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

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ABSTRACT

Background: Poultry is largest and rapidly growing sector of livestock in Pakistan. It is mainly influenced by viral pathogens such as Newcastle disease virus (NDV) and Avian influenza virus (H7N3). These viruses cause severe disease in poultry and leads to heavy economic losses throughout the world. The outbreaks of these pathogens have been increased in 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 vaccine of circulating serotype of virus to mitigate the rate of infection.

INTRODUCTION

Poultry sector is an important and major segment of livestock and contribute 26.8% in total meat production, 5.76% in agriculture, and 1.26% in national GDP of Pakistan¹. Due to rapid growth in population in urban as well as rural areas, there is high demand of perishable products like fruits, vegetables, dairy and meat products. In these conditions, consumers like to eat fish and poultry meat which contain adequate amount of omega 3 and milk hence, considered as a balanced ratio playing a vital role to fulfill protein demand. Livestock shows 3.43% growth per year and produce 58% of agriculture and 43% of total labor². Despite rapid growth of poultry in Pakistan, it is influenced by many viral infections specially, Newcastle disease (NDV) virus and Avian influenza virus (AIV) H7N3 causing poor weight gain, respiratory distress and drop in egg production³⁴.

In recent outbreaks, there is involvement of more than one viruses that pose a severe complication to control...
infections. To achieve proper prevention of these infections, most of the poultry producers are using live and inactivated vaccines for many years. Even though, live vaccine virus replicate in respiratory tract and can induce reaction known as post-vaccination reaction, however, to achieve required results, hyper immune yolk was developed to protect viral infections. Despite mixed infections, H7N3 is highly contagious that 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 virus when the hemagglutinin (HA) sequences differed by up to 13%.

It has been found that Pakistan suffered from 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, that 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.

Meanwhile, in early 2003, avian influenza H7 sub-type caused pandemics in Pakistan in commercial breeder because of inactivated mass vaccination.

The diagnosis and detection of virus circulating in the fields pose a severe problem and has a 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 and 10,000 humans infected. Research has found that low pathogenic Avian influenza viruses causes infections to wild and captive birds, especially in chickens that may acquire high virulence on infection from one flock to another. However, poultry industry is a 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 infections and outbreaks of various viral and bacterial disease, specially NDV and H7N3. Likewise, the commercialized poultry is a chain for movement of live poultry, facilitating the transmission, dissemination of infection and re-infection. Therefore, this study is designed to know 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 at 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 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 virus, while absence of agglutination shows negative sample.

**Agar Gel Precipitation Test (AGPT)**

Agar gel precipitation was done as described by Okwor, 2011 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 in 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 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 after every 24hrs till 72hrs. A line of precipitation was observed and noted.

**RNA Isolation**

The RNA was extracted by using TRizol® reagent as per 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 Hot Start RT-PCR kit (Thermo scientific) as per 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' TGCCCCATTGAACTGAAA 3’, and ND-forward 5’ GGGAGGCATACACAGGACA 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 have revealed that 116/200 samples were found positive for various viruses, while 84/200 samples were negative. Interestingly, 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>
<thead>
<tr>
<th>S. No.</th>
<th>No. of Samples</th>
<th>HA Positive</th>
<th>HA Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>200</td>
<td>116</td>
<td>84</td>
</tr>
</tbody>
</table>

All samples with positive virus isolation were then subjected to 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>
<thead>
<tr>
<th>S. No.</th>
<th>Type of Infection</th>
<th>No. of Samples</th>
<th>AGP Positive</th>
<th>% of Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>NDV</td>
<td>200</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>02</td>
<td>H7 Subtype</td>
<td>200</td>
<td>16</td>
<td>08</td>
</tr>
<tr>
<td>03</td>
<td>NDV+H7N3</td>
<td>200</td>
<td>76</td>
<td>38</td>
</tr>
</tbody>
</table>

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

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

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

![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.](image-url)
The results of RT-PCR have confirmed that 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).

Table 1: Prevalence of NDV and AI Virus among Different Breeds of Birds (100 samples). 

<table>
<thead>
<tr>
<th>Breed of Birds</th>
<th>NDV</th>
<th>AI Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler</td>
<td>30%</td>
<td>40%</td>
</tr>
<tr>
<td>Duck</td>
<td>20%</td>
<td>30%</td>
</tr>
<tr>
<td>Turkey</td>
<td>10%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Agar gel precipitation (AGP) revealed that out of 100% samples, 50% were found positive NDV (Table 2). Findings of 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.

Results of 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 live bird market was 23%.

Poultry industry is an emerging and dynamic field in Pakistan. In spite of this, poultry producers are facing problems such as 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 poultry industry. Therefore, this study was designed to know the prevalence of NDV and AIV (H7 subtype).

Virus isolation have 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. 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 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.

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 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 polyclonal IBV+NDV+AIV to get rid from emerging infections and heavy economic losses.
ACKNOWLEDGEMENTS

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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

REFERENCES

