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 (H3 and N2) for its sub typing as H3N2.

Key words: Avian Influenza virus, H3N2, 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 H5N1 in Pakistan during 1995-2003 and caused death of 3.2 million birds (Naem and Hussain, 1995; Capua and Alexandar, 2004; Rezza, 2004). The H5N1 is considered as low pathogenic (LPAI) and still persists in avian species. By 1999, H5N1 virus caused outbreaks of AI in young and laying birds in Pakistan (Naem et al., 1999; Bano et al., 2003). It causes mild signs of the disease but there are exceptions where H5N1 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 (H1, H2 and H5) and two N subtypes (N1 and N2) are circulating in human beings (De Jong and Hien, 2006; Peiris et al., 2007). There were repeated cases of avian H5N1 and H5N1 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, H5N1 virus infected birds were recorded first time in Pakistan in North-West Frontier Province in two commercial flocks (Naem et al., 2007). In February 2007, several outbreaks of H5N1 were reported among chickens in Rawalpindi, adjoining Islamabad and in peacocks in the North Western city of Mansehra. In February 2008, H7N1 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 H5N1 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 (Tolentino - 285; Lakshmi-110; Bhati- 100; Mugalpura-155; Samanabad-180; Sherwanwala gate-520 and Lohari-150) of Lahore, Pakistan. These samples were from ducks (45), poultry “bantam” (280), pigeons (30), doves (45), 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 (200 l) 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 10 l 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 l of complementary DNA, 1 l each of forward and reverse primers (H9(773)f: ATCTATCGTCCATGGTATGG, H9(1040)r: TGACCAACCTCCCTCTATGA, NP (1200)f: CAG(A/G)TACTGGGCC(A/T/C) ATAAG (A/G) AC, NP (1529) r: GCATTGTCCTC GAAGAAATAAG, N2 (478)f: CCAGCT CAAGGG GCCATGA, N2(940)r: GATCTCAGGTGTCGTC) and 9 l 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 (Sherwanwala gate), five (5/180) broilers (Saman abad) and nine (9/300) broilers (Sherwanwala). 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, H3N2 subtype was first time isolated from turkey with mild respiratory signs (Sabefar et al., 2007) and in 1997, H2N2 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 (H2N2) virus kills chicken embryo in 24 hours post inoculation (PI) (Kouwenhoven and Burger, 1986). H3N1 virus kills duck embryo 24 hours PI and chicken embryo 36 hours PI (Yaqub, 1998) and H2N1 virus causes embryonic death within 36-48 hours PI (Khan et al., 2010). It is advisable to inoculate AIV H3N2 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-haemagglutinating 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 H9N2 (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 H3 and H5) has the ability to mutate into a more devastating highly pathogenic avian influenza (HPAI). In Pakistan H9N2 subtype is continuously circulating in poultry. Wild birds are considered as major reservoir of H9N2 (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 (H9N2) 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 H9N2 in the Middle and Far East may mean that it is becoming an established endemic disease in those regions. The AI H9N2 multiplies in the carrier birds (migratory birds, water fowls, broilers and desi chiken). These carrier birds may act as mixing vessel for reassortment and development of new AI strains. Such strains may cause epidemic that may be serious health hazard for human beings (Butt et al., 2010).

It was concluded that AIV (H9N2) 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.

**REFERENCES**


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