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Short Communication

PREVALENCE OF AVIAN INFLUENZA SUBTYPE H9N2 IN BACKYARD POULTRY IN AND AROUND BAHAWALPUR CITY

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ABSTRACT

Avian influenza subtype H9N2 is endemic in Eurasia and responsible for huge economic losses to the poultry industry. Backyard poultry especially in the areas where modern poultry farming is less common provides a biological niche for the persistence and evolution of Influenza viruses. Current study was designed to bridge this gap by studying the prevalence of H9N2 in backyard poultry in the Bahawalpur city. Out of the ten randomly selected sites in and around the city, only one site was positive for H9N2 virus. The positive site had 22.22% (20/90) prevalence and the overall prevalence rate of the Bahawalpur city was 4% (20/500). This provides a space for viral existence as well as mutation in the viral genome. Backyard poultry sector has close interaction with humans; therefore, Avian Influenza is a continuous threat to humans as a zoonotic agent.

Key words: Avian Influenza H9N2, Bahawalpur, Prevalence, Backyard Poultry
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INTRODUCTION

Besides other viral diseases, Avian Influenza is responsible for inhibiting the progress of poultry sector causing annual production losses of approximately 2.2 billion rupees (Ayaz *et al*., 2017). Avian Influenza subtype H9N2 is endemic in chickens worldwide (Pusch and Suarez 2018). It belongs to type A Influenza viruses that have a high potential of genetic mutation (genetic drifts) and reassortment (genetic shifts). These viruses are known to cross the species barrier, causing infection and adaptation in new hosts (Kausar *et al*., 2018). The first outbreak of H9N2 was reported in northern areas of Pakistan in 1998 (Lee *et al*., 2016; Ayaz *et al*., 2017), since then the virus has been circulating in region among the commercial and backyard poultry. Subtype H9N2 is categorized into Low Pathogenic Avian Influenza (LPAI) viruses (Sarwar *et al*., 2013) with high morbidity rate up to 100% and low mortality rate 5-30% causing huge annual economic losses globally, due to loss of production (Li *et al*., 2005; Parvin *et al*., 2014; Ayaz *et al*., 2017). In affected birds, there is hyperemic trachea along with congested lungs while in complicated cases there are also lesions suggestive of hepatitis, peritonitis and enteritis (Cheema *et al*., 2011). This is a zoonotic virus capable of causing respiratory illness in human beings. Symptoms of the disease include mild upper respiratory tract infections, fever and cough (Ahad *et al*., 2013; WHO, 2018). Many studies have been conducted to find the prevalence of this virus in areas where the modern poultry farming has been adopted while the low poultry farming areas of Punjab have been neglected. This provides a suitable environment for the virus to circulate in the poultry population and emerge as a new variant (Umar *et al*., 2016). The current study was conducted to evaluate the prevalence of H9N2 virus in backyard poultry of Bahawalpur City. Most of the area of this region consists of desert with hot climatic conditions so that most of the people in rural areas depend on subsistence farming and there is poor availability of veterinary services for their livestock and poultry (Farooq *et al*., 2007; Sadef *et al*., 2015). The common poultry breeds reared in this area are Fayoumi, Desi, Rhode Island Red, Naked Neck, Plymouth Rock and Aseel. Due to free-living nature of farming, crosses of these breeds are also common (Sadef *et al*., 2015). The purpose of targeting backyard poultry was that they are mostly non-vaccinated, more exposed to wild and migratory birds as compared to commercial poultry and they are reared as more than one breeds at a time along with other species of animals like cattle and goats in the same place (Farooq *et al*., 2007). Inadequate biosecurity measures adopted by the farmers in this area make the birds more prone to harbor new infections and become reservoir of avian influenza zoonosis (Hadipour *et al*., 2011).

MATERIALS AND METHODS

Sample collection and transportation: A total of 500 samples of backyard poultry were collected from 10 different randomly selected locations in and around Bahawalpur City (Table 4) on convenient basis from November 2019 to February 2020. The virus transport medium (VTM) was prepared from CRITERON® Brain Heart Infusion Broth (BHI) according to the manufacturer's instructions and autoclaved. Gentamycin

was added (0.5mg/ml of BHI) after cooling. Oral swabs were collected from H9N2 suspected live birds, labeled properly, dipped in BHI and transported in ice box to the Influenza Laboratory, Department of Microbiology, UVAS for further processing.

Sample processing: Swabs were placed in microfuge tubes along with VTM and vortex. Then, the samples were centrifuged at 10,000 rpm for 15 minutes and supernatant was collected in microfuge tubes. Nystatin was added at a dose rate of 2.5mg/ml and tubes were placed in incubator at 37ºC for one hour.

Virus Propagation and Harvesting: After incubation, the processed samples were inoculated in 9 days old viable embryonated chicken eggs via chorio-allantoic sac route using standard procedure (Khalili *et al*., 2013). After inoculation the eggs were sealed with wax and incubated at 37ºC for 48 hrs (OIE., 2005; Abid *et al*., 2017). Candling of embryonated eggs were done after 24 and 48 hours in order to check post inoculation death. After 48 hours, eggs were chilled at 4 ºC overnight prior harvesting the Allantoic Fluid (AF). After that, AF was collected in 15ml falcon tube from each egg separately under sterilized conditions with the help of 5mL micropipette and labeled properly.

Haemagglutination Test (HA) and Haemagglutination Inhibition (HI) Assay: Haemagglutination activity of the virus in the harvested AF was checked by HA test. Haemagglutination test was performed by using 1% washed chicken RBCs and protocol was performed as described (OIE., 2012). The 4HA units of virus were prepared by following the procedures described by (Villegas and Purchase 1998). The HA positive samples were proceeded for HI test using commercially available sera against H5, H7, H9 and Newcastle Disease Virus (NDV). The HI assay was performed according to the method described (OIE., 2012).

RNA Extraction and cDNA Synthesis: Total RNA was extracted from HI positive AF against H9 anti-serum using QIAmp Viral RNA Extraction Mini Kit following the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was synthesized from extracted RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific®) according to manufacturer's instructions.

PCR and Agarose Gel Electrophoresis: For Polymerase Chain Reaction (PCR) reported primers of H9 and N2 genes were used following (Ali *et al*., 2018) and PCR products were analyzed on a 1.2% agarose gel and visualized in a Gel Doc system (Bio Rad System US). A brief description of primers used for virus identification and their PCR conditions are mentioned in below (Table1, 2, 3).

Table 1: Primer detail for first half gene amplification of H9 and N2 genes.

Sr. No.	Primer Name	Primer Sequence	Product size (bp)
1	NA-F-10	5-GCAGGAGTGAAAATGAATCCAAATC-3	833
	NA-R-10	5-ATGCTGAGCACTTCCTGACAGT-3	
2	HA-900-F	5'-TGACAACAAGCAATGCAGAC-3'	945
	HA-900-R	5'-GGTAGTGTACTGTTTAAGCCAC-3'	

Table 2: Thermal conditions for first half segment of neuraminidase gene amplification (NA-10).

Sr. No.	PCR steps	Temperature (ºC)	Time	Cycles
1	Initial denaturation	95	5min	1
2	Denaturation	94	30sec	35
3	Annealing	57.2	30sec	
4	Extension	72	60sec	
5	Final Extension	72	10min	1
6	Hold	4	10min	1

Table 3: Thermal conditions for first half segment of hemagglutinin gene amplification (HA-900).

qSr. No.	PCR steps	Temperature (ºC)	Time	Cycles
1	Initial denaturation	95	5min	1
2	Denaturation	94	30sec	35
3	Annealing	53	30sec	
4	Extension	70	60sec	
5	Final Extension	70	10min	1
6	Hold	4	10min	1

RESULTS

A total of 500 samples were collected from Akhtarabad (n=92), Mari Lachi (n=08), Chak 13 BC (n=90), Tailwala (n=05), Chak 12/DRB (n=69), Mithra bangla (n=19), Chak 14/DRB (n=43), Chak 37/DNB (n=62), Chak 38/DNB (n=77) and Head Rajkan (n=35). Out of 500 samples, 65 (13%) were positive for HA test (Table 4; Figure 2). HI assay using specific anti-sera against H5, H7, H9 and NDV did not reveal avian influenza subtypes H5 and H7. An overall prevalence of 4% (20/500) for H9 and 9% (45/500) for ND was detected (Table 5).

Table 4: Prevalence of H9N2 in Backyard poultry at different locations of Bahawalpur City.

Area of sampling	No. of samples (n)	Samples positive for HA	Samples positive for HI (against NDV)	Samples positive for HI (against H9)	Percent positive of H9 (%)
Akhtarabad	92	15	15	0	0
Mari Lachi	08	0	0	0	0
Chak 13 BC	90	20	0	20	22.22
Tailwala	05	0	0	0	0
Chak 12/DRB	69	05	05	0	0
Mithra bangla	19	0	0	0	0
Chak 14/DRB	43	05	05	0	0
Chak 37/DNB	62	10	10	0	0
Chak 38/DNB	77	05	05	0	0
Head Rajkan	35	05	05	0	0
Total	**500**	**65**	**45**	**20**	

Table 5: Prevalence of H9N2 and ND in Bahawalpur City.

	Total samples tested	Number positive	Prevalence
H9N2	500	20	4%
ND	500	45	9%

According to our results, ND is more prevalent than Avian Influenza among backyard poultry of the Bahawalpur City. Highest isolation of H9N2 was observed at Chak 13 BC while no any other case of avian influenza was observed at any other location of Bahawalpur City during the study period (Figure 1). Furthermore, molecular testing of H9 positive samples of Chak 13 BC through conventional reverse transcriptase PCR by using specific primers targeting H9 and N2 genes confirmed the subtype as H9N2 of Avian Influenza (Figure 2).

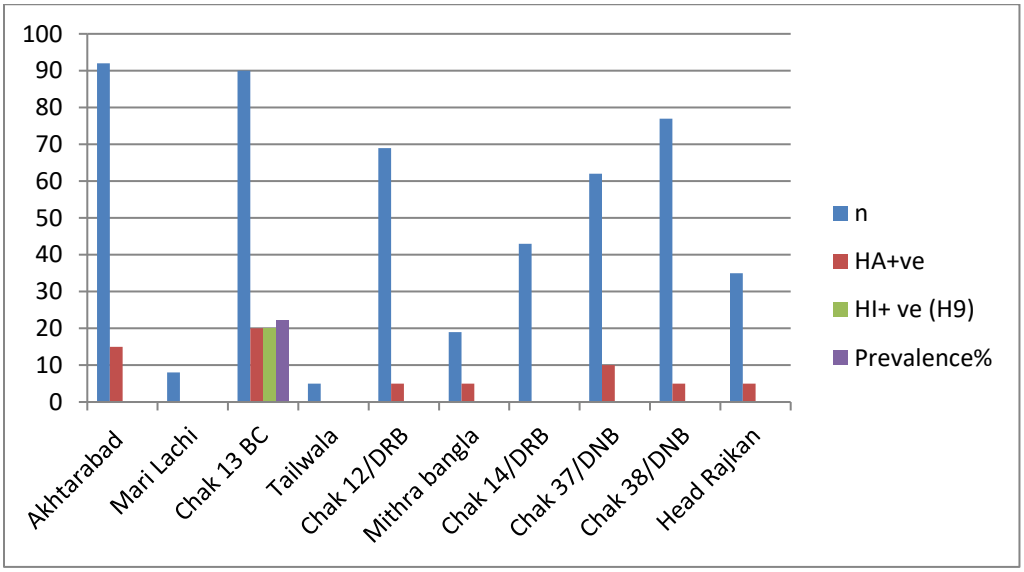

Figure 1: Percentage positivity of H9N2 in Backyard Poultry from different localities of Bahawalpur City

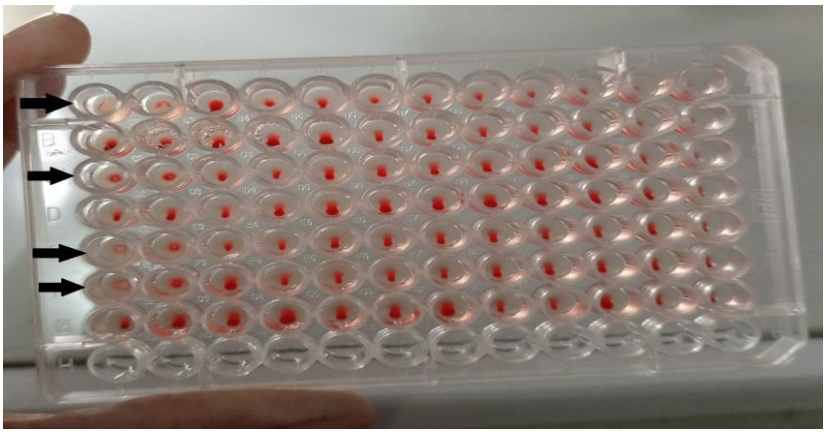

Figure 2: Haemagglutination positive samples

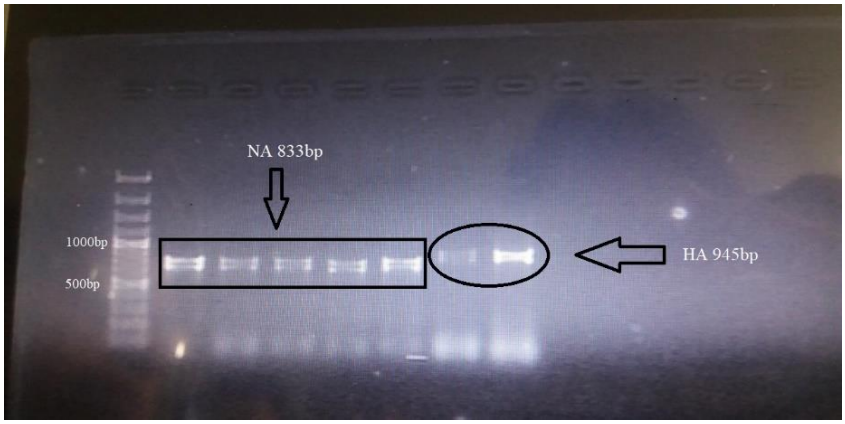

Figure 3: Agarose gel showing first half of H9 and N2 gene amplicons

DISCUSSION

RNA viruses are more prone to mutation (genetic drift) and among them viruses possessing segmented genome have more chances to evolve into a new variant in short period of time by genetic shift (Quinn *et al*., 2011). Avian Influenza viruses are the RNA viruses segmented genomes are continuously evolving so there is a need of regular surveillance to monitor their prevalence in a specific area (Chen and Deng., 2009). Poultry industry is the second largest industry of Pakistan providing livelihood to about 1.5 million peoples directly and indirectly (Economic survey of Pakistan 2019-2020). Subtype H9N2 of avian influenza is being endemic in the country is a continuous threat to the poultry industry (Lee *et al*., 2016). Even though it causes low mortality in the birds (5-30%) but due to high morbidity (100%) birds show loss of production and as a result there is huge economic loss to the farmers (Liu *et al*., 2003). A study conducted in Gujranwala district of Pakistan in which sero-prevalence of H9N2 calculated in commercial poultry farms. Three sites (out of 10) were found to be positive with a prevalence of 20%, 50% and 30% on Wazirabad road, Pasroor road and Lahore road respectively (Cheema *et al*., 2011). Hazara region which is comprised to hilly area (with an altitude ranging from 1700ft to 4000ft above the sea level). A study conducted in this region for the prevalence of H9N2. Oral and cloacal swabs were collected from commercial poultry farms of five districts of this region including; Abbottabad, Mansehra, Battagram, Haripur and Kohistan which showed the prevalence rate of 50%, 27.3%, 4.5%, 13.7% and 4.5% respectively (Ayaz *et al*., 2017). Bahawalpur is situated in Cholistan desert having hot climatic conditions with prolonged summer (Farooq *et al*., 2007). This makes the area less suitable for commercial poultry farming. Therefore most of the people living in this area prefer backyard farming (Sadef *et al*., 2015). Backyard poultry in this area consists of various local breeds of chicken which can survive in harsh climatic conditions (Padhi 2016). But lack of veterinary facilities in remote areas, ignorance and non-adherence regular vaccination schedule make the backyard poultry and livestock of this area more vulnerable to various diseases (Farooq *et al*., 2007). Current study provides evidence on the presence of H9N2 influenza virus in backyard poultry with a prevalence rate of 4% in Bahawalpur City, which has a

zoonotic potential for the farmers (Ahad *et al*., 2013). However, the presence of the other subtypes H5 and H7 was not detected in the area. In addition, NDV was also found to be circulating in the region and had more prevalence (9%) than H9N2 on the basis of HI assay. Our results suggest that, the local breeds may be more resistant to influenza viruses when compared to NDV. A similar finding was reported in a study in Iran (Broomand *et al*., 2018). Another study showed that H9N2 is more common than the NDV in commercial poultry because of effective vaccination against the later one (Ali *et al*., 2018).

Conclusion: Avian Influenza viruses are circulating not only in commercial poultry but also in the backyard poultry. Therefore, strategies should be developed to control avian influenza in both commercial and backyard poultry.

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