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DETECTION OF HIGHLY PATHOGENIC AVIAN INFLUENZA (H5N1) IN APPARENTLY HEALTHY DUCKS (*Anas sparsa sparsa*) IN LIVE BIRD MARKETS, NIGERIA

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SUMMARY

Nigeria reported the first outbreak of Highly Pathogenic Avian Influenza (HPAI) in Africa, February 2006. Since then effort by relevant authorities to control the spread and persistence of the disease has been effective, with only sporadic resurgence in backyard and live bird markets. Surveillance for HPAI was carried out in live bird markets (LBM) between May and June 2008 in ducks among other species. A total of 4,707samples including sera and swabs of trachea and cloaca from live birds, and parenchymatous organs from dead or moribund birds were collected from 11 states of the country where HPAI has not been previously reported. Tissues were processed for virus isolation in embryonating chicken eggs, sera analyzed by Agar Gel Immuno Diffusion test (AGID) and Haemaglutination Inhibition (HI) tests with standard monoclonal antisera to H5 and the swabs by RT-PCR using gene specific matrix and H5 primers. Two isolates of HPAI were recovered from the tracheal swab samples from apparently healthy ducks.

Keywords: Waterfowls, Live bird markets, HPAI Detection

INTRODUCTION

Influenza A viruses particularly the low pathogenic avian Influenza (LPAI) are found predominantly in waterfowls, in which all the 16 subtypes co-exist in perfect harmony with their host (Stallknecht and Shane, 1988; Alexander, 2000). The virus hardly causes mortality in these natural hosts, and they remain in evolutionary stasis, with minimal changes over a long period of time. This benign equilibrium between the influenza virus and its hosts has changed with changes in nature, ecology, agricultural practices and trade which allow mixing and interaction of species. This has enhanced co-circulation of pathogens especially subtypes of Avian influenza (Webster *et al.*, 2006; Monne *et al*., 2008).

The precursor of the H5N1 influenza A virus that spread to humans in 1997 was first detected in Guangdong, China in 1996, when it caused a moderate number of deaths in geese and attracted little attention (Tang *et al.*, 1998). This goose virus acquired internal gene segment from influenza viruses later found in quail (A/Quail/HK/G1/97 (H9N2) and also acquired the neuraminidase gene segment from a duck virus

(A/Tenl/HK/W312/97(H6N1) and became widespread in live poultry markets in Hong Kong where it killed 6 of 18 infected persons (Sims *et al.,* 2003). This H5N1 virus was eradicated by the culling of all domestic poultry in Hong Kong. Different reassortant of this virus however continued to emerge from goose and duck containing the same H5 haemagglutinins glycoprotein but had various internal genes and spread to different regions (Guan *et al.,* 2002). Highly Pathogenic Avian Influenza also caused respiratory disease and deaths in humans for the first time in 1997 in Hong Kong (Yuen *et al*., 1998). It is this influenza virus that appeared most threatening, acquiring unprecedented and disturbing capability to infect humans; to cause neurotropic disease and a high proportion of death in water fowls in nature and to cause death and be transmitted among wild species, including domestic cats (Kuiken *et al.,* 2004). These changes have intensified concern over H5N1 virus pandemic potential. Before 1997, no evidence had indicated that H5 influenza viruses could infect humans and cause fatal disease. But as at April 2010, Human cases of HPAI H5N1 was positively confirmed in 15 countries, accounting for 493 illnesses and 292 deaths (WHO 2010)

Highly Pathogenic Avian Influenza H5N1 appeared for the first time in Nigeria, in February, 2006. The source of the initial outbreak is still shrouded in mystery. However, it is becoming clearer that wild birds could be responsible (Ducatez *et al*., 2006). The virus subsequently spread to all the agro-ecological regions of the country and to date over 1.2 million domesticated birds have been killed by the virus or culled to stop its spread, with one human fatality and over 5 million US dollars paid in compensation to affected farmers (Monne *et al*., 2008; Joannis *et al*., 2008).

Waterfowls were suggested as a vector because the virus spread through areas that had no record of any virus presence and coincided with migration of wild water birds between these areas. HPAI H5N1 was also detected in many wild waterfowls, often in areas where no outbreaks had been detected among intensively surveyed poultry (Keawcharoen *et al*., 2008).Yet it is often argued whether wild waterfowls are long distance vectors of HPAI because the birds where the virus were identified were either dead or sick, and could not possibly be fit enough to carry the virus for a long distance (Olsen *et al*., 2006), but over time evidence has shown that certain wild duck shows abundant virus excretion without clinical or pathologic evidence of debilitating disease (Keawcharoen *et al*., 2008)

During the period under review, the virus circulated in both intensive and rural poultry flocks enabling it to re-assort, and the sub lineages also circulated over a period (Monne *et al*., 2008). In an effort to control HPAI and unravel its transmission dynamics, a number of surveillance programmes were implemented, among this was a targeted surveillance in live bird markets in 11 states of Nigeria that were previously uninfected by HPAI with the aim of detecting reservoirs of the infection.

MATERIALS AND METHODS

Samples were collected randomly in 22 live bird markets in Nigeria; two markets in each of the 11 states where the surveillance was carried out. These states, namely Abia; Akwa-Ibom; Bayelsa; Cross-River; Ebonyi; Gombe; Imo; Kebbi; Kogi; Ondo and Osun were selected on the basis that they had not reported any case of HPAI as at the time of the study. A total of 43 ducks and 1899 other avian species were sampled. A total of 4,707samples including tracheal/cloacal swabs, sera from

live birds and parenchymatous organs from dead or moribund ducks (*Anas sparsa sparsa*), were collected. Tissues were processed for virus isolation in 9-11day old specific antibody negative embryonated chicken eggs. Sera collected from gallinaceous birds were analyzed by Agar Gel Immuno Diffusion test (AGID) whereas those collected from non gallinaceous were first heat treated at 56^0C in a water bath and with 10% chicken rbc to remove non specific precipitin and agglutinin in the serum before testing by haemagglutination inhibition with standard monoclonal antisera to H5 and 1% chicken rbc as indicator (OIE reference laboratory for Avian influenza and Newcastle disease, Padova Italy). Antigen and antisera were also sourced from OIE Reference laboratory for Avian Influenza and Newcastle diseases, Padova, Italy. Ribose nucleic acid (RNA) extraction and reverse transcription polymerase chain reaction (RT-PCR) were performed starting with the matrix (M) gene and for every positive matrix gene, RT-PCR for Haemagglutination (H) gene for subtype H5 was carried out using the following oligonucleotide primers: M forward 5'-AGA TGA GTC TTC TAA CCG AGG TCG-3' rev. 5'-TGC AAA AAC ATC TTC AAG TCT CTG-3'. H5 forward 5'-CCT CCA GAR TAT GCM TAY AAA ATT GTC-3' rev. 5'-TAC CAA CCG TCT ACC ATK CCY-3'. As described in Joannis *et al.,* (2008). Amplicons were detected conventionally by agarose gel electrophoresis with ethidium bromide staining and the products captured in a gel documentation system. Virus isolation attempt were also carried out from tracheal or cloacal swabs that were positive by RT-PCR by inoculation in 9-11 day old chicken embryonated eggs. Further molecular characterization and sequencing of isolates was carried out at the OIE Reference Laboratory for Avian influenza and Newcastle disease in Padova Italy.

RESULTS

Two isolates of Influenza A virus were detected from 2 out of 43 ducks sampled by virus isolation. They were both positive for Influenza A matrix gene and H5 gene by RT-PCR. The 320bp amplicon was correctly identified in the gel electrophoresis which corresponds with the positive control (Fig. 1). The duck isolates were from tracheal swab samples from apparently healthy ducks in a live bird market in Gombe State in North East Nigeria. Further molecular characterization and sequencing was carried out at the OIE Reference laboratory Padova, Italy. The full-length genome sequence for A/duck/Nigeria/3724-2/2008 and the sequence of the haemagglutinin (HA) segment for A/duck/Nigeria/3724-10/2008 were obtained. Sequences of the 8 gene segments of A/duck/Nigeria/3724-2/2008 were submitted to the Global Initiative on Sharing Avian Influenza Data public database (accession nos. EPI161701–EPI161708). Serology did not detect avian influenza antibody in both gallinaceous and non gallinaceous birds.

DISCUSSION

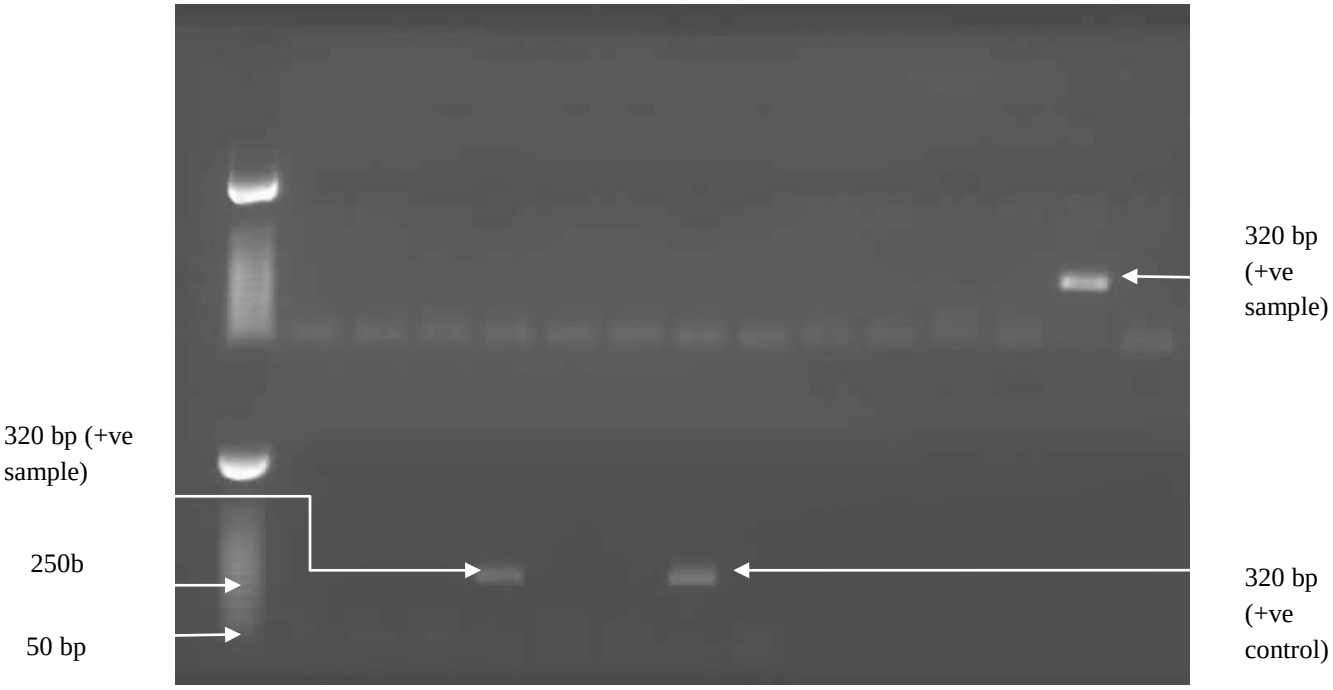
The evidence of circulation of HPAI in apparently healthy waterfowls shows the importance of these species in the maintenance and transmission of the virus. Virus contamination in LBMs is usually associated with movement from outbreak areas and attempt by poultry farmers to sell infected birds in an effort to reduce their economic loss.

Waterfowls are reported to be less susceptible to HPAI than chickens (Stallknecht and Shane, 1988; Keawcharoen *et al.,* 2008) and are thus able to shed the virus as healthy carriers in live bird markets. Most of the waterfowls being sold in LBMs are from rural areas

and backyard farms where they usually share the same water and wetland areas with both residential and migratory birds as observed in this study. Live bird market has been a source of HPAI outbreaks in the past especially in Asia (Wang *et al.*, 2006). Effective control and eradication of HPAI therefore would require minimizing contact between susceptible hosts and healthy carriers like waterfowls in poultry flocks and live bird markets. In addition to other interventions like biosecurity, the LBM should be re-organized to discourage the practice of mixing species in the same cages. This must begin with poultry farms, where multi-age birds of different species, sources and breeds are flocked together. So that the least susceptible species like waterfowls will not be a source of transmission to the more susceptible species like chickens and turkeys that are the main economy birds in the poultry industry (Adene and Oguntade, 2006).

Because of the role of quail in the transmission and pathogenicity of HPAI in Hong Kong, 1997 (Lau *et al*., 2007), there was a ban in its sale along with other live poultry which positively impacted on HPAI control. Waterfowls, ducks and geese are recognized as the natural reservoir of influenza viruses, ducks especially have high virus isolation rates (Shortridge, 1992) it may be necessary to discourage the sale of live waterfowls in markets to reduce HPAI contamination and transmission. Waterfowls are evidently re enacting their peculiar role in the transmission dynamics and genetic evolution of HPAI. A cardinal intervention in the control of HPAI would be reduction or elimination of contact between waterfowls and domestic birds especially in the LBMs as they seem to bridge the gap between migratory birds and domestic poultry population in the transmission of the virus.

Figure1: Amplicon size of the avian influenza isolate.



H5 gene – 320bp

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