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ORIGINAL ARTICLE

Prevalence of Newcastle Disease Virus and Avian Influenza Virus (H7N3) in Poultry at Karachi

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ABSTRACT

Background: Poultry is the largest and rapidly growing sector of livestock in Pakistan. It is mainly influenced by viral pathogens such as the Newcastle disease virus (NDV) and Avian influenza virus (H7N3). These viruses cause severe disease in poultry and lead to heavy economic losses throughout the world. The outbreaks of these pathogens have been increased in the last few decades. Therefore, the study about antigenic prevalence is needed to know about the emergence of these pathogenic viruses and to get rid of severe ailments associated with reduced poultry production.

Objectives: To determine the prevalence of Newcastle disease virus (NDV), Avian influenza virus (H7N3) and co-infections in poultry flocks at Karachi.

Methodology: For detection of NDV and H7N3, a total of 200 tracheal swabs were collected and tested through Virus Isolation (V.I); the sample with positive virus isolation were tested through Agar gel precipitation (AGP) and then the RNA was isolated through TRI Reagent, which was further tested through Reverse transcription-polymerase chain reaction (RT-PCR).

Results: The virus isolation showed that 58% of samples were positive for various viruses. Agar gel precipitation (AGP) revealed that the occurrence of NDV, H7N3 and ND+H7 were 50%, 8% and 38%, respectively. RT-PCR for F and HA gene of NDV and H7N3 confirmed the presence of NDV and H7N3 in the poultry.

Conclusion: It is concluded that NDV and H7N3 are circulating in the flocks causing co-infections, therefore it is important to know the field challenge of viruses and to prepare a vaccine of circulating serotype of the virus to mitigate the rate of infection.

Keywords
Poultry, NDV, H7N3, AGP, Livestock, RT-PCR.

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Article info.
Received: December 11, 2019
Accepted: July 26, 2020

Cite this article: *Channa AA, Kalhoro NH, Nizamani ZA, Mangi AH, Soomro J. Prevalence of Newcastle Disease Virus and Avian Influenza Virus (H7N3) in Poultry at Karachi. RADS J Biol Res Appl Sci. 2020; 11(1):9-14.*

Funding Source: *Nil*
Conflict of Interest: *Nil*

INTRODUCTION

The poultry sector is an important and major segment of livestock and contributes 26.8% in total meat production, 5.76% in agriculture, and 1.26% in the national GDP of Pakistan[1]. Due to rapid growth in population in urban as well as rural areas, there is a high demand for perishable products like fruits, vegetables, dairy and meat products. In these conditions, consumers like to eat fish and poultry meat which contain an adequate amount of omega 3 and milk hence, considered as a balanced ratio playing a vital role to fulfil protein demand. Livestock shows 3.43% growth per year and produces 58% of agriculture and 43% of total labor[2]. Despite the rapid growth of poultry in Pakistan, it is influenced by many viral infections especially, the Newcastle disease (NDV) virus and Avian influenza virus (AIV) H7N3 causing poor weight gain, respiratory distress and drop in egg production[3,4].

In recent outbreaks, there is the involvement of more than one viruses that pose a severe complication to control

infections. To achieve proper prevention of these infections, most poultry producers are using live and inactivated vaccines for many years. Even though live vaccine virus replicates in the respiratory tract and can induce a reaction known as a post-vaccination reaction, however, to achieve the required results, the hyperimmune yolk was developed to protect against viral infections[5]. Despite mixed infections, H7N3 is highly contagious which leads to heavy economic loss. A regular and sudden mutation in these viruses could cause minor antigenic differences between the isolates, likely contributed to decreased vaccine protection and shedding of a virus when the hemagglutinin (HA) sequences differed by up to 13%[6].

It has been found that Pakistan suffered from a heavy economic loss of about 5.4 billion in 2004-5. These losses are associated with epidemics of circulating serotypes of NDV and AIV. Additionally, the outbreaks of Avian influenza viruses have been increasingly occurring in the last few decades in Asian counties, which leads to the major economic defeat of commercial poultry farming. It has also been reported previously that the infection might be transmitted from infected birds to other healthy birds. However, it has not been reported to have birds to human transmission and no human case has been observed[7]. Meanwhile, in early 2003, avian influenza H7 sub-type caused pandemics in Pakistan in commercial breeder because of inactivated mass vaccination[8].

The diagnosis and detection of virus circulating in the fields pose a severe problem and has key importance. Since 2003, highly pathogenic avian influenza viruses (HPAIVs) H5N1 have infected poultry as well as humans in more than 62 countries in Asia, Africa and Eurasia which leads to 400 million deaths of birds[9,10,11]. Research has found that low pathogenic Avian influenza viruses cause infections to wild and captive birds, especially in chickens that may acquire high virulence on infection from one flock to another[12]. However, the poultry industry is the fastest-growing field throughout the world and act as a vehicle for transportation of birds either from peri-urban to urban areas or from one live bird market (LBM) to another, that cause infection and outbreaks of various viral and bacterial disease, specially NDV and H7N3. Likewise, the commercialized poultry is a chain for the movement of live poultry, facilitating the transmission, dissemination of infection and re-infection. Therefore, this study is designed to know the prevalence of avian influenza virus H7- subtype and NDV.

MATERIALS AND METHODS

Collection of Samples and Transportation

A total of 200 samples including tracheal swabs were collected from suspected birds located in Karachi. Samples were transferred in sterile Eppendorf's containing phosphate-buffered saline, transported to Sindh Institute of Animal Health, Karachi and stored at -70°C for further processing.

Virus Isolation

The samples (tracheal swabs) collected were mixed gently with 1ml of normal saline and centrifuged for 10min at 10,000rpm. The upper transparent fluid was separated and dispensed in a new Eppendorf which was mixed with streptomycin (40µl/ml). After that, 100µl of fluid was inoculated into 5 Embryonated Chicken Eggs (ECE) of 10 days' age. Moreover, the eggs were placed in an incubator at 37°C for 72hrs, candled after 24hrs and the dead eggs were chilled at 4°C. The amnioallantoic fluid was collected from dead eggs and tested through rapid Hemagglutination Assay (HA). After rapid HA, the positive samples were tested through Agar gel precipitation (AGP) and confirmed by RT-PCR.

Rapid Haemagglutination Assay

A volume of 500µl harvested amnioallantoic fluid was placed on a rapid HA plate, 2-3 drops of 7% chicken RBCs were poured on it and the plate was then incubated at room temperature for 10min and shaken slowly. The agglutination of chicken RBCs indicated the presence of a virus, while the absence of agglutination shows a negative sample.

Agar Gel Precipitation Test (AGPT)

Agar gel precipitation was done as described by Okwor, 2011[13] such as a volume of 10ml noble agar (1%) was prepared in distilled water containing 8% sodium azide and the pH was adjusted up to 7.2 ± 0.1. The agar was then poured into petri dishes and incubated for 24hrs. After that, a well of 4mm diameter was cut in a circle of 6 wells surrounding an internal well. The internal (middle) well was filled with a known serum of NDV and H7 while

peripheral wells were filled with amnioallantoic fluid. After that, the plates were incubated at 37°C and observed every 24hrs till 72hrs. A line of precipitation was observed and noted.

RNA Isolation

The RNA was extracted by using TRIzol® reagent as per the manufacturer's protocol. Briefly, a volume of 250µl of tissue/fluid was mixed with 750µl of TRI® reagent, centrifuged for 10min at 10,000rpm. The supernatant was mixed with 200µl chloroform, incubated for 15min and then centrifuged at 10,000rpm for 15min. After that, the supernatant was transferred to a sterilized Eppendorf, mixed with 500µl of chilled isopropanol and centrifuged for 10min at 10,000rpm. The upper fluid portion was discarded, and the RNA pellet was precipitated with ethanol (100%). The RNA was diluted with 30µl of nuclease-free water and stored at -80°C for further experimental analysis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

It was performed through the Hot Start RT-PCR kit (Thermo Scientific) as per the manufacturer's protocol. A volume of 50µl of RT-PCR reaction mixture was prepared by mixing verso enzyme (1µl), master mix (25µl), RT enhancer (2.5µl), forward primer (1µl), reverse primer (1µl), RNA template (1-5µl) and nuclease-free water (17µl). The primers include H7-forward 5'CAGGCGGAATTGATAAGGAG 3' and H7 reverse 5' TGCCCCATTGAAACTGAAAG 3', and ND-forward 5' GGGAGGCATACAACAGGACA 3' and ND-reverse 5' TGGTTGCAGCAATGCTCTC 3'. The thermal cycling was carried at 45°C for 15min (1 cycle for cDNA synthesis) with initial denaturation at 95°C for 15min (1 cycle). After that, 40 cycles including denaturation (95°C for 20sec), annealing (58°C for 30sec), extension (72°C for 1min.) and final extension (72°C for 5min)[3] was performed.

RESULTS

In this study, the analysis of virus isolation of pooled (n=200) samples has revealed that 116/200 samples were found positive for various viruses, while 84/200 samples were negative. Interestingly, the virus isolation test explored that the positive samples either contains Newcastle disease virus (NDV), Avian influenza virus (H7 subtype) or co-infection caused by both NDV and H7N3 viruses as mentioned in Table **1**.

Table 1. Prevalence of Different Viruses by Virus Isolation in Poultry at Karachi.

S. No.	No. of Samples	HA Positive	HA Negative
01	200	116	84

All samples with positive virus isolation were then subjected to an agar gel precipitation test for conformation. The analysis of results has found that NDV is most prevalent in the fields causing serious illness and occur in 50 samples out of 100, followed by mixed infections of Avian influenza virus (H7 subtype) and NDV that occurs in 38 samples out of 100, and then H7 subtype that was found positive in 8 samples out of 100 (Table **2**).

Table 2. Prevalence of ND, H7 Subtype and ND+H7 Through Agar Gel Precipitation.

S. No.	Type of Infection	No. of Samples	AGP Positive	% of Positive samples
01	**NDV**	200	100	50
02	**H7 Subtype**	200	16	08
03	**NDV+H7N3**	200	76	38

After Agar gel precipitation, the samples were subjected to RT-PCR for a reliable diagnosis. The results of RT-PCR have found that the Newcastle disease virus (NDV) was positive with clear bands at 238bp (Fig. **1**).

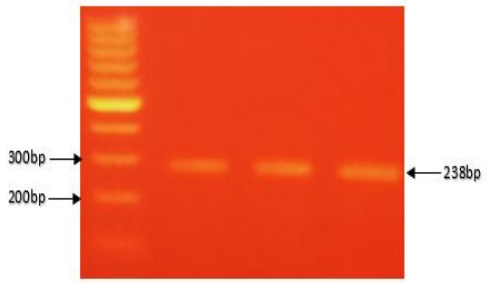

Figure 1. RT-PCR analysis of Newcastle disease virus (NDV). Lane 1 is DNA marker 100bp, Lane 2 positive control, Lane 3 & 4 = field sample.

The results of RT-PCR have confirmed that the Avian influenza virus H7N3 subtype was circulating in local flocks that cause severe damage to poultry and found positive with expected product size 407bp (Fig. **2**).

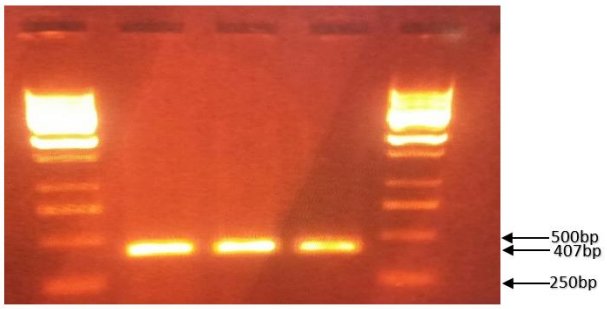

Figure 2. Amplification of NP gene of H7-subtype. An expected product size is 407bp. Lane 1 & 5 (DNA marker 1kb), Lane 2 = positive control, Lane 3 & 4 = field samples.

DISCUSSION

The poultry industry is an emerging and dynamic field in Pakistan. Despite this, poultry producers are facing problems such as the use of improper vaccination, medication and disposal of dead birds that may lead to the chance of infection, re-infection, dissemination of infectious agents and severe outbreaks of bacterial as well as viral diseases. Mixed infections of NDV and H7 cause heavy economic losses to the poultry industry. Therefore, this study was designed to know the prevalence of NDV and AIV (H7 subtype).

Virus isolation has revealed that most of the circulating viral pathogens in poultry in Sindh, Pakistan could be due to NDV and H7 subtype that have shown 58% HA positive per 100 sample (Table **1**). Similarly, the prevalence of H9 subtype in commercial poultry farms of Thatta, Karachi and Mirpurkhas districts of Sindh was found to be 97%, 86% and 89%, respectively, as compared to 31%, 41% and 53% for H7N3 subtype[14]. The high prevalence could be due to improper vaccination, disposal of dead birds and frequent mutation in these viruses. According to WHO, highly pathogenic AIV severely invaded poultry, transmit to humans, and caused 442 human cases leading to 262 deaths until September 2009 in Azerbaijan, Lao people's Democratic Republic, Thailand, Bangladesh, Myanmar, Nigeria, Pakistan, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Turkey and Vietnam[12].

Agar gel precipitation (AGP) revealed that out of 100% samples, 50% were found positive NDV (Table **2**). Findings of the current study are in agreement with other findings, who have reported that the prevalence rate of Newcastle disease was comparatively higher in local breeds of birds i.e., 55.0% while it was found in layer, broiler and duck i.e.; 37.5%, 32.5% and 27.5%, respectively[15].

Results of the current study have found that the prevalence of mixed infection of NDV and H7 was 38% and 8% with H7 (Table **2**). Correspondingly, others have reported that the prevalence of AIV in the live bird market was 23%[14]. Similarly, it has been reported that infections of the Avian influenza virus and Newcastle disease virus have been increased in Pakistan in the last few decades[15,16]. Likewise, the outbreaks of the Avian influenza virus (H7N3) in Pakistan during 2003 caused the death of 3.2 million birds[17]. The reason for the higher prevalence of AIV might be either the transportation of birds from the local market to the urban areas or due to the improper vaccination and disposal of dead birds. Moreover, the zoonotic infections caused by Avian influenza viruses such as H5NX, H7NX, H9N2 and H10N8 are sporadically detected[18,19,20]. The pathogenicity of Avian influenza virus H7 Subtype is associated with polybasic cleavage site i-e, PEKRRKR/G at position 322 to 329 amino acids[21].

The RT-PCR have shown that NDV was positive with the clear band at 238bp, similarly, the H7 virus is positive with 409bp (Fig. **1 & 2**). The primer set tested in this research work might show homogeneity endemic nature of circulating virus in Pakistan[17]. The increasing outbreaks either be due to migratory birds, exotic birds or imported vaccines.

CONCLUSION

It is concluded that the NDV and H7N3 are circulating in the poultry flocks causing co-infections. However, the rate of co-infections of NDV and H7N3 is highest followed by the Newcastle disease virus. Therefore, it is important to isolate the circulating strains of NDV and H7 subtypes to prepare vaccines such as monovalent NDV/H7N3, divalent NDV+H7N3 or polyvalent IBV+NDV+AIV to get rid of emerging infections and heavy economic losses.

ACKNOWLEDGEMENTS

We are highly thankful to the DG/ Executive director of Sindh Institute of Animal Health Karachi, for his kind support and gratitude.

LIST OF ABBREVIATIONS

AGP	Agar Gel Precipitation
AIV	Avian Influenza Virus
HA	Hemagglutination Assay
HPAIVs	Highly Pathogenic Avian Influenza Viruses
NDV	Newcastle Disease Virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction
VI	Virus Isolation

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