl of RNase inhibitor, 0.5 µl of dNTPs, 10 µl of RNase free water, 1 µl of primer, 5 µl of RNA template was added and vortexed for 5 s. Then the tube was placed in thermocycler for incubation at 50°C for 45 min and second temperature was incubation at 85°C for 5 min.

PCR analysis

For PCR, total volume for reaction was 25 µl. First microtube was taken to add 12 µl of master mix, 7 µl of distilled water, 1 µl of each primer, 4 µl of sample and set in PCR machine for 1 h and 30 min. The details of primers are shown in *Table 1* (see also *Table 2*).

Gel electrophoresis

After PCR, Gel Electrophoresis was performed to determine bands. Agarose gel powder of 0.82 g and TAE-buffer of 35 ml was taken in beaker to mix them well. Heat on burner and boiled for 2-3 min and EtBr of 5 µl was added and pour in tray to solidify. After solidify, the gel was placed in apparatus and filled with TAE buffer. Samples of amount 7 µl was added to each well and covered with lid to attach wires to start electrophoresis for 30 min at 120 V.

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Table 1. *Primers used for avian influenza virus (H5, H7, H9)*

Name of viruses	Primer sequence	Product size	Reference
Avian influenza H5: Forward Reverse	 5'-ACTATGAAGAATTGAAACACCT-3' 5'-GCAATGAAATTTCCATTACTCTC-3'	251	Ng et al., 2006
H7: Forward Reverse	 5'-ACATACAGTGGGATAAGAACC-3' 5'-TCTCCTTGTGCATTTTGATGCC-3'	481	Pasick et al., 2005
H9: Forward Reverse	 5'-ATGGGGTTTGCTGCC-3' 5'-TTATATACAAATGTTGCACCTG-3'	490	Rashid et al., 2009

Table 2. *Thermal condition for amplification*

Sr. No.	Steps of PCR	Temperature	Time		Cycles
1	Initial denaturation	94℃	5 min		
2	Denaturation	94℃	30 s		
3	Annealing	51℃	30 s		
4	Extension	72℃	50 s		
5	Final extension	72℃	5 min		
6	Holding	4℃	10 min		30 cycles

Results

Total 400 blood samples were collected from selected areas, i.e. Abbottabad (n = 109), Havelian (n = 105), Lora (n = 88), and Lower Tanawal (n = 98). Different breeds were selected such as Aseel, Desi, Fancy chicken, Golden Fayoumi for sampling purpose.

Overall statistics of infected birds with AIV

Analysis done by using SPSS-11.0 for Windows 7 and Graph pad Prism version 5.0. Variables were given percentages and ratios. The analysis of data regarding birds during current study revealed that the minimum flock size was 2 whereas maximum flock size studied during current was as large as 354 with a median value of 14. The mean flock size remained 24.76 with a standard deviation value of 35.88. According to reports, Desi and Aseel breeds are second and third most prevalent in Pakistan, with indigenous Golden Fayoumi birds dominating flocks (Ghoname et al., 2022). This study found that 94% of backyard chickens were Golden Fayoumi. Farmers of the local area prefer the local Golden Fayoumi breed because it can adapt to a local environmental condition. Other investigations from Thailand and Bangladesh revealed 10 to 37 birds included in their study (Javed et al., 2003). The analysis of data regarding the age range of the birds from different flocks analyzed during current study revealed that the minimum bird age was 2 weeks whereas maximum age of the bird study during current study was reported to be 123 weeks with a median value of 45. The mean age remained 46.14 weeks with a standard deviation value of 23.12 as shown in *Tables 3* and *4* (see also *Figs. 1* and *2*).

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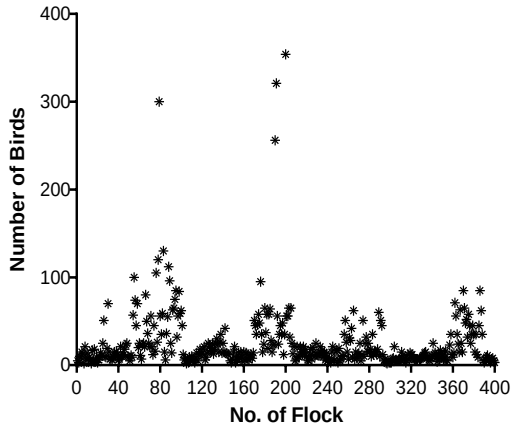

Figure 1. *Statistics of number of flock and birds*

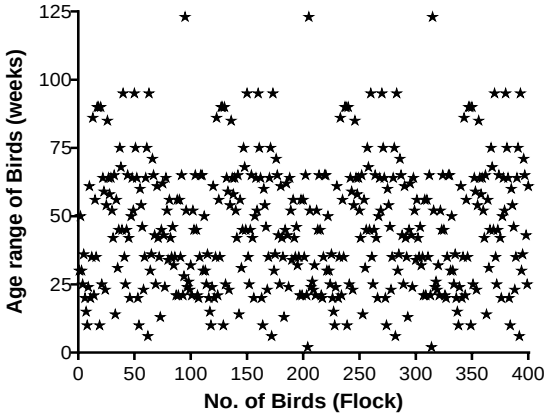

Figure 2. *Age range of birds according to flock size*

Table 3. *Column statistics of the flock size of birds*

Number of values	**400**
Minimum	2.000
25% Percentile	8.000
Median	14.00
75% Percentile	25.00
Maximum	354.0
10% Percentile	5.000
90% Percentile	56.90
Mean	24.76
Std. deviation	35.88
Std. error of mean	1.794
Lower 95% CI of mean	21.24
Upper 95% CI of mean	28.29
Coefficient of variation	144.9%
Geometric mean	15.24
Sum	9905

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Table 4. *Age statistics of birds studied during current of the study*

Number of values	**400**
Minimum	2.000
25% Percentile	25.00
Median	45.00
75% Percentile	64.00
Maximum	123.0
Mean	46.14
Std. deviation	23.12
Std. error of mean	1.156
Lower 95% CI of mean	43.87
Upper 95% CI of mean	48.41
Coefficient of variation	50.11%
Geometric mean	39.55
Geometric SD factor	1.844
Sum	18455

Sero-surveillance of avian influenza subtype (H5, H7, H9):

It was observed from current study that the prevalence of AI strains has been affected by various factors. The area wise sampling was performed and it was observed that the geographical location of birds affected the occurrence of AI.

The outbreak was recorded in different regions of Pakistan which leads to spread of avian influenza in other birds. In our study we estimated sero-surviellance of avian influenza subtypes H5, H7 and H9 in backyard poultry of Abbottabad region which was 60.85% as shown in *Table 5*. AIV occurs in both summer and winter seasons but in colder season it shows more positivity because the virus survives mostly in colder environment (Morin et al., 2018).

Table 5. *Overall percentages of H5, H7 and H9*

Samples processed (n)	**Positive samples AI H5, H7 (MT) log2> 5.0**	**Negative samples log2< 5.0**	**Strain of avian influenza detected**	**Positive samples (%) (MT) log2>5.0**	**Negative samples (%) (MT) log2 <5.0**
400	60.85	39.15	HI for H5	73.16	26.84
			HI for H7	9.39	90.61
			HI for H9	80.10	--

Backyard birds are not vaccinated properly which indicates high titre of H9 in birds. Sero-surveillance of H9 was 80.10% in Abbottabad but in District Lahore it was recorded as 62% because of colder environment of Abbottabad city. High variation occurs due to presence of inactivated vaccines commercially which results in mortality rate in chicken. Chicken infected with H5 and H7 die and number of seropositive cases increases in backyard. On other hand, H9 was low pathogenic in Pakistan but now H9 have high frequency and mortality rate.

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Since 1995, Pakistan has been affected by five waves of Avian Influenza and causative subtypes were H5, H7, and now H9 is at peak seropositivity. Birds purchased from local markets are at higher risk of getting infection and spread by congested cages (Rahman et al., 2018). Furthermore, our results show high rate of H9 and H5 which mean co-infection of both subtypes whereas H7 was much rare. In neighboring counties such as China faced higher rate of infection (Flora et al., 2021). Our study shows risk factors and titre of avian influenza leads to its spread because these birds are rich source of protein and vitamins and mostly used by rural population.

Sero-surveillance of H5

In tehsil Abbottabad, Avian Influenza Strain H5 was present with a prevalence of 9.25% where as in Tehsil Lora Havelian and Lower Tanawal, H5 was absent. The effect of Area on occurrence of H5 was found highly Significant with a P-value of 0.00. The effect of chicken specie on occurrence of AI H5 was assessed and it was noticed that it was more prevalent in Golden Fayoumi birds (15.32) followed by Aseel birds (7.80), Desi (Non-descript) 5.88%. While H5 reported negative in Fancy chicken. The role of chicken specie was found highly significant (p-value 0.010) on occurrence of H5. The detail is presented in the *Table 6*.

Table 6. *Sero-surveillance of H5 in various tehsil*

Variables	Category	Prevalence (%) of H5	Total	$\chi 2$ value	P value
Tehsil	Abbottabad	9.25	109	108.848	0.000
	Havelian	0	105		
	Lora	0	88		
	Lower Tanawal	0	98		
Chicken species	Aseel	7.80	141	11.302	0.010
	Desi (non descript)	5.88	85		
	Fancy chicken	0	37		
	Golden Fayoumi	15.32	137		
Flock size	Less than 20	6.56	259	9.233	0.010
	20 to sixty weeks	5.01	109		
	More than 60 weeks	2.70	32		
Age	< 20 weeks	16.66	48	17.745	0.000
	20 to 60 weeks	12.34	235		
	> 60 weeks	0	117		

Sero-surveillance of H7

The area wise sampling of backyard poultry revealed that the geographical location of bird affected the occurrence of AI. Like in tehsil Abbottabad, H7 was present with a prevalence rate of 10.09% where as in Tehsil Lora it was 27.27%, Havelian 22.85% and in Lower Tanawal it was 10.20%.

The effect of Area on occurrence of H7 was found highly Significant with a P-value of 0.001. The effect of chicken specie on occurrence of AI H7 was assessed and it was noticed that it was more prevalent in Fancy chicken 21.62% followed by Golden Fayoumi birds 19.70%, Aseel birds 19.14% and Desi (Non-descript) was 8.23%. The effect of chicken specie was found highly significant (p-value 0.010) on occurrence of H7. In November 2003, H7 materialized in Karachi area, with an abrupt boost in

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mortality at some farms with an index of 2.8 which affect a commercial layer (Naeem and Siddique, 2006). The detail is presented in the *Table 7*.

Table 7. *Sero-surveillance of H7 in various Tehsil*

Variables	Category	Prevalence (%) of H7	Total	χ2 value	P value
Tehsil	Abbottabad	10.09	109	15.827	0.001
	Havelian	22.85	105		
	Lora	27.27	88		
	Lower Tanawal	10.20	98		
Chicken species	Aseel	19.14	141	6.271	0.010
	Desi (non descript)	8.23	85		
	Fancy chicken	21.62	37		
	Golden Fayoumi	19.70	137		
Flock size	Less than 20	16.21.5	259	1.832	0.010
	20 to sixty weeks	21.5	109		
	More than 60 weeks	12	32		
Age	< 20 weeks	10.41	48	42.370	0.000
	20 to 60 weeks	27.23	235		
	> 60 weeks	0	117		

Sero-surveillance of H9

The sampling from birds originating from different areas analyzed and it was observed that the geographical location of birds affected the occurrence of AI. Like in tehsil Abbottabad, H9 was present with a prevalence of 76.15% where as in Tehsil Lora 69.32%, Havelian 75.24% and in Lower Tanawal it was 72.45%. The effect of Area on occurrence of H9 was found non-significant. The effect of chicken specie on occurrence of AI subtype H9 was assessed and it was noticed that it was more prevalent in Golden Fayoumi 76.64% followed by Aseel birds 75.89%, Fancy birds 72.97% and in Desi (Non-descript) it was 64.71. The effect of chicken specie was found non-significant on occurrence of H9. The detail is presented in *Table 8*.

Table 8. *Sero-surveillance of H9 in various Tehsil of district Abbottabad*

Variables	Category	Prevalence (%) of H9	Total	χ2 value	P value
Tehsil	Abbottabad	76.15	109	1.401	0.705
	Havelian	75.24	105		
	Lora	69.32	88		
	Lower Tanawal	72.45	98		
Chicken species	Aseel	75.89	141	4.487	0.213
	Desi (non-descript)	64.71	85		
	Fancy chicken	72.97	37		
	Golden Fayoumi	76.64	137		
Flock size	Less than 20	72.97	259	0.111	0.946
	20 to sixty weeks	74.31	109		
	More than 60 weeks	75	32		
Age	< 20 weeks	87.5	48	224.785	0.000
	20 to 60 weeks	96.17	235		
	> 60 weeks	22.22	117		

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Molecular characterization of AIV samples

RNA was extracted from positive samples to perform RT-PCR of AIV subtypes. Specific primers were used to target HA genes. The PCR products consist of 251 bps of H5, 481 bps of H7 and 490 bps of H9 were visualized by Agarose Gel Electrophoresis (shown in *Fig. 3*).

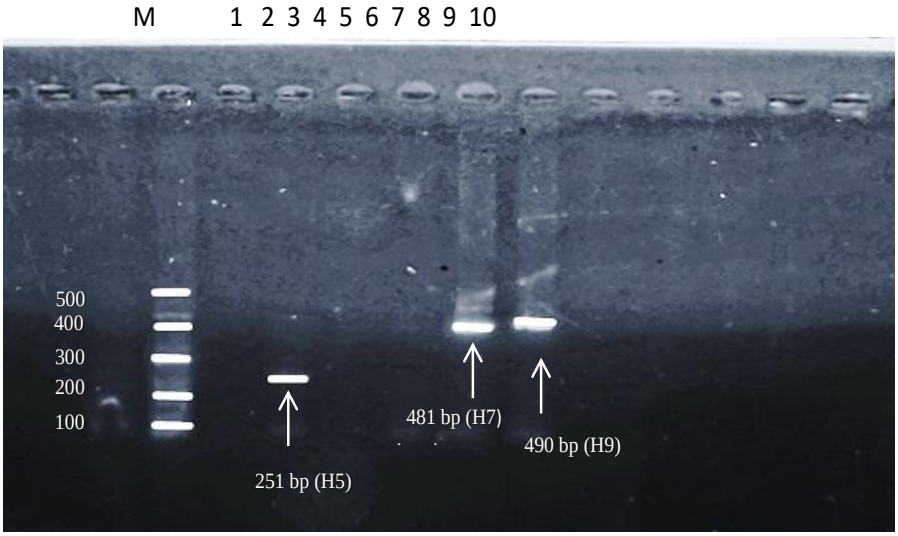

Figure 3. *RT-PCR for HA gene of AIV (H5, H7, H9) from different samples*

Discussion

In current study, results confirmed the presence of subtypes H5, H7 and H9 due to mutation, weather changes and lack of awareness in workers, owners or poultry dealers. Results and evidences gave also been provided which confirm the co-infection of H5 and H9 in backyard poultry of Abbottabad region. The highest prevalence was observed in Golden Fayoumi birds followed by Desi, Aseel and Fancy. The highest prevalence of H5 of 9.25% was found in Abbottabad and Golden Fayoumi was more prevalent of about 15.32% followed by Aseel and Desi. The effect of Area on occurrence of H5 was found highly Significant with a P-value of 0.00. While another study from five different districts of Pakistan (Mansehra, Haripur, Abbottabad, Islamabad and Rawalpindi) confirmed significant value ($P < 0.05$) of highest Sero-prevalence of the infection in Abbottabad and lowest in Mansehra (Fatima et al., 2017). H7 was found more prevalent in Lora of about 27.27% and target Fancy chicken of about 21.62%. In November 2003, H7 materialized in Karachi area, with an abrupt boost in mortality at some farms with an index of 2.8 which affect a commercial layer (Naeem and Siddique, 2006). H9 was found highly prevalent in Abbottabad of about 76.15% and chicken breed Golden Fayoumi has highest prevalence of 76.64%. From Hilly Hazara region (with an altitude ranging from 1700 ft to 4000 ft above the sea level) Oral and cloacal swabs were taken from commercial poultry farms in five districts: Abbottabad, Mansehra, Battagram, Haripur, and Kohistan. The prevalence rates were 50%, 27.3%, 4.5%, 13.7%, and 4.5% correspondingly (Ayaz et al., 2017). Vaccination prevents and controls the infection. Vaccinated hens have antibodies that combat viral antigens and prevent illness (Sitohy et al., 2022). Backyard poultry had greater H9 antibody titers than H5 and H7. The PCR products consist of 251 bps of H5, 481 bps of H7 and 490 bps of H9 were visualized by

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Agarose Gel Electrophoresis, whereas RT-PCR for HA gene of AIV (H9, H7, H5) from different field samples was performed and expected PCR product size 499, 490 and 221 was detected from Karachi (Channa et al., 2021).

Conclusions

In the current study, results confirmed the presence of subtypes H5, H7, and H9 due to mutation, weather changes (31-34°F), and lack of awareness among workers, owners, or poultry dealers. Results and evidence have also been provided which confirm the co-infection of H5 and H9 in backyard poultry of the Abbottabad region. The highest prevalence was observed in Golden Fayoumi birds followed by Desi, Aseel, and Fancy. The highest prevalence of H5 9.25% was found in Abbottabad and Golden Fayoumi was more prevalent at about 15.32% followed by Aseel and Desi. H7 was found more prevalent in Lora at about 27.27% and target Fancy chicken at about 21.62%. H9 was found highly prevalent in Abbottabad at about 76.15% and chicken breed Golden Fayoumi has the highest prevalence of 76.64%. Proper surveillance of backyard poultry helps to reduce the risk of disease in the population. The presence of infected birds would threaten human life as well and birds are in frequent contact with circulation, farmers, or by eating processes to population. The sanitary system and water supply to chickens should be properly cleaned to avoid AIV infection.

Recommendations

Vaccination of birds is an important factor in preventing the Avian Influenza virus and avoiding inactivated vaccines that cause high mortality rates in birds. Avoid the purchase of birds from local markets because they spread the virus in congested cages. Awareness of farmers and people who deal with chickens to avoid the spread of the virus and its migration from one place to another. Try to avoid semi cages and the intermingling of birds with each other. Water contamination and feed also lead to the spread of the virus by weakening the immune system of birds.

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