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Original article

Etiology and pathology of epidemic outbreaks of avian influenza H5N1 infection in Egyptian chicken farms

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Abstract

Epidemic outbreaks of avian influenza (AI) virus H5N1 have been frequently reported in Egypt during the last nine years. Here we investigate the involvement of AI H5N1 in outbreaks of acute respiratory disease that occurred in several commercial chicken farms in Egypt in 2011, and we describe to the pathology caused by the virus in the course of the outbreak.

Twenty-one chicken farms with history of acute respiratory symptoms and high mortalities were screened for AI H5N1. Virus identification was based on hemagglutination inhibition test, and PCR detection and sequencing of the hemagglutinin and neuraminidase genes. Virus distribution was determined by immunohistochemical staining of AI antigens in organs of infected birds. Standard H&E staining was performed for histological examination of affected organs.

Eighty-one % of the examined birds, representing 100% of the screened farms, were positive for AI H5N1 virus. Phylogenetic analysis of the hemagglutinin and neuraminidase genes of the isolated virus reveals its affiliation to clade 2.2.1. Viral antigens were localized in the endothelial cells of the heart, liver, lungs and skin, where pathological lesions including congestion, hemorrhages, multifocal inflammation and necrosis were concurrently observed. According to the pattern of the viral antigen and lesion distribution in the visceral organs, we suggest cardiovascular and circulatory failures as the probable cause of death during these outbreaks. In conclusion, the present study further confirms the epidemic status of AI H5N1 virus in Egypt and reveals the highly pathogenic nature of the local isolates.

Key words: avian influenza, H5N1, pathology

Introduction

Highly pathogenic avian influenza (AI) virus of the subtype H5N1 is a contagious pathogen that causes severe respiratory disease with high mortality in poultry. The H5N1 subtype has first emerged in China in 1996 and is currently circulating in Asia, Europe and Africa (Xu et al. 1999, Chen et al. 2005). In Egypt, the first outbreak of AI H5N1 was reported in 2006 across more than 20 governorates and resulted in high mortalities and severe economic losses to the poultry industry (Aly et al. 2006). Despite the control efforts of the Egyptian health authorities, which included culling of more than 34 million birds and the mandatory application of a vaccination program, the virus has become enzootic in the country (Abdelwhab et al. 2010). Thus, during the last 8 years, outbreaks of AI H5N1 infection have been occasionally reported in Egypt (Abdelwhab et al. 2010, Arafa et al. 2012).

Despite the enzootic presence of such a highly pathogenic subtype of AI in Egypt, the pathology caused by the local virus isolates in commercial chicken has not been investigated. Furthermore, the previous reports describing the pathology of AI H5N1 elsewhere are based on experimental infections rather than natural outbreaks (Acland et al. 1984, Nakatani et al. 2005, van Riel et al. 2009). In the present study, we identified AI H5N1 as the causative pathogen responsible for outbreaks of severe acute respiratory disease in Egyptian chicken farms during 2011. Additionally, we describe the pathology caused by the virus in the course of the outbreak.

Materials and Methods

Virus surveillance and sampling

Twenty-one commercial chicken farms (14 broiler and 7 layer farms), located in the Delta territory in north Egypt, were screened for AI H5N1 in 2011. These farms experienced severe respiratory symptoms including nasal and ocular discharges, dyspnea, coughing, sneezing, in addition to signs of reduced growth, huddling, diarrhea, and swelling of the head, comb and wattles. These clinical symptoms were accompanied with high mortality rates, which reached up to 80%. A total number of 107 freshly dead chickens were collected and examined in the course of this study. For AI virus detection, tracheal and cloacal swabs or tissue samples from trachea, lung, liver, spleen, pancreas, heart and skin were collected from examined birds, kept in sterile phosphate buffered

saline (PBS) containing streptomycin (100 µg/ml), fungizon (2 µg/ml) and pencillin (100 IU/ml), and stored at -20°C. After thawing, swabs and tissue specimens were vigorously suspended in the PBS and centrifuged thereafter. The resultant supernatants were filter-sterilized and either stored at -80°C or directly used for virus detection.

Influenza virus propagation and identification

Supernatants obtained from swabs or tissue samples were inoculated into the allantoic sac (three embryos per sample) of 11-day-old pathogen-free embryonated-chicken-eggs with an inoculum of 0.2 ml per egg. Four days after inoculation, the amnio-allantoic fluids were harvested and tested by hemagglutination (HA) and hemagglutination inhibition (HI) tests according to the standard protocols of the World Health Organization for detection of viral particles using chicken erythrocytes (World Health Organization 2002). H5N1-specific hyperimmune antisera were kindly provided by The Veterinary Serum and Vaccine Research Institute, Abbassia, Egypt.

Total RNA was extracted from 140 µl of allantoic fluid using the GeneJET™ RNA Purification Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions. The extracted RNA was subjected to reverse transcriptase-PCR using sequence-specific primers targeting the hemagglutinin (HA) (Rashid et al. 2009) and neuraminidase (NA) (Wright et al. 1995) genes of the H5N1 subtype as previously described. The amplification was carried out using the One-Step RT-PCR kit (Qiagen, Valencia, CA) in Eppendorff thermal cycler (Eppendorf, Hamburg, Germany). The reaction consisted of cDNA synthesis at 50°C for 30 min, an initial denaturation at 95°C for 2 min, then 40 cycles of amplification; each consisted of a denaturation step at 95°C for 20 sec, an annealing step at 50°C for 30 sec, an extension step at 72°C for 60 sec, followed by a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining.

The possible presence of some other prevalent non-AI respiratory pathogens in swabs or tissue samples of examined birds was tested by PCR as described elsewhere (Roussan et al. 2008). These pathogens included infectious bronchitis virus, Newcastle disease virus, avian pneumovirus and *Mycoplasma gallisepticum*, which might manifest clinical and/or pathological features similar to those of AI (Roussan et al. 2008).

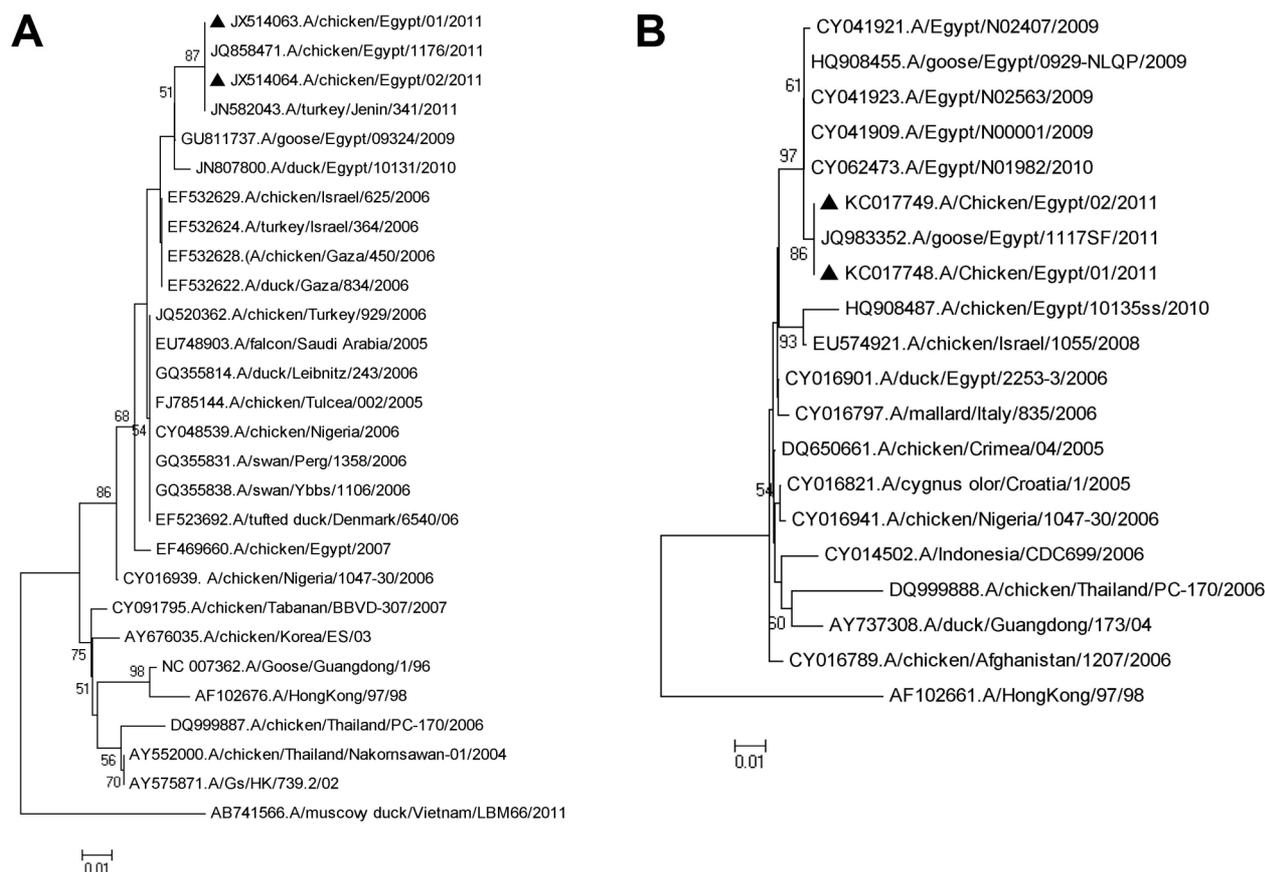


Fig. 1. Phylogenetic tree of isolated AI virus based on partial nucleotide sequences of hemagglutinin glycoprotein (A) or neuraminidase glycoprotein (B) genes of AI virus. The trees are constructed with multiple alignments of 456- and 615-base nucleotide sequences of HA and NA genes, respectively using Neighborhood Joining method in MEGA5.0 with bootstrap values calculated for 1000 replicates. Sequences of the isolates from this study are marked with solid triangles.

Nucleotide sequencing and genetic identification

Nucleotide sequences of the PCR-amplified H5 and N1 genes were determined using a BigDye Terminator Kit (Applied Biosystems, Foster, Canada) on a 3130 Genetic Analyzer (Applied Biosystems, Foster, Canada). The forward and reverse nucleotide sequences were aligned with the published H and N gene sequences using MEGA 5.0 software by Clustal W method. Consequently, a phylogenetic tree was created using Neighbor-Joining method (1000 replicates for bootstrap).

Immunohistochemistry and histopathology

Tissue specimens from skeletal muscles, liver, heart, spleen, trachea, lung, intestine, pancreas, skin, proventriculus, gizzard, kidney, thymus, bursa of Fabricius and brain were collected from ten AI-positive birds. These specimens were fixed in 10% neutral

buffered formalin and paraffin sections of 3-5 μ m thickness were prepared from each tissue. For immunohistochemistry, an initial step of antigen retrieval was carried out by adding proteinase K enzyme solution to the sections for 10 min, followed by addition of 3% H_2O_2 for 15 min to block the endogenous peroxidase. Goat serum was added at a 1:10 dilution and incubated for 15 min to block nonspecific binding sites. Subsequently, slides were incubated for 45 min with a 1:200 dilution of mouse monoclonal antibody, specific to influenza A virus nucleoprotein (Meridian Life Science Inc., Saco, USA, clone: B1740M). Slides from RT-PCR positive cases of swine influenza virus were used as a positive control and slides incubated with mouse IgG were used as negative control. Immunoreactivity was detected using 3-Amino-9-Ethylcarbazole and the slides were counterstained with Mayer's hematoxylin. For histopathological analysis, sections were stained with the standard H&E staining (Vollmer et al. 1983).

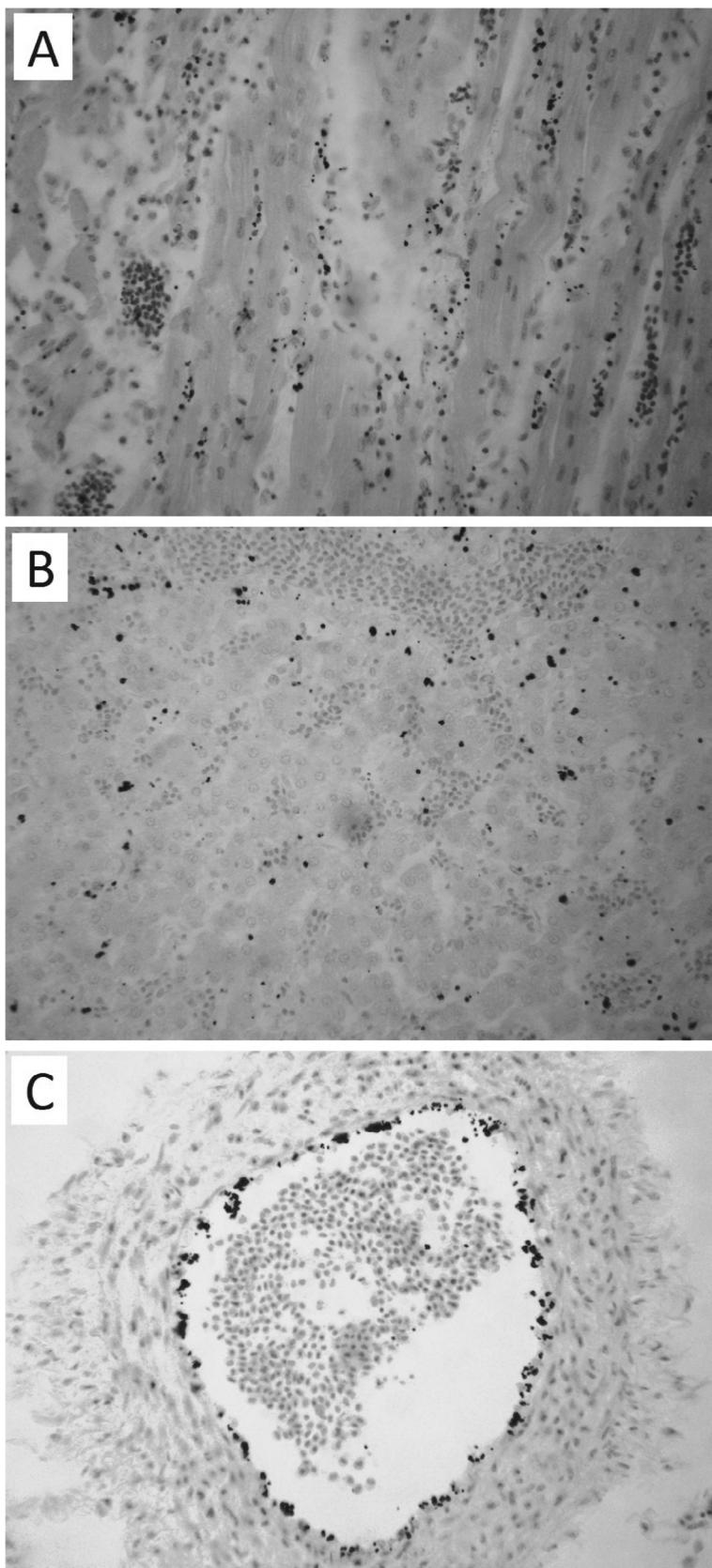


Fig. 2. Organ distribution and antigen localization of AI H5N1 virus in infected birds. The virus antigens were observed in the parenchymal myofibers and the capillary endothelium of the heart (A), the hepatocytes and the sinusoidal endothelium of the liver (B), and the pulmonary endothelium of the lungs (C). Immunoreactivity was detected using 3-Amino-9-Ethylcarbazole and the tissues were counterstained with Mayer's hematoxylin. Original magnifications are x40 for A and B, and x60 for C.

Results

Virological Surveillance

Eighty-seven chickens, representing 81% of the total examined chickens, and 100% of the total screened farms, were positive for AI virus as confirmed by HA and HI assays and by RT-PCR showing specific HA and NA bands. Nucleotide sequencing of the amplified HA and NA genes showed that the virus isolates from all the infected birds clustered into two isolates, which were designated KC017748/Egypt/Chicken/01/2011 and KC017749.A/Egypt/Chicken/02/2011. Sequencing data obtained from these two isolates were submitted to NCBI GenBank with the following accession numbers: JX514063 and JX514064 for the H5 gene, respectively, and KC017748 and KC017749 for the N1 gene, respectively. The phylogenetic analysis of the identified H5 and N1 were performed for 456 and 615 bp truncated sequences by neighbor-joining analysis with bootstrapping (1000), respectively. H5 and N1 gene sequences of the isolated viruses showed homology ranging between 94-100% and 99.7-100% with the published sequences of the homologous genes of the AI H5N1 virus subtype, respectively. Additionally, phylogenetic analysis of the identified H5 and N1 gene sequences shows the affiliation of the H5N1 isolates to the genetic clade 2.2.1 (Fig. 1A and 1B, respectively). Analysis of the amino acid sequences adjacent to the HA cleavage site reveal the presence of multiple basic amino acids ($^{HA1-6}KRRKRR/GLF^{HA2+3}$), which is a molecular marker for the high pathogenicity of avian influenza H5N1 viruses. Only five of the examined birds were positive for one or more of the prevalent non-AI respiratory pathogens, three of which were concurrently positive to AI virus. None of the birds with concurrent infections was selected for the subsequent pathological investigations.

Gross pathology

The examined birds showed the presence of petechial and ecchymotic hemorrhage and congestion in visceral organs, severe subcutaneous hemorrhage, edema in head and neck with hemorrhagic spots on the leg shanks. Bursa of Fabricius and thymus appeared to be atrophied with pale discoloration. Severe swelling was observed in kidneys, spleen and pancreas with the presence of diversified hemorrhagic and necrotic foci in the latter. In few cases, there were hemorrhagic spots on the proventriculus and ventriculus.

Virus distribution and antigen localization

AI virus antigens were detected in the trachea, lungs, heart, liver and skin, mainly in the endothelial cells and in alliance with the degenerative changes and/or peripheral to the necrotic zone. In the majority of the examined specimens, viral antigens were disseminated in the endothelium and in between the cardiac fibers (Fig. 2A), in between hepatocytes and within hepatic sinusoids (Fig. 2B), in pulmonary endothelium (Fig. 2C) and in the dermis of the wattle. Interestingly, no viral antigens were detected in the spleen, intestine, proventriculus, gizzard, bursa of Fabricius, and thymus.

Histopathology

The organs in which the AI viral antigens were detected were further subjected to histological examination. In the trachea, there was desquamation of the lining epithelium with extensive hemorrhage, congestion and lymphocytes, macrophages, plasma cells and heterophils infiltration in the lamina propria (Fig. 3A). In lungs, heterophilic interstitial pneumonia consisted of thickening of the interlobular septa with inflammatory cells infiltration and edema was found. The cytoplasm of the pneumocyte of the air capillaries was laden with hemosidrin pigment. In the liver, hepatocytic necrosis was evident, besides infiltration of heterophils, lymphocytes and macrophages in the sinusoids and portal areas (Fig. 3B). Additionally, congestion of the hepatic sinusoids and parenchymatous hemorrhage and hemosiderosis were observed (Fig. 3C), together with desquamation of the bile duct epithelium. In the heart, there was severe necrosis and destruction of the myocardium with high infiltration of heterophils and macrophages in between the destructed myocytes and in the epicardium (Fig. 3D). Skin of the comb and wattles markedly showed severe cellulitis, which was associated with congestion, edema and mild heterophilic infiltration in the dermis and subcutis.

Discussion

Since the first epidemic outbreak of AI H5N1 virus infection in poultry was reported in Egypt in 2006, the virus has become enzootic in the country (Abdelwhab et al. 2010, Arafa et al. 2012). In the present study we report the involvement of AI H5N1 virus in outbreaks of severe acute respiratory disease, which occurred in many commercial chicken farms in Egypt during 2011. Thus, our report further highlights

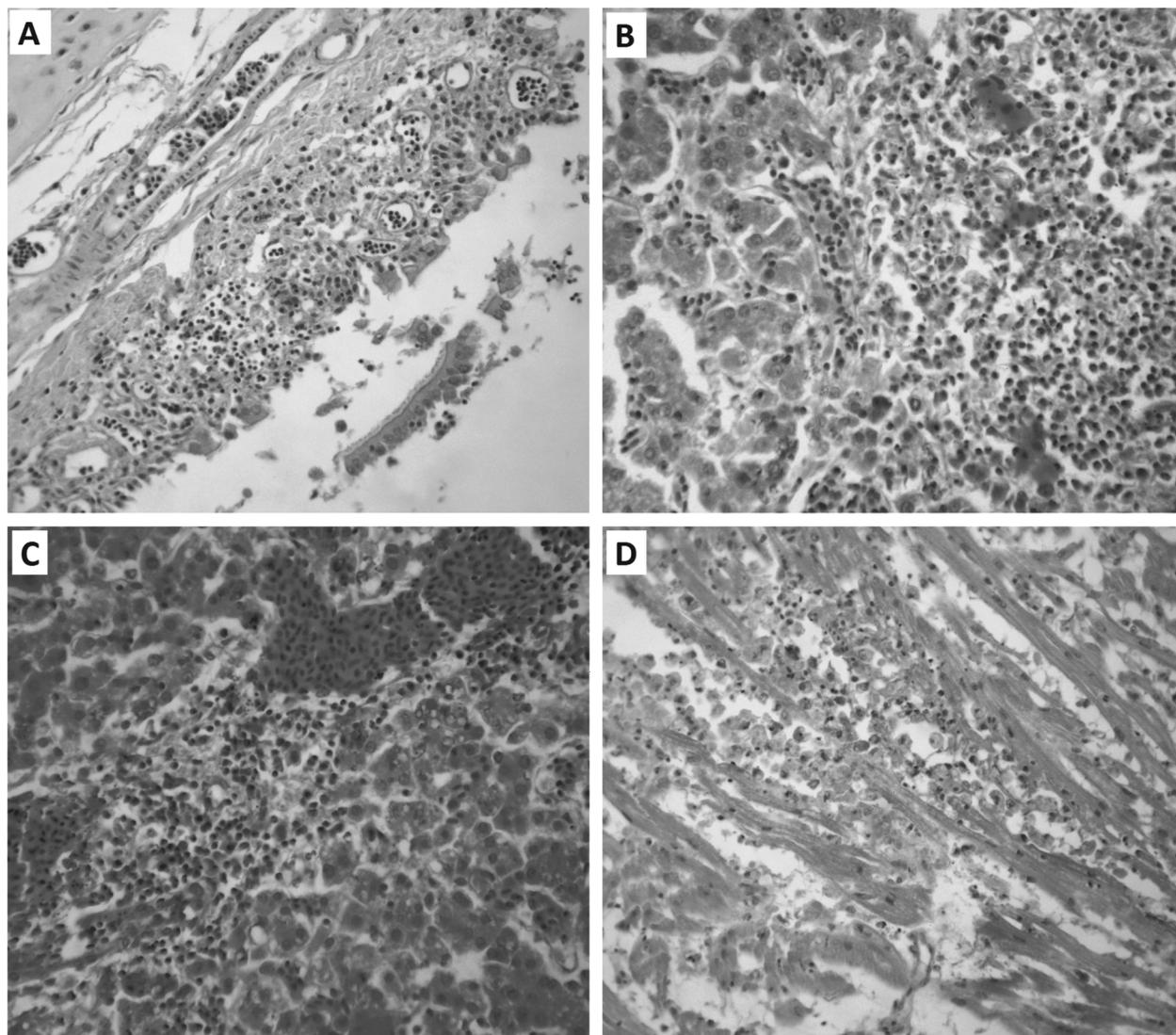


Fig. 3. Histopathological picture of the organs positive for AI H5N1 antigens. Trachea (A): Partial desquamation of the tracheal epithelium with increasing capillary bed with leukocytic infiltration in the lamina propria. Liver: parenchymatous necrosis and infiltration of lymphocytes and macrophages (B) and severe peri-sinusoidal hemorrhage and hemosiderosis (C). Heart (D): Severe necrosis in the myocardial fibers with leukocytic infiltration and edema. Slides stained with standard H&E staining. Original magnification is x60 for all subfigures.

the reemerging risks on public health, animal welfare and the poultry industry due to AI H5N1 infection. Additionally, in this study we evaluated the pathology caused by the virus in infected chicken during the course of the outbreak. Studying the pathology of AI viruses in naturally infected birds can be beneficial for assessing the virus transmission routes and identifying the causes of mortality (Nakatani et al. 2005). However, limited reports are available to describe the pathology of natural infection with highly pathogenic AI H5N1 in chickens, which are the most industrially common poultry species (Acland et al. 1984 and Nakatani et al. 2005).

The virus was isolated and identified in tissues and tracheal swabs of about 81% of the examined birds.

The molecular identification of the virus was based on the amplification of the HA and NA genes using RT-PCR and gene sequencing. In agreement with the report of Brojer et al. (2009), we found that RT-PCR is more sensitive to the detection of AI H5N1 in biological samples and tissues (data not shown). Phylogenetic analysis of the HA and NA gene sequences showed the affiliation of the isolated AI H5N1 virus to clade 2.2.1. The latter is the classical clade that has been reported during the first outbreak of AI H5N1 in Egypt in 2006, and continued to cause disease outbreaks throughout the country (for a comprehensive review, see Arafa et al. 2012). Noteworthy are the other variants of this classical types, which have also been associated with AI outbreaks in Egypt,

namely clade 2.2.1.1 which was first reported in 2007 (Arafa et al. 2012), and clade 2.2.1.2 which was recently reported in 2015 (Arafa et al. 2015).

The H5N1 subtype of AI viruses is known for its high pathogenicity, and therefore, is commonly known as highly pathogenic AI. Senne et al. have previously found a relationship between the presence of multiple basic amino acids adjacent to the HA protein cleavage site and the aggravated pathogenicity of AI H5N1 (Senne et al. 1996). Amino acid sequence analysis showed the presence of such a putative pathogenicity marker in our AI H5N1 isolate. This finding certainly coincides with the acute clinical picture and the high mortality observed in the investigated chickens as well as to the presence of various pathological lesions in vital systemic organs, such as the heart and lungs, which altogether indicate the high virulence of the AI H5N1 virus isolate.

The infected birds showed a typical clinical picture of acute influenza infection, which as previously described, includes signs of respiratory distress, edema, swelling and hemorrhages on the head, neck and leg shanks, and necrotic foci and cyanosis of the comb and wattles (Kobayashi et al. 1996, Pantin-Jackwood and Swayne 2009). At the organ level, gross lesions including petechial and ecchymotic hemorrhage were mostly observed, while the histological lesions mainly included leukocytic infiltration, necrosis and tissue degeneration, hemorrhage and vascular damage. In accordance with the histopathological picture, AI viral antigens were mostly localized in the endothelial cells of these systemic organs, which may affect the integrity of the blood vessels and cause hemorrhage in these organs. Thus, in light of these findings we suggest the cardiovascular failure as the most probable cause of death during these outbreaks. Although other organs such as the spleen, the proventriculus, the intestine, and the bursa of Fabricius showed lesions of discoloration, atrophy, necrosis and petechial hemorrhage (data not shown), we are not in favor of attributing them to the AI infection, since no viral antigens were detected in these organs. In previous studies that investigated the pathology of AI viruses during natural infection, a dichotomy of the virus antigen localization and tissue injury was frequently reported (Brojer et al. 2009, van Riel et al. 2009). The authors attributed this dichotomy to the inadequacy of virus antigens for immunohistochemical staining in necrosed tissue or to the effect of another pathogen. Similarly, in our study we did not detect AI antigens in necrosed tissues, albeit the antigens were detectable in the surrounding viable tissue, which indicates the presence of the virus in the examined organ. Additionally, in our investigation we found no evidence for the presence of concurrent in-

fections with other respiratory pathogens in tissues of the examined birds. Although this further supports the association of AI H5N1 to the lesions in the tissues where AI antigens were concurrently detected, we do not fully preclude the involvement of other pathogens that specifically target the bursa of Fabricius or the gastrointestinal tract. After all, the pathological lesions described in our study are consistent with those previously reported in natural cases of AI H5N1 infection in chicken (Acland et al. 1984, Nakatani et al. 2005).

Conclusion

In the present study, we report the association of AI H5N1, but not other prevalent respiratory pathogens, to outbreaks of respiratory disease that occurred in several Egyptian chicken farms in 2011. The phylogenetic identification of the virus isolate reveals its affiliation to the genetic clade 2.2.1. Thus, this study, together with those previously done by others, corroborates the enzootic status of this AI H5N1 subtype in Egypt. Our findings on the virus distribution in the systemic organs of infected birds reveal a wide tissue tropism, which involved vital organs such as the heart and lungs. Additionally, we clearly observed a preferential localization of the virus in and around the vascular endothelium. Consistently, the pathological picture of the affected tissues and organs included congestion, hemorrhage and necrosis. Altogether, these findings reveal the highly pathogenic nature of the isolated H5N1 virus, while the cause of death appears to be a result of the cardiovascular and circulatory failure.

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Author contributions

All authors have substantially contributed to the conception and design of the study, as well as the acquisition, analysis and interpretation of the data. Bakr Ahmed has additionally written the manuscript and composed the figures. All authors have approved the final version of the manuscript.

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