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PREVALENCE OF AVIAN INFLUENZA VIRUSES IN LIVE BIRD MARKETS OF LAHORE

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ABSTRACT

Prevalence of Avian Influenza Viruses (AIV) in live bird markets of Lahore was determined. A total of 1500 cloacal swabs were collected from seven live bird markets of Lahore, Pakistan for surveillance of the viruses. The samples were tested for virus isolation in chicken embryos. Allanto-amniotic fluid (AAF) of each of the embryos was used for Haemagglutination (HA) test. Eighteen (18/1500) samples were positive for HA activity. AIV Antigen Rapid Test Kit differentiated 4 HA virus as AIV and other 14 as Newcastle disease virus. Haemagglutination Inhibition test also proved four HA positive isolates as AIV, while remaining fourteen as NDV, using specific sera. All the AI virus isolates were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) using a universal nucleoprotein (NP) primer set specific to the virus NP gene and serotype specific primers (H_9 and N_2) for its sub typing as H_9N_2.

Key words: Avian Influenza virus, H_9N_2, live bird markets, RT-PCR

INTRODUCTION

Avian influenza (AI) is a highly contagious disease caused by either of the subtypes of influenza virus. The influenza viruses mostly infect birds and mammals and are classified into three subtypes A, B and C on the basis of nucleoprotein (NP) and Matrix protein (MP). The type A virus is further classified into subtypes on the basis of its surface glycoproteins: hemagglutinin (H) and neuraminidase (N). Total 16 H and 9 N serotypes are present in aquatic wild birds (Fouchier *et al*., 2005). Wild waterfowl is a natural reservoir for these viruses and introduces the viruses into poultry (Kawaoka *et al*., 1988). All of the subtypes of avian influenza virus are not highly pathogenic (HPAI) but arise from low pathogenic avian influenza (LPAI), viruses introduced into poultry flocks from wild birds (Kawaoka *et al*., 1988).

There were several out breaks of highly pathogenic subtype H_7N_3 in Pakistan during 1995-2003 and caused death of 3.2 million birds (Naeem and Hussain, 1995; Capua and Alexandar, 2004; Rezza, 2004). The H_9N_2 is considered as low pathogenic (LPAI) and still persists in avian species. By 1999, H_9N_2 virus caused outbreaks of AI in young and laying birds in Pakistan (Naeem *et al*., 1999; Bano *et al*., 2003). It causes mild signs of the disease but there are exceptions where H_9N_2 influenza virus had caused high morbidity rates, diarrhea, depression and reduced egg production in China. Mortality rate was approximately 5-30% depending on the husbandry based study from 1995-2002 in China (Liu *et al*., 2003).

Three H subtypes (H_1, H_2 and H_3) and two N subtypes (N_1 and N_2) are circulating in human beings (De Jong and Hien, 2006; Peiris *et al*., 2007). There were repeated cases of avian H_9N_2 and H_5N_1 influenza viruses in human beings since 1997 in Southern China, Thailand, Vietnam and Indonesia (Guan *et al*., 1999; Xu, 1999; Mukhtar, 2007). Avian influenza virus has raised the concerns for public health, posing a pandemic threat to the whole world. In March 2006, H_5N_1 virus infected birds were recorded first time in Pakistan in North-West Frontier Province in two commercial flocks (Naeem et al., 2007). In February 2007, several outbreaks of H_5N_1 were reported among chickens in Rawalpindi, adjoining Islamabad and in peacocks in the North Western city of Mansehra. In February 2008, H_5N_1 virus outbreaks were reported in Southern port city of Karachi (Khan *et al*., 2010).

Many conventional diagnostic tests have been developed for identification of the avian influenza viruses but AI isolation via chicken embryos (CE) remains the gold standard (Alexander, 2000). The H and N based subtypes of avian influenza viruses are mostly identified by hem-agglutination inhibition (HI) test, neuraminidase inhibition tests and reverse transcriptase polymerase chain reaction (RT-PCR) (Nicholson *et al*., 1998; Steininger, 2002).

Most of the research work is conducted on avian influenza A viruses isolated during outbreaks. In markets, healthy birds are aggregated from different sources and kept in close proximity. Lack of biosecurity in such markets can contribute to avian influenza subtype H_9N_2 persistence and dissemination. The present study was therefore carried out to investigate the prevalence of AIV

type A in apparently healthy birds of different species, sources and locations at live bird markets of Lahore, Pakistan.

MATERIALS AND METHODS

Sampling plan: Total 1500 cloacal swabs were collected from the seven live bird markets (Tolenton -285; Lakshmi-110; Bhati- 100; Mugalpura-155; Samanabad-180; Sheranwala gate-520 and Lohari-150) of Lahore, Pakistan. These samples were from ducks (45), poultry "bantam" (280), pigeons (30), doves (40), peacocks (25), broilers (980), Indian green parrots (35), finches (30) and quails (35). The swabs were collected in five ml tubes containing BHI broth containing Penicillin (1000 IU/ml), Streptomycin (1 mg/ml) and gentamycin (200µg/ml) (Lindh *et al*., 2008).

Serological identification: Each tube containing swab was vortexed and centrifuged at 1500 rpm for 10 minutes. The supernatant was filtered from 0.20 um porosity and inoculated in 10 days old chicken embryos (200µl) through allantoic cavity route in triplicate (Khan *et al*., 2010). The embryos were incubated, chilled and opened aseptically. Allantoic amniotic cavity fluid (AAF) of each embryo was harvested and used for hemagglutination (Allan *et al*., 1978). The HA positive samples were detected through Avian Influenza Virus (AIV) Antigen Rapid Test Kit, manufactured by ANIGEN, animal Genetics, Inc, for differentiation between AI and ND viruses. The isolates declared positive for AI were confirmed by RT-PCR (Steininger, 2002; Sarwar *et al*., 2012).

Molecular identification: Viral RNA was extracted from each AAF with TRIZOL® reagent (Gibco, UK) according to the manufacturer's instructions. After extracting RNA, cDNA was prepared by Reverse transcription using cDNA synthesis kit (Revert Aid™ Cat No. K1662. Fermentas). For RT-PCR, 25µl reaction mixture was prepared by 10µl of 2X AmpliTaq Gold® Fast PCR Master Mix (Applied Bio systems) containing hot start Taq polymerase dNTP mixture, reaction buffer and 4mM MgCl2, in a 0.2 ml micro-centrifuge tube along with 4 µl of complementary DNA, 1µl each of forward and reverse primers {H9(773)f: ATCTAATCGCTCCATGGTATGG, H9(1040)r: TGACCAACCTCCCTCTATGA, NP (1200)f: CAG(A/G)TACTGGGC(A/T/C) ATAAG (A/G) AC, NP (1529) r: GCATTGTCTCC GAAGAAATAAG, N2 (478)f: CCAGCT CAAGTT GCCATGA, N2(940)r: GATCTCTGCAGTTGCTGCT} and 9µl of Nuclease free water (GIBCO, Invitrogen, USA). The amplification of reaction mixture was carried out according to following program; Initial denaturation at 94°C for 7 minutes, denaturation and annealing at 94°C and 58°C for 1 minute and extension at 72°C for 60 sec up to 30 cycles, followed by a final extension at 72°C for 7 min.

RESULTS AND DISCUSSION

Avian influenza virus sometimes persists as carrier form in migratory or domestic birds. Only eighteen out of 1500 (0.012%) cloacal swabs from apparently healthy live birds showed presence of Haemagglutinating virus. The haemagglutinating viruses persist in respiratory and intestinal tracts of the carrier birds while high antibody titers against these viruses are present in circulation (Swayne and Suarez, 2000). Avian viruses bind and replicate in the cell lining of the gastrointestinal tract of duck and water fowl (Nicholson *et al.,* 1998). The droppings contaminate water and might be taken up by other birds via drinking water (Shortridge, 1999). Spreading of Influenza virus from one host to another within a host population depends on its transmissibility. Avian Influenza viruses exist in birds, particularly in wild waterfowl (Suarez *et al.*, 2004) as potential natural reservoirs for poultry (Kawaoka *et al*., 1988).

The haemagglutinating virus was detected in one of the cloacal swabs (1/35) from ducks (Tolintin market), one (1/10) desi chickens (Lakshmi), two (2/280) desi chickens (Sheranwala gate), five (5/180) broilers (Saman abad) and nine (9/300) broilers (Sheranwala). However, such viruses were not detected in cloacal /tracheal swabs of pigeon, doves, peacocks, parrots and quails from either of the live birds markets. Different species have different resistance to influenza virus infection. Migratory water fowls and wild ducks are the most resistant to infection and usually asymptomatic carrier but shed the virus in their droppings (Swayne and Suarez, 2000). These carrier birds are source of infection for susceptible birds in rural / farm birds. In 1996, H_9N_2 subtype was first time isolated from turkey with mild respiratory signs (Saberfar *et al.*, 2007) and in 1997, H_9N_2 influenza viruses were found in other regions such as Northern China, Korea, India, Saudi Arabia, Europe and South Africa (Guo *et al.,* 2000; Guan *et al*., 1999; Liu *et al*., 2003).

The cloacal swabs from live healthy birds when inoculated in nine days old chicken embryos did not kill the embryos up to 12th day of embryonic age. This indicates that virus isolates were low pathogenic. However, avian influenza (H_5N_2) virus kills chicken embryo in 24 hours post inoculation (PI) (Kouwenhoven and Burger, 1986), H_7N_3 virus kills duck embryo 24 hours PI and chicken embryo 36 hours PI (Yaqub, 1998) and H_5N_1 virus causes embryonic death within 36-48 hours PI (Khan et al., 2010). It is advisable to inoculate AIV H_9N_2 on 9th day of chicken embryonic life that may result embryonic death on 12th day of age. In this way maximum AAF may be obtained for vaccine production or diagnostic HA antigen of the virus. The agglutinating viruses have ligand molecules on their surface and avian/ mammalian erythrocytes have receptors for the ligand molecules. The mixing of both results in virus mediated

clumping / agglutination. This test can differentiate haemagglutinating and non-haemagglutinaing viruses (Yaqub, 1998; Khan *et al*., 2010). Four out of eighteen HA virus isolates were differentiated as AI viruses. While, remaining fourteen were NDV isolates. The NDV isolates could be vaccinal strains normally used as vaccine in broilers and desi chickens.

The AI viruses were tested through reverse transcriptase polymerase chain reaction (RT-PCR) using universal primers NP and serotype was confirmed by serotype specific primers (H9, N2). All isolates detected as AI viruses were confirmed as H_9N_2 (Fig. 1 and 2). The NP primers yielded amplicon of 330, H9 and N2 of 287 and 483 bps, respectively. Annealing Temperatures 58°C for NP and H9 primers, 57°C for Newcastle primers and 51°C for N2 primer set were found to be optimal. Avian Influenza viruses have segmented RNA genome and can easily mutate. Circulating LPAI (especially H_5 and H_7) has the ability to mutate into a more devastating highly pathogenic avian influenza (HPAI). In Pakistan H_9N_2 subtype is continuously circulating in poultry. Wild birds are considered as major reservoir of H_9N_2 (Khawaja *et al*., 2005). Outbreaks are not uncommon in the vaccinated poultry flocks. Frequent antigenic changes (mutation) in field viruses could be incriminated as a cause of failure of the immunoprophylaxis. LPAI viruses (H_9N_2) outbreaks have been reported in Pakistan, China, Korea, Iran, Germany, Italy, Ireland, South Africa, Hungary, Hong Kong and countries of the Middle East (Naeem *et al.,* 1999 and Alexander, 2000). The continued presence of H_9N_2 in the Middle and Far East may mean that it is becoming an established endemic disease in those regions. The AI H_9N_2 multiplies in the carrier birds (migratory birds, water fowls, broilers and desi chiken). These carrier birds may act as mixing vessel for re-assortment and development of new AI strains. Such strains may cause epidemic that may be serious health hazard for human beings (Butt *et al*., 2010).

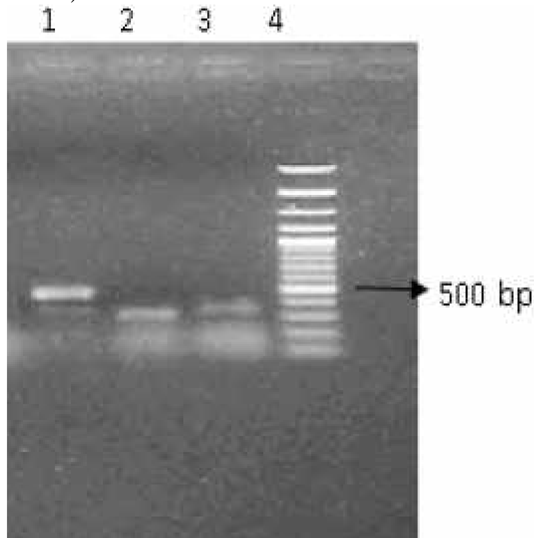

Fig-1: Optimization of RT-PCR for the NP, N_2 and H_9 primers

Lane 1: Amplification product (483bp) of H_9 cDNA with N_2 primer set

Lane 2: Amplification product (330bp) of H_9 cDNA with NP primer set

Lane 3: Amplification product (287bp) of H_9 cDNA with H_9 primer set

Lane 4: Marker (DNA ladder 100bp)

It was concluded that AIV (H_9N_2) subtype was prevailing in domestic birds of live bird markets in Lahore, Pakistan which is a constant source of infection in healthy birds. Continuous screening of live bird markets in major cities for AIV may help scientists to control outbreaks by preventive measures.

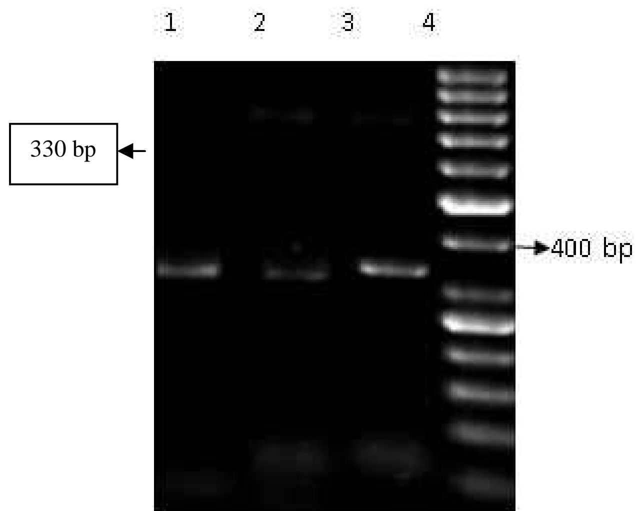

Fig-2: Amplification of H_9N_2 live bird market samples cDNA with NP primers Set

Lane 1: H_9N_2 control positive cDNA (Positive control)

Lane 2 and Lane 3: H_9N_2 Positve Samples cDNA (330bp)

Lane 4: Marker (50bp DNA ladder)

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