

Molecular Detection and Histopathological Evaluation of Naturally Occurring HPAI Virus in Chicken in Sudan

Wegdan H. A. *, Khair S. A. M., Zakia A. M.

Veterinary Research Institute (VRI), Khartoum, Sudan

Abstract

During the epidemic of highly pathogenic avian influenza (HPAI) virus in 2006 several outbreaks of severe fatal diseases with 100% mortality and morbidity occurred in some poultry farms in Khartoum and Elgazera States, Sudan. The present study deals with the molecular and histopathological evaluation of these outbreaks. The most striking gross and histopathological changes were vasculitis, severe generalized systemic haemorrhages, and congestion. Mild to severe diffuse inflammatory reaction and necrosis were detected in brain, visceral organs and subcutaneous tissue of skin of combs and wattles. Virological results indicate that allantoic fluids of the inoculated samples were positive for type A avian influenza. Allantoic fluids sent to OIE, FAO (the National Reference Laboratory for Newcastle Disease and Avian Influenza, Padova, Italy) for confirmation. The isolates were analysed using reverse transcriptase-polymerase chain reaction (RT-PCR), real-time PCR, and the amino acid sequence of the H5 gene is positive to highly pathogenic AI subtype H5N1. In conclusion: The results obtained confirmed the presence of highly pathogenic AI virus subtype H5N1 in Sudan.

Keywords

Avian Influenza, H5N1 Molecular, Histopathology, Domestic Fowl, Sudan

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1. Introduction

Highly pathogenic avian influenza (HPAI), previously known as Fowl plague (Shinya *et al.*, 2010) induces an acute generalized fatal infectious disease. It represents a threat to poultry industry worldwide and is a public health hazard. The disease is caused by avian influenza virus (AIV) type A which belongs to the family *Orthomyxoviridae* of RNA virus (Pringle, 1998). These viruses are classified into sub-types according to their haemagglutinin (HI-H16) and neuraminidase (N1-N9) (OIE, 2015). Based on virulence, the AIVs are classified into two groups, highly virulent viruses which cause 100% mortality and designated as Highly pathogenic avian influenza (HPAI) viruses and Low pathogenic avian influenza (LPAI) viruses that cause a mild

respiratory infection (Alexander, 2000). HPAI due to H5N1 first occurred in poultry in Hong Kong in three chicken farms during March to May, 1997 and re-emerged in November (Shortridge, 1999). Since 1999, the number of outbreaks that has occurred worldwide increased significantly (Capua and Alexander, 2004). The virus has spread through trade of poultry or migratory birds (CIDRAP 2008). Since 2003, H5N1 has killed or forced the culling of more than 400 million domestic poultry and caused an estimated US\$20 billion in economic damage across the globe before it was eliminated from most of the 63 countries infected at its peak in 2006. The H5N1 HPAI virus remains endemic in few countries. The global number of H5N1 outbreaks dropped from 2003 to mid-2008, increased again from mid-2008 to mid-2011, and has dropped since then. (FAO, 2012).

* Corresponding author

E-mail address: wegdanhassan@hotmail.com (Wegdan H. A.)

The lack of in –depth data of the pathogenesis and the pathology of naturally occurring HPAI in chickens have stimulated the current investigation focusing on the clinical-pathological aspects of this disease in naturally affected chickens during outbreaks in 2006 in the Sudan

2. Materials and Methods

2.1. Case History

Twenty –four affected chickens (ages 12-32 weeks, with 100% morbidity and mortality rate of 100% in some farms) were obtained from layers flocks which were in the process of being depopulated because they had been declared to be infected with HPAI virus by the veterinary authority of the Ministry of Animal Resources and Fisheries, on the basis of history of mortality and clinical-pathological changes. The main clinical signs observed were dullness, ruffled feather, respiratory signs, swelling, cyanosis, haemorrhages, lacrimation and frothy discharge from the mouth. Tissue samples (organs) and swabs (tracheal and cloacal) were collected from eight farms; three (designated A, C and D) were from Khartoum North three from Khartoum (B, E and H) and two farms from Elgazera State (F and G).

2.2. Pathological Methods

At necropsy, specimens including brain, nasal passages, trachea, lung, kidney, liver, intestine, combs and wattles were collected from each chicken and fixed in 10% formalin, processed by conventional methods, for paraffin embedding. 5-6 μ m sections were cut and stained with haematoxylin & eosin (H & E).

2.3. Virological Methods

The presence of HPAI virus was determined using standard virological techniques, in brief:

Nine to eleven day old chicken embryonating chicken eggs were inoculated via the allantoic sac with 0.2 ml of tissue samples and swabs suspension. Eggs were examined daily by candling to detect embryo mortality. Four days post inoculation (PI) the allantoic fluid (AF) and CAMs were collected aseptically and stored at -20°C until used. For virus identification HA, HAI and AGID tests were performed as described by Thayer and Beard (1998).

2.4. Antigens and Antisera

Reference AI virus agar gel immunodiffusion (AGID) antigens and antisera to AI type A and sub-type H5 and ND virus (HA) antigens and antisera (Deventer- The Netherlands) kindly supplied by Detasi-company- Khartoum were used

2.5. Molecular Detection for AI Viruses

Allantoic fluids of eight samples A; B; C; D; E; F; G and H were sent to FAO-OIE National Reference Laboratory for Newcastle and AI (IZSVe) Italy for virus confirmation.

3. Results

3.1. Gross and Histopathological Changes

Grossly, the most common lesions were subcutaneous oedema, haemorrhages and congestion in skin of combs, wattles, periorbital and legs (hocks and shanks). Other gross lesions included haemorrhages and congestion in the mucosa of nares, oral cavity and trachea, petechiation, in abdominal fat, serosal surfaces of visceral organs, peritoneum and ecchymosed haemorrhages in the gizzard proventriculus junction. Livers and kidneys were enlarged and fragile; the lungs were congested.

The histopathological changes were consistent with the gross lesions. The most prominent changes were severe generalized haemorrhages, congestion and vasculitis which evidenced by swelling vaculation and denudation of lining endothelial cells (Figure 1). Other lesions compromising mild to severe multi-organ inflammation which was more marked in the brain, respiratory tract and subcutaneous tissue of the head. The brain displayed congestion of blood vessels, haemorrhages, and local to scatter gliosis, aggregation of lymphoid cells in subventriculus area and occasionally formed cuffing around the blood vessels (Figure 2). Neuronal and chromatolysis were seen in cerebral hemisphere and cerebellum folia. The choroid plexus revealed oedema, lymphoid cells (Figure 3) infiltration and extravasations of erythrocytes. The most marked histopathological change in the nasal sinuses, nasopharynx, trachea and bronchia were thickening, vacuolation, necrosis and denudation of the epithelial cells lining the mucous membrane and the associated mucosal glands. There were oedema and intense infiltration of mononuclear cells the majority of which were lymphoid cells occasionally of lymphoid follicles in the lamina propria and sub mucosa.

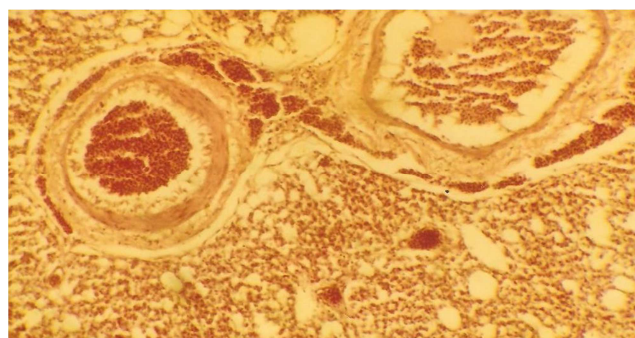


Figure 1. Lung. Note haemorrhages around blood vessels (H & Ex 10).

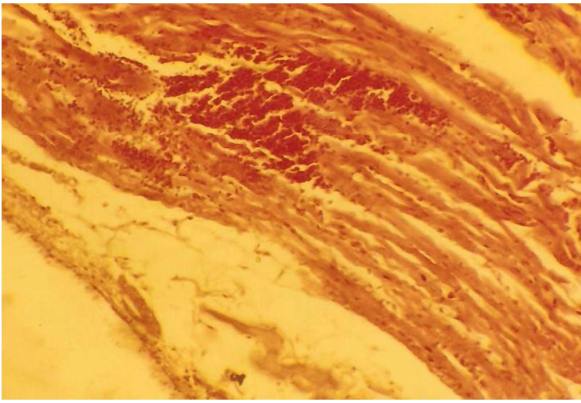


Figure 2. Heart. Note haemorrhage H & Ex10.

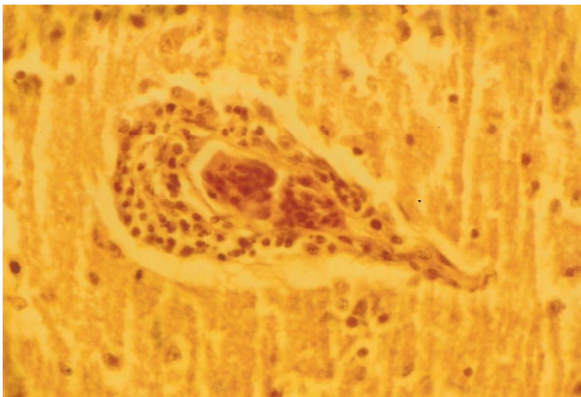


Figure 3. Brain. Note lymphoid cuffing. (H & Ex 10).

Extravasated erythrocytes were present in the lamina propria, sub mucosa and muscularis mucosa (Figure 4).

Sections of lung revealed congestion and a severe haemorrhage which was more evident around the blood vessels. Intense and diffuse effusion of lymphoid cells was seen in the sub-epithelial area of secondary and tertiary bronchi and around the respiratory atria. The lining epithelial of bronchi was hyperplastic and necrotic, in some areas there was complete obliteration of the normal lung tissue which was affected by lymphoid cells which was effaced by lymphoid cells (Figure 5).

Section of heart presented only extravasated erythrocytes between the cardiac muscles. (Figure 6).

The kidneys displayed congestion and haemorrhages especially around the blood vessels, degeneration and necrosis of some tubules and glomeruli. Many glomeruli showed segmentation, collapse and dilation of bowman's space.

Histopathological changes of the livers included swelling and vacuolation of hepatocytes, with collapsed sinusoids and infiltration of mononuclear cells in the periportal area and between the degenerated hepatocytes. The intestine showed necrosis and detachment of lining epithelium and accumulation of mononucleous cells infiltrating the

connective tissue lamina propria and sub-mucosa.

Skin of the combs and wattles revealed severe haemorrhage and congestion. The epidermal epithelial cells were vacuolated, necrotic and occasionally formed a micovesicle which was filled with necrotic tissue debris and extravasated erythrocytes.

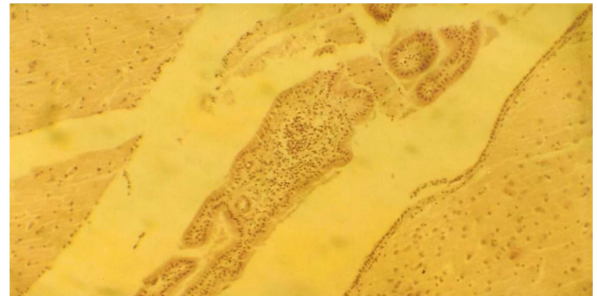


Figure 4. Brain. Note effusion of mononuclear cells in choroid plexus. (H & Ex 40).

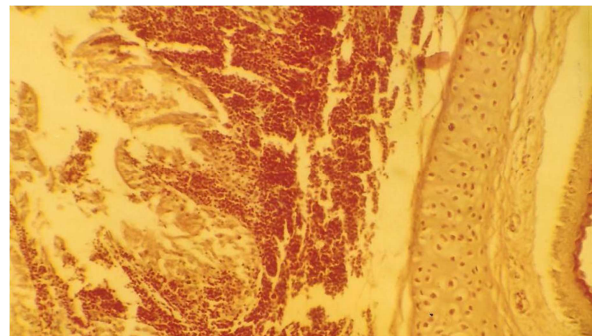


Figure 5. Nasal sinuses. Note, desquamation of lining epithelium and accumulation of extravasated erythrocytes (H & Ex 10).

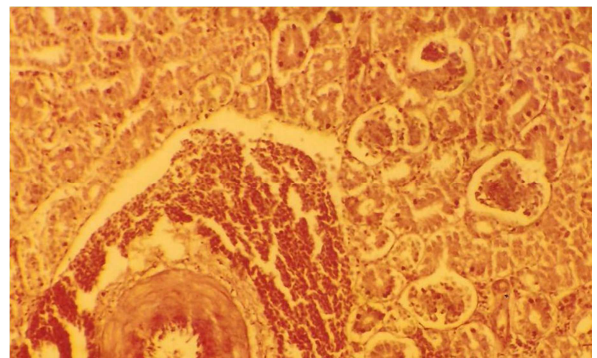


Figure 6. Kidney. Note, congestion and haemorrhage around blood vessel, tubular and glomerular necrosis. H & Ex 10.

3.2. Virological Results

The six samples designated A, B, C, D, E and F caused death of the embryos within 48 hours post inoculation . The allantoic fluids collected from these samples agglutinated chicken RBCs.

The HI results were negative when tested against standard reference ND antisera. AGID test, showed clear precipitation lines between the references AI antisera (type A and H5) in

the control wells and harvested CAM and AF in the peripheral wells after 48 hours of incubation.

3.3. Gene Analysis

Real time PCR for influenza type A (M gene) and H5 gene:

The M and H5 genes were detected in the allantoic fluids collected from the 6 samples, indicated that all isolates belonged to the influenza type A viruses subtype H5.

One step PCR for N1 gene and H5 gene:

The test indicated that the subtype of N and H genes are N1 and H5 gene; samples G and H were negative. (Figure. 7&8).

- *Nucleotide sequencing:*

The amino acid sequence of the H5 gene at the cleavage site was PQGEGRRKKRGLFGAIA (HPAI).

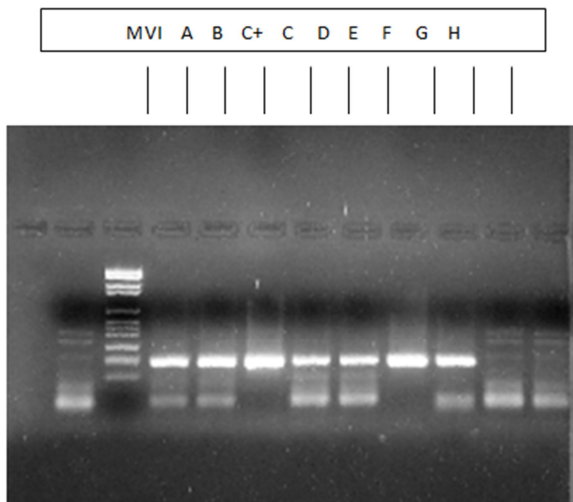


Figure 7. Gel N1 Sudan 26-05-2006 (Allantoic fluid).

Lane MV1 is the molecular marker, lane A, C and D allantoic fluids from Khartoum North, lane B, E and H allantoic fluids from Khartoum, lane F and G allantoic fluids from AlGazera, lane C+ is positive control

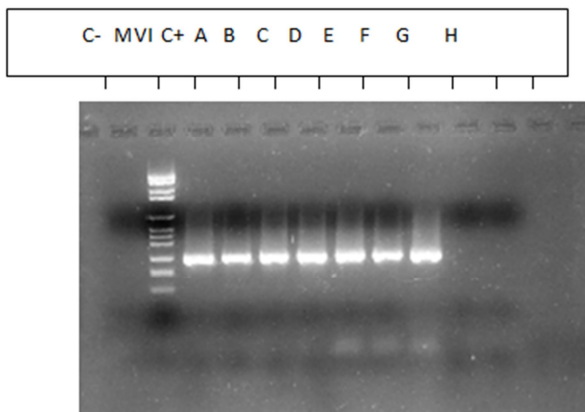


Figure 8. Gel H5 Sudan 26-05-2006 (Allantoic fluid).

Lane C- is negative control, Lane MV1 is the molecular marker, lane A, C and D allantoic fluids from Khartoum North, lane B, E and H allantoic fluids from Khartoum, lane F and G allantoic fluids from AlGazera, lane C+ is positive control.

4. Discussion

The highly pathogenic H5N1 avian influenza strain was responsible for the current epidemic (2005-2006) in which the virus has spread across Asia into Middle East, Europe and Africa (Salzberg *et al* 2007). Field investigation conducted during the period 2003-2006 in different locations in the Sudan indicated that AIV (H5N1) was not prevalent until early 2006. The main clinical signs observed in the present outbreaks are oedema, haemorrhages, and cyanosis of combs, wattles, periorbital and legs (hock and shank) are typical signs for HPAI infection in chickens (Acland, *et al* 1984, Kobayashi, *et al*, 1996; Swayne, 2000; Swayne and Halvorson, 2003; OIE, 2014 and Capua and Terregino, 2009). The gross and histopathological changes observed in the current investigation were similar to those reported previously (Mo, *et al*, 1997; Swayne and Halvorson, 2003; EuCouncil Director, 2005; and Capua and Terregino, 2009).

Oedema, haemorrhages, congestion and vasculitis in the aforementioned organs were suggestive for circulatory disturbance. This is in agreement with previous investigators (Brown, *et al* 1992; Kobayashi *et al*, 1996; Suarez *et al*, 1998) who have shown, using immunohistochemistry method, the presence of HPAI virus ribonucleoprotein in the endothelial cells throughout the body of chickens in the early stage of infection when using immunohistochemistry method and thereafter, the virus antigens were observed in perivascular parenchymal cells. This suggested that, the viral replication in the endothelial cells and its dissemination occurred following or during viraemia. Viral ribonucleoprotein immunoreactivity demonstrated generalized infection in the skin, brain, brain stem, thoracic and lumbar spinal cord and all visceral organs of large-billed crows experimentally infected with AI virus A/chickens/Indonesia/2003 (H5N1) (Tanimura *et al*, 2006). Other authors, observed the viral antigen in the acinar cells of pancreas and brain of pigs experimentally inoculated with AI virus A/chicken/Indonesia/2003/H5N1 (Klopfleisch *et al*, 2006) and Pekin ducks infected with AI virus A/Duck/Vietnam/12/05/H5N1 (Vascellari *et al*, 2007). Etiologically, HPAI viruses are restricted only to those of subtypes H5 and H7 which code for a furin-sensitive cleavage site in their haemagglutinin protein and therefore are capable of inducing systemic infection (Rott, 1992).

Virus isolation in egg embryos is a more sensitive and useful

technique for the diagnosis of viral infection when used with clinical specimens of good quality (WHO, 2002). In the present study, AIV was isolated by inoculation into 9-11 days embryo eggs via allantoic sac.; embryos died within 2 days PI. This agreed with the findings reported by Harder and Werner (2006) who stated that, eggs inoculated with HPAI died within 48h. The allantoic fluids harvested from infected embryos agglutinated chicken RBCs. Agglutination was inhibited by positive control serum prepared against AI but was not by hyperimmune serum against ND, indicated AI viruses. The isolates were further confirmed as influenza virus type A by AGID test which is a reliable test (Swayne *et al.*, 1998; OIE, 2015) and sub-typing indicated that all isolates were positive to AI subtype H5.

Since the conventional methods, such as virus isolation (VI), are usually slow and depend on the viability of the virus in the sample, many molecular- based techniques have been developed for diagnosis of AI (Spackman *et al.*, 2003). Real-time reverse transcriptase PCR (RRT-PCR) has several advantages including speed and elimination of the possibility of cross contamination of new samples with previously amplified products because the sample tube is never opened after PCR. In addition, the RRT-PCR product is detected with a sequence-specific probe and there is a guarantee that the correct target was amplified which reduces the chances for false positives. VI detects viable virus, whereas RRT-PCR detects intact viral RNA (Spackman *et al.*, 2002; Suriani *et al.*, 2004). In this study, RRT-PCR was used to detect influenza type A and H5 gene from extracted virus RNAs. In another investigation by Spackman *et al.* (2002) RRT-PCR was developed to detect type A and the avian H5 and H7 haemagglutinin subtypes. This assay provides a rapid and feasible alternate to VI in embryonating chicken eggs and subtyping by HAI as a flock or live bird market screening tool.

RT-PCR assay was used to determine haemagglutinin sub-type H5 and neuraminidase sub-type N1 from extracted virus RNA. Sequencing including the complete open reading frame (ORF) of the HA and NA gene segments of AIVs using the Big Dye terminator V3.1 cycle sequencing kit. Gene sequence analysis of the amino acid at the cleavage site indicated that the isolates are HPAI based on the presence of many alkaline amino acids. This finding is in agreement with OIE (2015). The high pathogenicity of H5 and H7 is associated with the presence of multiple basic amino acids (arginine or lysine) at the cleavage site of the haemagglutinin. In another investigation (Salzberg *et al.*, 2007) reported that influenza (H5N1) viruses isolated in Europe, the Middle East and Africa show a close relationship, despite the fact that they were collected from widely distant geographic regions, including Cote-d'Ivoire,

Nigeria, Niger, Sudan, Egypt, Afghanistan, Iran, Slovenia, Croatia and Italy. The shared lineage of the viruses suggests a single genetic source for introduction of influenza (H5N1) into Western Europe and northern and western Africa and their analysis places this source most recently in either Russia or Qinghai province in China.

5. In Conclusion

The present work has confirmed the presence of highly pathogenic AI subtype H5N1 in Sudan. Therefore it is recommended that further studies should be carried out on epidemiology of the disease with aim to control it because of its great economic and public health impacts.

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