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**Review Article**

## Isolation of avian influenza virus from backyard poultry population of tehsil Abbottabad, Khyber Pakhtunkhwa, Pakistan

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### ABSTRACT

The avian influenza virus is a highly contagious viral disease that predominantly impacts avian species, especially domestic birds like chickens, ducks, and turkeys. The study was conducted to isolate the avian influenza virus from domestic poultry breeds (Desi, Aseel, Fayoumi, and Golden) from backyard poultry population of the Tehsil Abbottabad from January to June 2023. A cross-sectional study was conducted in three rural and five urban areas of Tehsil Abbottabad Using sterile cotton swabs, 128 (tracheal and cloacal) swab samples were collected. The samples were labelled and stored in a brain-heart infusion medium, then transported to the National Reference Laboratory for Poultry Diseases in Islamabad using a thermostat shipment box. The samples were processed using a real-time reverse transcription polymerase chain reaction for avian influenza virus detection. This study concludes with a significant 9.38% prevalence percentage of avian influenza virus in Tehsil Abbottabad backyard poultry. The prevalence percentage of H9 was 83.33%, and that of H5 was 16.66%. The findings reveal the importance of implementing preventative measures to curb avian influenza outbreaks within the poultry population of the district. This study offers vital data for AIV research in 2023, guiding enhanced containment and prevention in backyard poultry to control avian influenza.

**Keywords:** Avian influenza virus; backyard chickens; golden; fayomi; aseel.

### INTRODUCTION

Small groups of domesticated birds are raised as backyard poultry in residential settings (Aslam et al., 2020). The domestic chicken, scientifically known as Gallus domesticus, is raised in Pakistan. There are four common breeds: Golden, Fayoumi, Desi, and Aseel, each with distinct physical and behavioral traits (Monika et al., 2018). Genus Alphainfluenzavirus and its family Orthomyxoviridae contain influenza viruses which are primary cause of respiratory tract infections (Lefkowitz et al., 2018). Annually, these viruses are responsible for approximately 3-5 million severe clinical infections (Carolien et al., 2012). In 412 BC, Hippocrates described symptoms like influenza, marking its earliest recorded history (Kuszewski et al., 2000). In the 19th century, AIV was responsible for numerous pandemics in humans, for example, the Spanish flu in 1918–1919, the Asian flu pandemic of 1957–1958, and the Hong Kong flu pandemic of 1968–1969, resulting in millions of deaths worldwide. The H5N1 AIV pandemic in 1997 caused severe respiratory illness in humans (Taubenberger & Morens, 2006). Compared to the investigation in Peshawar district, which reported a high seroprevalence of H9N2 AIV among backyard poultry (Rehman et al., 2021).

The H9N2 AIV pandemic in 1999 caused significant economic losses in the poultry industry (Cao et al., 2022). The H7N9 AI pandemic in 2013 ranged in shape and had a lipid envelope containing two surface glycoproteins, HA, and NA (Chang et al., 2023). The AIV genome has eight segments, and these segments encode 11 viral proteins (Suarez & Schultz, 2000). These viral proteins play essential roles in the replication, assembly, and pathogenesis of AIV (Cheng et al., 2019). A general composition of the virus includes the following components: The avian influenza virus is an RNA virus with a ribonucleic acid genome. The virus forms a lipid-bilayer envelope from the host cell, and the envelope contains glycoproteins and glycolipids with diverse carbohydrates (Resa et al., 2011). AIV is a contagious virus that causes seasonal outbreaks and occasional global pandemics in humans, birds, and animals (Alexander, 2003). The virus is categorized based on its surface proteins: HA and NA (Liu et al., 2022). Hemagglutinin is a glycoprotein that facilitates virus attachment and fusion with host cells (Guo et al., 2019). Neuraminidase is a glycoprotein that cleaves sialic acid receptors (Kargarfard et al., 2016). The classification of Type A influenza into two categories is based on its pathogenicity. The low-pathogenic avian influenza virus is only found in wild aquatic birds and usually results in mild or no symptoms. LPAI viruses H5N2 and H7N9 pose a low risk to human health (Chaudhry et al., 2017). Highly pathogenic avian influenza viruses (HPAI) are more severe and can cause high mortality rates in infected birds. The HPAI viruses are H5N1 and H5N8 viruses, and they pose a significant risk to human health (Swayneayne et al., 2017). The avian influenza virus can be transmitted through various means, including direct bird-to-bird contact, contaminated feed and water, and equipment and materials carrying the virus (Sarwar et al., 2013). The sources for the outbreak of the AIV are the following: the movements and interactions of water birds, which are the natural reservoirs for most AIVs (Wahlgren, 2011). Vaccination is crucial for controlling the spread of AIV (Swayne et al., 2014). Two types of vaccines are available: Whole viral vaccines stimulate a broad immune response. Subunit vaccines are based on specific viral proteins (Suarez & Schultz, 2000). The objective of the Tehsil Abbottabad research was to evaluate the distribution of avian influenza virus (AIV) subtypes and prevalence in backyard chickens.

## MATERIALS AND METHODS

### Study Area
The research was carried out in Tehsil Abbottabad, Khyber Pakhtunkhwa, Pakistan (2023).

### Sample size
A total of 128 backyard poultry samples (64 cloacal and 64 tracheal swab samples) were collected randomly from different households and Desi chicken shops in Tehsil Abbottabad (Table 1).

Table1. Description of the backyard poultry in Abbottabad Tehsil was utilized to collect the sample size.

| Species | Breed | Number of Bird Sample | Number of Samples | |
|---|---|---|---|---|
| | | | Tracheal Sample | Cloacal Sample |
| Chicken | Golden | 16 | 16 | 16 |
| | Desi | 16 | 16 | 16 |
| | Aseel | 16 | 16 | 16 |
| | Fayoumi | 16 | 16 | 16 |
| Total | 4 | 64 | 64 | 64 |
| Overall Total | | | 128 | |

### Study design
To conduct the study, we collected samples from both rural and urban areas of Abbottabad Tehsil. We selected three rural areas Namli Mera, Dahamtore, and Banda Qazi. Five urban areas, Nawanshehr, Mirpur, Salhad, Jhangi, and Kehal. From each of these areas, we collected a total of 16 samples of different breeds (Figure 1).

### Sampling Procedure
To collect swab samples, the mouth of the chicken (backyard fowl) was carefully opened. A sterile swab with a cotton tip was inserted into the mouth and gently guided towards the trachea. Once the swab reaches the trachea, it was rotated 18 times in total, with two rotations (clockwise and anticlockwise) in each direction while applying slight pressure to the mucosal surface. Similarly, the process was repeated for the collection of cloacal samples (Navid et al., 2022) (Figures
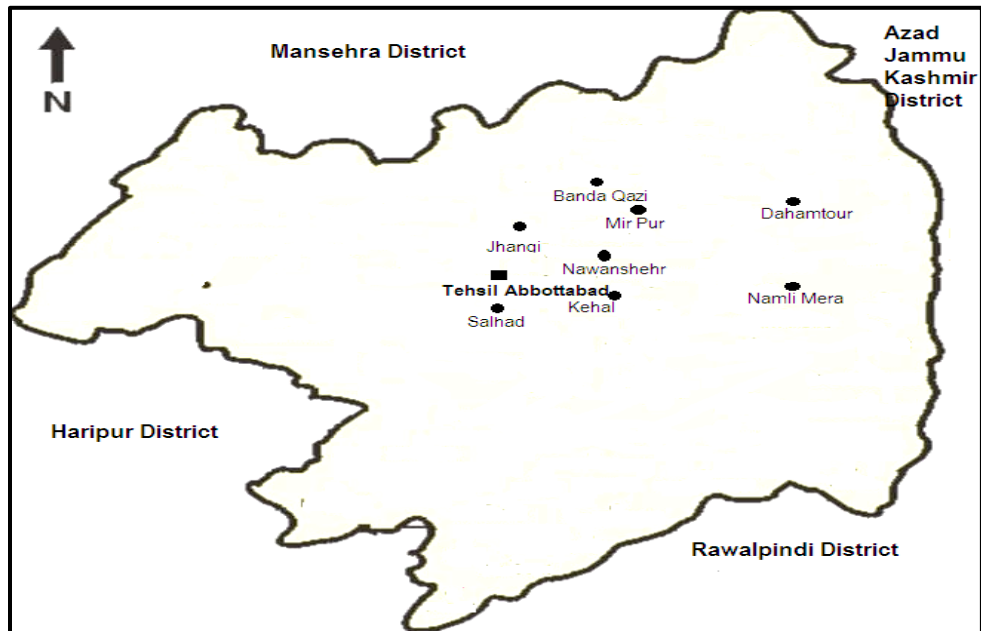
Figure 1. Tehsil Abbottabad, Khyber Pakhtunkhwa, Pakistan.

2-5). The sample was dipped in a sterile tube of brain-heart infusion (BHI) broth and given to the Poultry Research Institute (PRI) at Jaba, Mansehra. Once the swab was placed in the tube, the sample was labeled with the sample type and the information from the union council on the sample of each breed. To maintain the integrity of the sample, the tubes containing the samples should be stored in a thermal shipping box. The Poultry Research Institute (PRI) in Jaba was provided with an ice pack to ensure that the samples remain at the appropriate temperature during transportation. Swab samples can be kept for several weeks to months at -20 °C and up to one week at -40 °C (Chaudhry, 2020).



Figure 2. Tracheal sample collection from Desi and Golden.

Figure 3. Tracheal sample collection from Fayoumi.



Figure 4: Cloacal sample collection from Desi and Golden.

**AIV Isolation**

After the collection of samples, the next step was to ensure their proper transportation to the designated laboratory for further testing. The samples were then handed over to the PRI Jaba, which took responsibility for shipping them to the National Reference Laboratory for Poultry Diseases (NRLPD) in Islamabad. A special shipment box was used to transport them. For confirmation, reverse transcription polymerase chain reaction (RT-PCR) was used. Amplification of the influenza virus genome by RT-PCR. Using the McMaster RT Kit (Eppendorf TM, Germany), the RT-PCR procedure diagnosed the samples. The commercial RNA extraction kit was used to extract total RNA from swab samples, following the manufacturer's instructions (Spackman et al., 2002). One-step RT-PCR was then performed using a commercial kit with specific primers: forward primer:5'-AGATGAGTCTTCTAAC CGAGGTCG-3'.

Figure 5. Cloacal sample collection from Fayoumi.

Reverse primer: 5'-TCTACGCTGCAGTCCGAATC-3'. These primers are designed to amplify the AIV matrix \gene (Spackman et al., 2002). Amplification conditions included reverse transcription at 50°C for 30 min. Initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 sec. Annealing at 55°C for 30 sec. and extension at 72°C for 30 sec. Electrophoresis was performed on a 1% agarose gel to visualize the PCR products. In the presence of a 183-p band, a result was recognized by matching the AIV matrix gene (Spackman et al., 2002). The collected data was examined with Microsoft Excel, and the data was expressed in percentages. The Chi-square Test was used SPSS v29.0 software for statistical analysis.

### RESULTS
#### AIV Prevalence Percentage
The current study on Avian Influenza Virus (AIV) in Tehsil Abbottabad. The positive percentage of AIV in the backyard poultry population of Tehsil Abbottabad was 9.375%, and the negative percentage of AIV among the backyard poultry in Abbottabad was 90.625% (Figure 6).
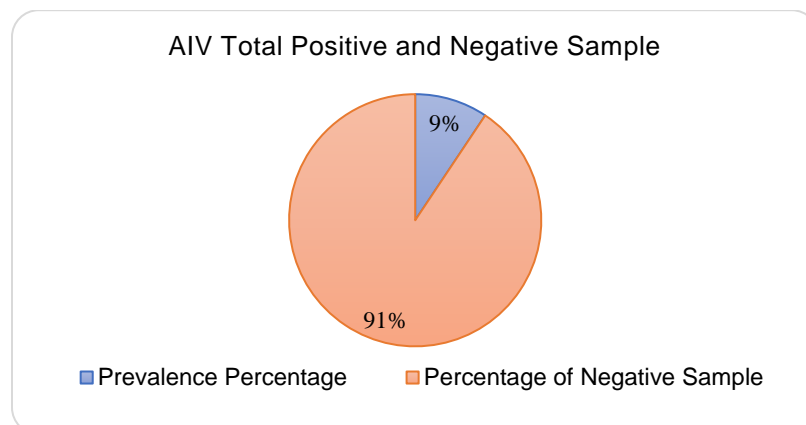


Figure 6. The AIV total positive and negative samples.

**Prevalence of Avian Influenza Virus in Tehsil Abbottabad**

To ensure a comprehensive analysis, we collected samples from both rural and urban areas within Abbottabad tehsil. Across all five urban areas, a total of 40 samples were collected, with 6 samples testing positive, resulting in a positivity rate of 15%, and no sample isolation from Jhangi. All 24 samples tested negative for AIV, indicating a 100% negative percentage in the rural areas of Tehsil Abbottabad (Figure 7).
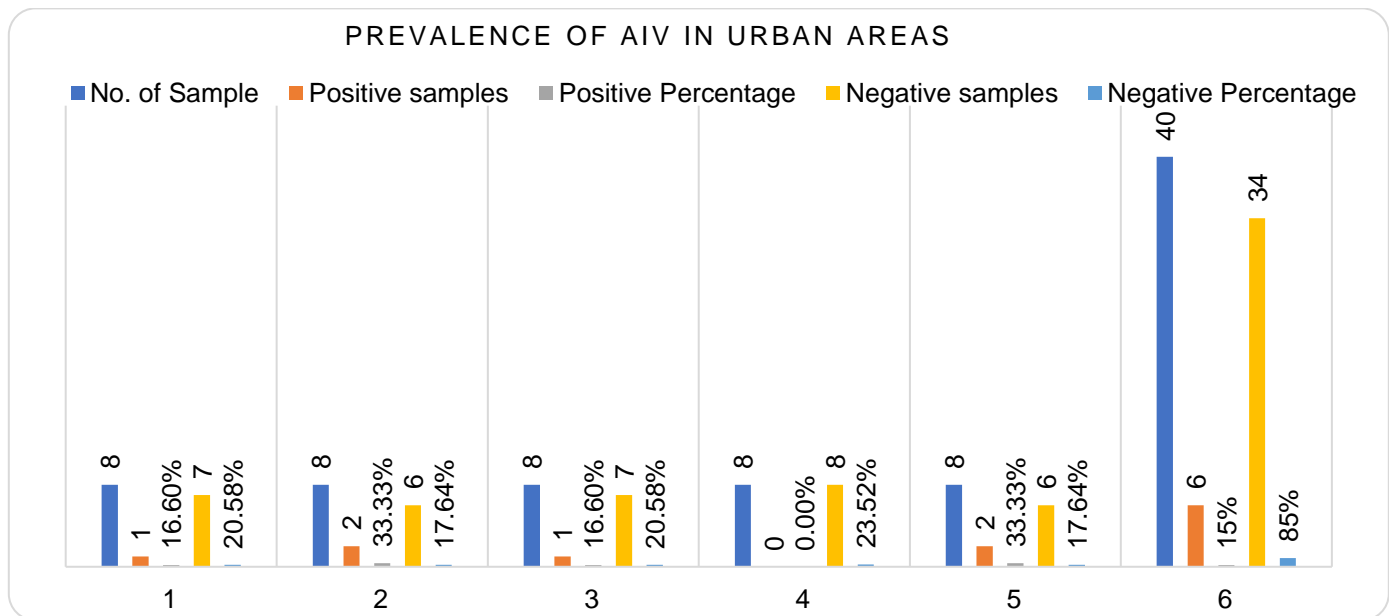


Figure 7. The Prevalence of Avian Influenza Virus in urban areas of Tehsil Abbottabad Pakistan: Sample results and prevalence percentage.

**Subtype Identification and Prevalence of AIV in the Backyard Poultry Population**

A total of 6 positive samples were obtained. Among these samples, only 1 sample tested positive for the H5 subtype, representing a percentage of 16.66%. Additionally, 5 samples were found positive for the H9 subtype, accounting for 83.33% of the positive samples. However, no samples were identified as positive for the H7 subtype, resulting in a percentage of 0%. Positive samples were isolated from all areas except Jhangi. Among samples, two samples isolated from Kehal were found to be positive for H9, one sample from Nawanshehr was positive for H5, two samples from Mirpur were positive for H9, and one sample from Salhad was positive for H9 (Figure 8).

**Prevalence of AIV in Different Backyard Poultry Breeds**

A total of 64 samples were collected, with 16 samples from each breed. The results revealed that 1 sample from the Desi breed, 3 samples from the Golden, 0 samples from the Aseel breed, and 2 samples from the Fayoumi breed tested positive for AIV (Figure 9).

**DISCUSSION**

Avian influenza virus, primarily infecting birds, a serious concern for both the poultry industry and public health is the risk of severe illness or death caused by the avian influenza virus (Chan et al., 2015). The objective of the Tehsil Abbottabad research was to evaluate the distribution of avian influenza virus (AIV) subtypes and prevalence in backyard chickens. Twelve of the 128 samples had positive AIV tests, meaning that the prevalence was 9.375%. With no H7 subtype found, the most common subtypes were H5 (16.66%) and H9 (83.33%). Even though the strains of AIV were different, Tehsil Abbottabad had a lower frequency of the virus than other areas like Mansehra and Multan. AIV positivity rates were greater in urban areas (16.6% to 33.33%) than in rural regions (no positive samples found). This discrepancy implies that urban environments may promote the spread of AIV because of things like population density and interactions with untamed avian species. Susceptibility differed throughout breeds as well; the Golden breed was the most sensitive, followed by the Fayoumi, Desi, and Aseel. The study in Mansehra district revealed a predominance of H9N2 avian influenza virus (AIV) in commercial poultry (Khalid, 2020), contrasting with findings in Tehsil Abbottabad, where both H5 and H9 subtypes were detected in backyard poultry, albeit in different proportions. Additionally, no H7
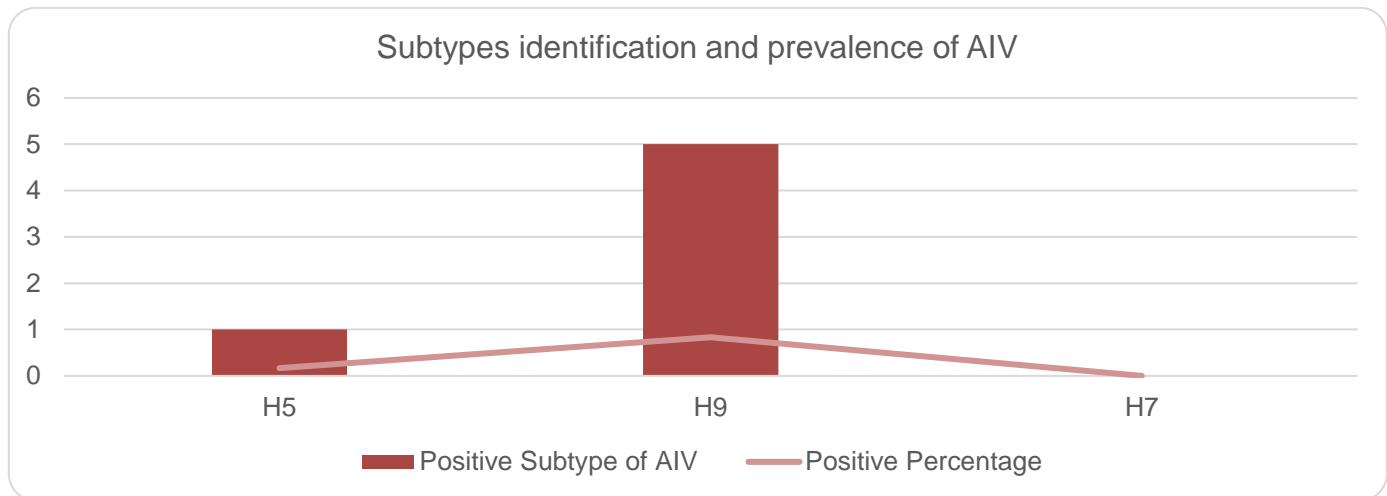
Figure 8. The Subtype Identification and Prevalence of AIV in the backyard poultry population.
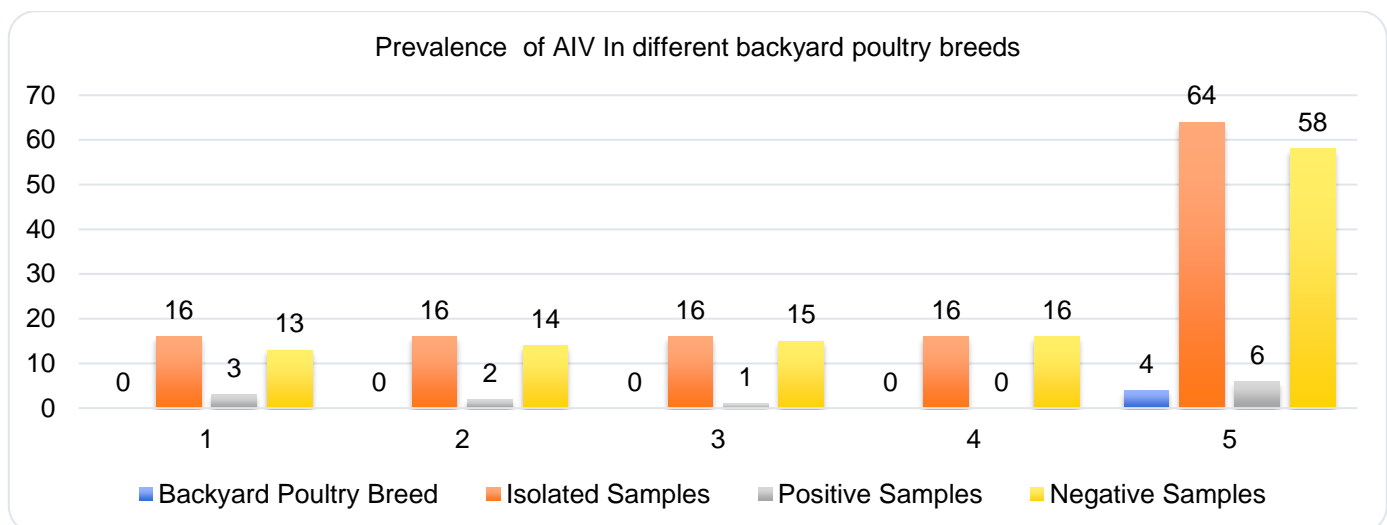


Figure 9. The isolation of AIV from backyard poultry breeds.

subtype was identified in the study. Compared to the investigation in Peshawar district, which reported a high seroprevalence of H9N2 AIV among backyard poultry (Rehman et al., 2021), study in Tehsil Abbottabad demonstrated a lower positivity rate. Similarly, the study in Multan district indicated a higher overall AIV prevalence, with a significant proportion of H9-positive birds (Navid et al., 2022), whereas findings in Tehsil Abbottabad suggested a lower prevalence, with diverse AIV strains detected, including H5 and H9. Contrasting with the substantial prevalence of H9N2 AIV found in Zhob district (Khan et al., 2022), study in Tehsil Abbottabad revealed higher prevalence rates in urban areas and none in rural areas, indicating potential differences in AIV distribution across regions. Furthermore, investigation into AIV distribution among different domestic poultry breeds in Tehsil Abbottabad highlighted varying susceptibility and exposure rates. While the Golden breed exhibited the highest positivity, the Aseel breed showed resistance, unlike findings from studies in Karachi, which reported varying seroprevalence among broilers, with H9 being the most common subtype (Channa et al., 2022). The study in Tehsil Abbottabad contributes insights into AIV prevalence, subtype distribution, and breed susceptibility in the region.

**CONCLUSION**
In conclusion, the study highlights the existence of avian influenza, particularly H5 and H9 subtypes, in Tehsil Abbottabad backyard poultry, underlining the need for continued surveillance and monitoring efforts. While the incidence rates are lower in some other areas, the diversity of strains and varied susceptibility across chicken breeds underline the importance of ongoing study and vigilant control measures to reduce risk to both animal and human health.

Furthermore, this analysis indicated that the Fayomi breed of tehsil Abbottabad had a higher susceptibility to AIV infection. AIV was detected in all urban areas except Jhangi, while no detections were made in rural areas. Preventative measures should be applied to the poultry population to reduce the occurrence of avian influenza outbreaks.

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