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Isolation of Highly Pathogenicavian Influenza Virus (H5N1) from Poultry in Sudan in 2006

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Abstract: This is first report isolation of Highly Pathogenic Avian Influenza (HPAI) subtype (H5N1) from natural outbreaks in Sudan in 2006. From 2003 to 2005,1000 samples(organs, tracheal and cloacl swabs) were collected from different locations in Sudan, Central (AlGazeera and Khartoum), Westeran(Nyala) and Eastern Sudan (Kassala and AlGadarif) all samples were negative by Virus Isolation (VI) in egg embryo. In early 2006 an outbreak of HPAI was occurred in AlGazeera and Khartoum samples were tested by VI in department of virology the result is positive for type A Avian Influenza (AI), allantoic fluids were sent to) OIE, FAO and National Reference Laboratory for Newcastle Disease andAvian Influenza, Padua, Italy for advance confirmation Isolates were analyses using RT-PCR, VI and Sequencing positive to subtype H5N1.

Key words: Pathogenic, influenza, virus isolation, allantoic, padua

INTRODUCTION

AI is a contagious viral infection of many avian species including domestic poultry wild and exotic birds, shore birds and migaratory water fowl (Wenhuakang *et al*., 2006). Influenza also occurs in pigs, horses and humen. Recently H5N1 has been recognized in cats, tigers and leopards (IDSA, 2005). Avian Influenza Virus (AIV) is an enveloped RNA virus with eight segments of single stranded negative sense RNA (Wenhuakang, *et al*., 2006). Influenza A virus classified into subtypes according to their heamagglutinin (H1-H16) and neuraminidase (N1-N9) OIE Manual (2005). HPAI in poultry is characterized by a sudden onset, sever illness of a short duration and mortility approaching virtually 100% (Timm and Ortrud, 2006).

MATERIALS AND METHODS

History: Early 2006 highly pathogenic strain of AIV caused sever disease in domestic poultry with mortility rate reaching 100% in different farms in Khartoum and Algazeera. Clinical signs were rough feather, swollen head and comb, haemorrhages of the leg shanks and feet, cynosis of comb and wattle, oculonasal discharges, yellwish diarrhea, frothy fluid from mouth, drop in egg production, recombency and death.

Collection of samples: Organs (trachea, lung, intestine and spleen). Tracheal and cloacl swabs were collected from six farms in Khartoum and two farms from Algazeera, for (VI)and RTPCR.

Chicken embryonated eggs: Day-old embryonated eggs used for VI, were obtained from CVRL poultry farm (Khartoum, Sudan) and were incubated in an ordinary incubator at 37°C till they were nine-day-old.

Flu detect test strip: Is an *In-vitro*, rapid immunochromatographic assay used to detect influenza type. A viruses in tracheal and cloacl specimen from symptomatic birds or flocks.The test was performed as described by manufactured (Fabriqu par, Synbiotics corporation, France). The test was carried.

Eight drops of extraction buffer were placed into test tube,swabs to be tested were put into collection tube, swabs were discarded then the test strip were placed directly into the test tube containing the sample and left incubated at room temprture for 15 min, after that the test strip was removed and read.

Isolation of the virus: Samples were inoculated in 9-day-old embryonated chicken eggs with 0.2 mL via the allantoic cavity. Inoculated eggs were incubated at 37°C. Eggs candled daily,embryos that died within 24 h were discarded. Allantoic fluid was collected from embryos

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that died more than 24 h Post-Inoculation (PI) and then tested for the presence of influenza virus by the Haemagglutination (HA) and Haemagglutination Inhibition (HI) tests.

HA and HI tests: The HA and HI tests were carried out for detection of influenza viruses using standard procedures with 4 HA units (Thayer and Beard, 1998). For HA tests harvested allantoic fluid from inoculated chicken embryos were tested with chicken erythrocytes. For HI tests allantoic fluids were used as antigen aginst reference Newcastle antisera.

Agar Gel Immunodiffusion Test (AGID): Allantoic fluids as unknown samples antigens and positive antigen control were tested with reference AI serum for AIV by AGID using standard procedures(David *et al*., 1998).

Confirmation of AI isolates: Allantoic fluids were sent to FAO,OIE and National Reference Laboratory for Newcastle and AI (IZSVe) Italy for more confirmation, according to the (IZSVe) protocols as follows.

Qualitative real time PCR(M gene): This test used to detect type A AIV using 2 primers;
Forword M +25:AGA TGA GTC TTC TAA CCG AGG TCG
Reverse M-124:TGC AAA AAC ATC TTC AAG TCT CTG
Probe:FAM M+64:FAM -5-TCA GGC CCC CTC AAA GCC GA-3-TAMRA.

Qualitative real time PCR (H5 gene): It used to identify sub types (H5 gene) using 2 primers and probe
Primers;
1 Forword H5 F: TTA TTC AAC AGT GGC GAG
2 Reverse H5 R:CCA GAA AGA TAG ACC AGC T
Probe
FAM H5:FAM-5-CCC TAG CAC TGG CAA TCA TG -3-TAMRA

KHA one step RT-PCR(H5 gene): Also to identify subtype (H5gene) and sequencinge.
Primer:
H5-kha – 1:CCT CCA GAR TAT GCM TAY AAA ATT GTC
H5-kha – 3:TAC CAA CCG TCT ACC ATK CCY TG

One step RT-PCR (N1gene): The test used to detect (N1gene).
Primers
1 Forword 171 for:5-GCGCGCGGCCGCCAGGAGTTAAAATGAATCCAAAT-3
2 Reverse pN1 rev:5-AGGAATTGCCCGCTAAT-3

RESULTS

The flu detect test strip: More than half numbers (80) of tracheal and cloacal swabs showing presence of influenza type A viruses two pink purple bands (control line and test line) were present on the test strip.

Virus isolation: The embryos developed haemorrhages. The isolates caused death of most embryos within 48 h PI. The allantoic fluids had Haemagglutination Activity (HA).The HI test results showed that all allantoic fluid were negative when tested aginst positive ND antisera. The allantoic fluids were positive for type A AIV using AGID.

CONFIRMATION

Gene analysis: Real time pcr.(M gene) This test indicate that all isolates were belong to the influenza type A viruses Real time pcr.(H5 gene) and amino acid sequencing.

This test indicate that the subtype of heamagglutinin is H5 and the amino acid sequence around the cleavage site is PQGEGRRKKRGLFGAIA(HPAI).

One step RT PCR (N1 gene): The test showing that the subtype of N gene is N1gene.

DISCUSSION

Outbreakes of HPAI (H5N1) were occurred in allover the world Timm and Ortrud, 2006.Inspite of the largest chickens population in Sudan a few studies on AI were done.

In the present studies, a field investigation from (2003-2006) in different location in Sudan indicated that AIV were not isolated until early 2006.In addition,virus isolate from different outbreaks in Khartoum and ElGazeera,with clear clinical signs which included;drop in egg production,yellowish diarrhea, frothy fluid from mouth lachrymation, nasal discharge,oedema, cynosis of the comb and wattle, oedema in the head, haemorrhages of the leg shanks and feet, ruffled feathers, recombination and death such as mentioned by OIE Manual (2005), David (2000).

The development of direct tests such as antigen detection (The flu detect test strip) which we used in this assy is easy and rapid for diagnosis of avian influenza viruses within 15 min. The disadvantage is that it may lack sensitivity (OIE Manual, 2005).

Virus isolation in egg embryo to identify the virus is more sensitive and very usefull technique for the diagnosis of viral infections when used with clinical

specimens of good quality (WHO manual, 2002). AIV is isolated by inoculation of the samples into 9-11 days embryo. Eggs died within 2 days PI and this agree with finding by Timm and Ortrud, 2006. who stated that eggs inoculated with HPAI die within 48 h. Also all samples were found positive in virus isolation (IZSVe) FAO\OIE reference lab.RTPCR technique was used to detect influenza type A and H5 gene and PCR for N1 gene,sequence analysis of the amino acid around the cleavage site is PQGEGRRKKGLFGAIA (HPAI) and this indicated that the isolates is HPAI according to presense of many alkaline amino acid at the cleavage site and this finding of Wenhuakang *et al.* (2006), OIE Manual (2005).

This study was the first report of isolation of highly pathogenic AI subtype H5N1 in Sudan.

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