RESEARCH ARTICLE

Acute Outbreak of Co-Infection of Fowl Pox and Infectious Laryngotracheitis Viruses in Chicken in Egypt

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ABSTRACT

The current study reports an outbreak of a high mortality within an 86 days old chicken flock. The birds showed severe respiratory manifestations associated with obstruction of the upper tracheal tube with diphtheritic lesions. Collected tracheal samples were examined using direct electron microscopy (EM), polymerase chain reaction (PCR), virus isolation in embryonated chicken eggs (ECE) and histopathological examination. Electron microscopy findings revealed intracytoplasmic dumbbell-shaped FPV particles within fat inclusions. Histolopathologically, the tracheal sections revealed fibrinous tracheitis, associated with presence of intracytoplasmic eosinophilic inclusion bodies (Bollinger bodies). Virus isolation showed generalized thickening with white opaque pock lesions on the Chorioallantoic membranes (CAMs) of the inoculated eggs. PCR results were positive for Fowl Pox virus (FPV) and Infectious Laryngotracheitis virus (ILTV). In conclusion, dual infection of both FPV and ILTV viruses should be taken in consideration in fibrinous tracheitis lesions. Moreover, PCR was sensitive in the detection of ILTV virus than histopathology and electron microscopy.

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INTRODUCTION

Fowl pox (FP) is an infectious viral disease of domestic and wild birds characterized by mild to severe lesions with significant levels of morbidity and mortality (Zhao *et al*., 2014). Fowl pox virus (FPV) belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Avipoxvirus* which is a large oval-shaped enveloped virus. The viral particle measures 270×350 nm and is composed of an electron dense, centrally located core with two lateral bodies (Weli and Tryland, 2011). The viral genome consists of double stranded DNA ranging in size from 260 to 365 kb (Diallo *et al*., 2010). The genes located within the central region are conserved among poxviruses and are involved in the basic replication mechanisms. In contrast, the terminally located genes encode proteins involved in host range restriction (Tulman *et al*., 2004).

The disease is primarily found in two main forms, cutaneous and diphtheritic forms. The cutaneous form is usually mild and characterized by multifocal cutaneous pock lesions consisting of epithelial hyperplasia producing wart-like projections mostly on the non-feathered skin such as legs, feet, eyelids and the base of the beak (Tripathy *et al*., 1997). The diphtheritic (wet) form is characterized by sneezing, gasping, head-shaking and fibrous necrotic proliferative lesions on the mucous membranes of the upper respiratory tracts as well as the mouth and esophagus leading to relatively high mortality rates usually resulting from asphyxiation (Eo *et al*., 2011).

FPV replicates easily in the cytoplasm of infected avian cells producing a characteristic cytopathic effect (CPE) within 4 to 6 days post infection represented with proliferative pock lesions on the chorioallantoic membrane (CAM) of embryonated chicken eggs (Weli and Tryland, 2011). Transmission of the virus can occur commonly through biting insects such as mosquitoes and mites (Kane *et al*., 2012), or by direct methods such as ingestion of contaminated food or water (Niemeyer *et al*., 2013), or via respiratory aerosols or by contact with affected birds or contaminated surfaces as perches and nests (Ruiz-Martı́nez *et al*., 2016).

Infectious laryngotracheitis virus (ILTV, gallid herpes virus 1) causes an acute upper respiratory tract disease in chickens. Severe epizootic forms of ILT show a great respiratory distress, gasping, expectoration of bloody mucus, and high mortalities (Hidalgo, 2003; Ali *et al*., 2015).

Although, FPV and ILTV are members of two different viruses families, there is a great resemblance of the clinical and postmortem signs of both diseases (Tadese *et al.*, 2007). In addition ,natural dual infection with both FPV and ILTV were reported among chicken flocks (Fatunmbi *et al.*, 1995; Davidson *et al.*, 2015).

Herein, we investigated a severe outbreak of wet form of diphtheritic or membranous tracheitis. We reported a case of FPV mixed with ILTV infection in a commercial chicken flock which was clinically diagnosed as FPV-infected and with high mortality rates. The affected flock had not been vaccinated against FPV or ILTV.

MATERIALS AND METHODS

Source and collection of samples: On January 2016, an acute outbreak of the wet form of fowl pox was recorded in an 86-days-old commercial chicken flock in Kafrelsheikh province, in the northern part of the Nile Delta of Egypt. Forty six tracheal samples were fixed in 10% neutral buffer formalin for histopathological examination. Specimens taken for viral isolation were immediately stored at -80°C.

Histopathology: After fixation in formalin, the samples were dehydrated in ascending series of ethanol, cleared in xylene and embedded in paraffin wax. The paraffin-embedded specimens were sectioned into 4-μm samples for hematoxylin and eosin staining.

Electron microscopy: Tracheal samples were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 24 h at room temperature. Several changes were applied overnight with 0.1 M sodium phosphate buffer, PH 7.4 at 4ºC. Then, the samples were post-fixed in 2% osmium tetraoxide in 0.1 M sodium phosphate buffer. Specimens were dehydrated in ascending grades of cold ethanol and propylene oxide, and then embedded in Spurr's resin. Ultrathin sections of 50 nm thickness were prepared and double stained with 3% aqueous uranyl acetate for 10 minutes in lead citrate. Samples were then observed under a JEM, 100CXII electron microscope, Assiut University.

Virus isolation: Tracheal samples were pooled and minced in PBS and the resulting 10% suspensions were centrifuged at 1000 x g for 10 min at 4°C. The supernatants were treated with (2000 IU of penicillin and 10 mg of streptomycin/ml) stock solution. Then 0.2 ml of tissue suspensions were inoculated into the CAMs of 10-days-old SPF eggs (Tripathy and Reed, 1998). The eggs were incubated at 37°C for 5-7 days and checked daily for abnormalities.

DNA extraction and PCR amplification: DNA extraction was done according to the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Briefly, 200 μl of the samples suspension, 10 μl of proteinase K and 200 μl of lysis buffer were incubated at 56°C for 10 min. Then, 200 μl of absolute ethanol was added to the lysate. The DNA was eluted with 100 μl of elution buffer and stored at -20°C.

PCR was conducted in an applied biosystem 2720 thermal cycler using 2 sets of primers (Table 1). The cycling conditions were similar in both viruses unless the annealing temperature of ILTV was 52°C for 1 min. Commercial FPV vaccine and ILT (LT-IVAX. Shering-Plough, USA) vaccines were used as control positive. A negative control containing only PCR master mix, primers and PCR grade water was also included.

Purification and sequencing of PCR products: The 578-bp PCR product of FPV 4b gene was purified using QIA quick PCR product extraction kit (Qiagen, Valencia) and sequenced in an automated sequencer (Applied Biosystems 3130 genetic analyzer) (HITACHI, Japan) using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer).

Alignment and phylogenetic analysis: Resulting sequences were analyzed using the nucleotide Basic Local Alignment Sequence Tools (BLASTn) using reference GenBank sequences. The phylogenetic tree was analyzed by the neighbor-joining (N-J) tree method using MEGA version 6 software.

RESULTS

Clinical and gross findings: The affected birds were suffering from severe asphyxia and gasping manifestations. High mortality rates (up to 68%) were recorded. The trachea of normal examined birds showed normal mucosal lining (Fig. 1A), while the affected birds demonstrated severe fibrino-necrotic obstructive lesions mostly within the upper third of tracheal tube associated with occasional hemorrhage within the tracheal tube (Fig. 1B).

Histopathology: The trachea of normal bird showed normal lining epithelial layer consisted of ciliated pseudostratified squamous epithelium (Fig. 2A). While, oropharynx, larynx and upper third of trachea of the infected birds revealed severe thickening of the mucosal layer associated with hyperplasia of the lining epithelium, which is covered by fibrino-necrotic sheet and are mixed with many bacterial colonies (Fig. 2B). The lining epithelia of the glands were markedly vacuolated with characteristic large intracytoplasmic eosinophilic inclusion bodies (Bollinger bodies) (Fig. 2C). The submucosa revealed hemorrhage and severe inflammatory reaction consisted mainly from heterophilic infiltration. The lower tracheal portion demonstrated erosion, ulcer of the mucosal layer and severe hemorrhagic tracheitis.

Table 1: Primer sequences, target genes and amplicon sizes used in this study

Target gene	Primer sequences	Amplified segment	Reference
4b core protein gene of FPV	CAGCAGGTGCTAAACAACAA CGGTAGCCTTAACGCCGAATA	578 bp	Prukner-Radovcic *et al.*, 2006
Thymidine Kinase (TK) gene of ILTV	ACGATGACTCCGACTTTC CGTTGGAGGTAGGTGGTA	647 bp	Pang *et al.*, 2002
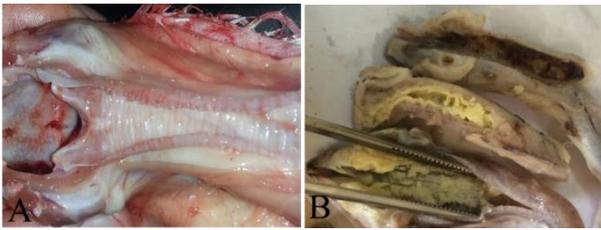
Fig. 1: (A) Trachea of normal bird (B).Trachea of infected bird showing severe obstruction of the airways with fibrino-necrotic sheet.
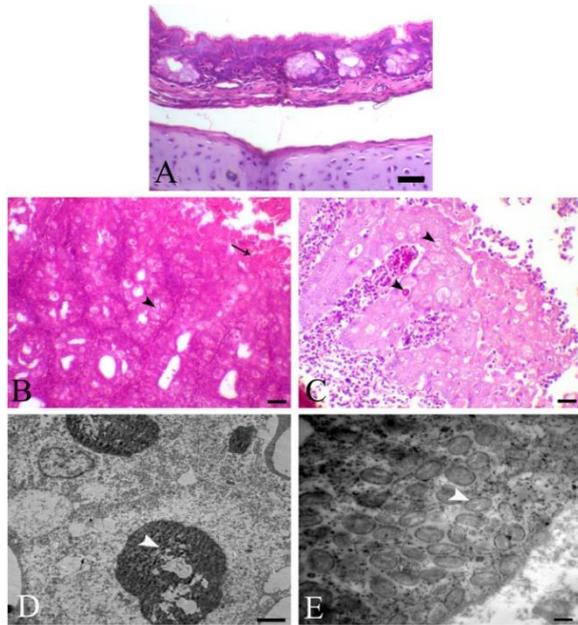
Fig. 2: (A) Histological section of normal bird showing normal covering epithelium upon the tracheal cartilgenous ring. (B). Trachea of infected bird showed severe obstruction of the airways with fibrino-necrotic sheet. Trachea of the upper tracheal portion showed hyperplasia of the mucosal layer covered with fibrinous membrane mixed with bacterial colonies (arrow), arrowhead indicates intracytoplasmic Bollinger's bodies, H&E, bar = 50 µm. (C). The middle tracheal portion revealed mucosal hyperplasia with presence the cytoplasmic inclusion bodies, H&E, bar = 25 µm. (D). EM showing presence of fat inclusion laden with virus particles, bar = 2 µm. (E). Characteristic dumbbell-shaped virus particle bar = 500 nm.
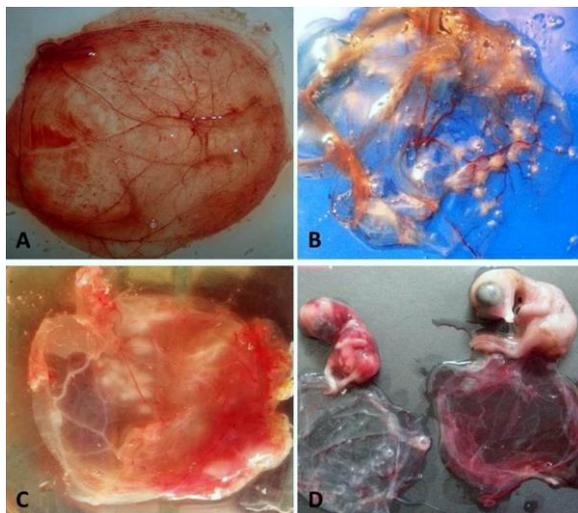
Fig. 3: (A) CAM of a normal non infected chicken embryo (B). CAM of ECE inoculated with tracheal homogenate collected from infected chicken showing white opaque pock lesion 4 days post infection after 1st passage. (C). CAM shows thickening and white pock lesions 7 days post infection after 1st passage. (D). Normal non infected 17-days-old chicken embryo (right), congested and stunted infected embryo (left).
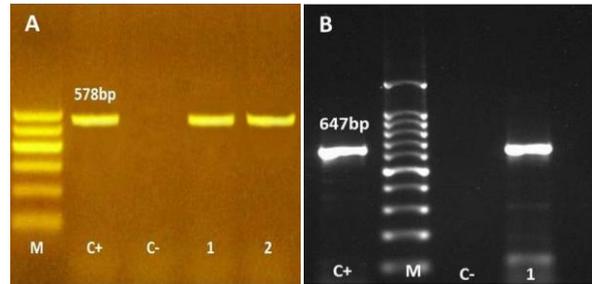
Fig. 4: (**A**) PCR amplification of 578 bp of the FPV 4b gene from infected field samples. Lane M; 100bp DNA size marker. Lane C$^+$; Control positive DNA extracted from FPV vaccine. Lane C-; Negative control. Lanes 1 showed positive amplification of 4b gene from tracheal homogenate of infected cases. Lanes 2 showed positive amplification of 4b gene from infected CAMs. (**B**). PCR amplification of 647bp of the ILTV TK gene from infected field samples. Lane M; 100bp DNA size marker. Lane C$^+$; Control positive DNA extracted from ILTV vaccine. Lane C-; Negative control. Lane 1 showed positive amplification of TK gene from tracheal homogenate of infected cases.
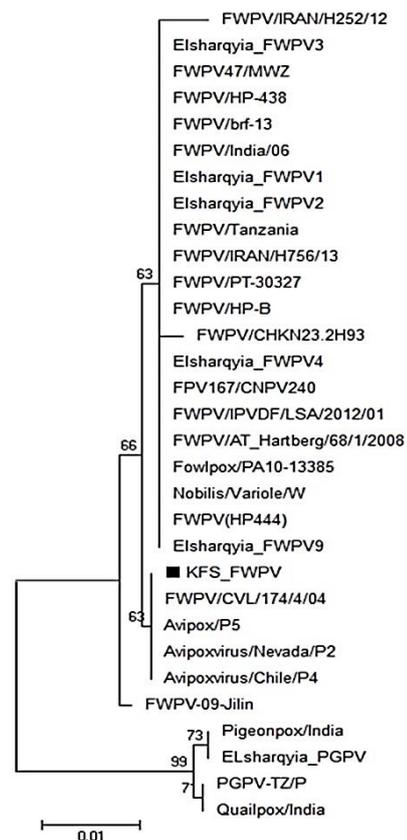
Fig. 5: Phylogenetic anal$^+$ysis of KFS_FWPV isolated from Kafrelsheikh province, Egypt, based on 4b gene nucleotide sequences of previously published sequences. Phylogenetic tree was constructed via multiple alignments of 578 bp nucleotide sequence and analyzed using N-J analysis with bootstrapping (1000).

Transmission electron microscopy: Virus particles were seen within lipid droplets (Fig. 2D). There were numerous characteristic dumbbell-shaped FPV particles (Fig. 2E). ILTV particles couldn't be detected by EM.

Virus isolation: In comparison with normal CAM of non-infected egg (Fig. 3A), diffuse thickening associated with the presence of white opaque pock lesions on the CAMs of the inoculated eggs on the 4th (Fig. 3B) and 7th days post infection (Fig. 3C) and the embryos showed severe congestion (Fig. 3D).

PCR and sequencing: Tracheal suspensions and the infected CAMs were positive for the expected amplicon size of 578 bp corresponding to a portion of the major core protein (4b) gene of the FPV (Fig. 4A). The amplicon was sequenced. The obtained 4b gene sequence was submitted to GenBank (accession no. KU947037). ILTV was also co-detected by amplification of (TK) gene with an expected amplicon size of 647 bp (Fig. 4B).

Alignment and phylogenetic analysis: Analysis of the nucleotide sequence of the 4b gene amplicon of our field isolate KFS_FWPV (GenBank KU947037) was illustrated in (Fig. 5), which revealed 90-99% sequence identity to most of Avipox viruses isolated from different countries. Our isolate showed 99% sequence homology with five Egyptian FPV isolated in 2013 (GenBank accession numbers JX464820, JX464821, JX464819, JQ665838, and KJ809101); 2 Tanzanian isolates (GenBank accession numbers KF722858 and KF722860); FPV from Asian and Europian countries. On the other hand, KFS_FPV showed 90% nucleotide sequence identity to the Egyptian pigeon pox virus (Elsharqyia_PGPV), the Tanzanian (PGPV-TZ/P) and the Indian (Pigeonpox India) (GenBank accession numbers JQ665840, KJ913659 and DQ873811 respectively). Phylogenetic analysis revealed that our isolate was also closely related to the FWPV/CVL/174/4/04 strain (Gen Bank AM050377), Avipox/P5 strain (pheasants) (GenBank KC017964), Avipoxvirus/Nevada/P2 strain (Domestic turkey) (GenBank KC017961) and Avipoxvirus/Chile/P4 strain (Parrot) (GenBank KC017963).

DISCUSSION

We report an acute outbreak of severe respiratory symptoms associated with high mortality rate in a commercial chicken flock aged 86-days-old. Post mortem examination of the affected chickens revealed caseous plugs occluding the trachea which is suggestive for both FPV and ILT. Fowl pox particularly the virulent wet form is characterized by diphtheritic lesions which might be easily confused with ILT. Therefore it is strongly recommended in cases of respiratory diseases as well as severe decrease in the egg production in chickens (Hafez, 2010) to exclude both of these diseases (Fatunmbi et al., 1995).

FPV infection is mostly associated with low mortality rate in chickens, but high mortality rates up to 65–100% were also recorded (Abdallah and Hassanin, 2013). The main causes of the uncommon high mortality rates might be attributed to mixed infection of the APV with other viruses such as avian papillomavirus (Williams et al., 2014), chicken infectious anemia virus (Islam et al., 2012), ILTV (Fatunmbi et al., 1995; Davidson et al., 2015) or in rare cases due to contamination of FPV vaccines with other viruses such as Reticuloendotheliosis virus (Zhao et al., 2012).

Histologically, there was fibrinous tracheitis associated with presence of intracytoplasmic inclusion bodies. Unfortunately, intranuclear inclusion bodies of ILTV couldn't be detected in the tracheal section which is in consistent with (Fatunmbi et al., 1997), who suggested that the detection of intranuclear inclusion bodies of ILTV in the trachea of affected chickens mostly occurs within the early stage of infection before necrosis and desquamation of the epithelial cells takes place. Also, the inclusion bodies are present only in the early five days of infection then they disappear as the cellular damage progresses (VanderKop, 1993). Hidalgo (2003) demonstrated that diagnosis of ILT based on detection of intranuclear inclusion bodies in tissues has been shown to be less sensitive than other techniques.

Intracytoplasmic inclusion bodies (Bollinger bodies) are histologically confirmative of an avian poxvirus infection (Mubarak and Mahmoud, 2000; Manarolla et al., 2010; Williams et al., 2014), however other confirmatory techniques such as EM and PCR should be applied to conserve a clear diagnosis of the disease (Abdel-Moneim et al., 2014).

Direct EM examination of tracheal specimens showed only FPV particles. Failure of detection of ILT viral particles in the examined samples is in accordance with Hughes and Jones (1988) who observed that detection of ILTV by EM is successful only when large numbers (minimum titer of 3.5 log10) of virus particles are present in clinical samples.

Isolation of infectious viruses via the CAMs of ECE results in characteristic white pock lesions by the 1st egg passage which is characteristic for both FPV and ILTV (Diallo et al., 2010). In addition it suggests that the viruses are well adapted to chicken embryos (Manarolla et al., 2010; Abdallah and Hassanin, 2013). However some strains of Avipox viruses (APV) do not grow well on chicken embryos (Weli and Tryland 2011).

Molecular identification of the isolated viruses was done using 2 sets of primers which specifically amplify a 578 bp DNA fragment of 4b gene and a 647 bp DNA fragment of TK gene of the FPV and ILTV respectively. PCR results were positive for both genes, which indicate a natural co-infection of FPV and ILTV in the infected chicken flock.

Nucleotide sequence alignment of a 578 bp 4b gene of our isolate revealed a close relationship (99%) with most of FPVs recorded in the GenBank, which indicates that the P4b gene is highly conserved among FPV isolates (Masola et al., 2015; Henriques et al., 2016). Phylogenetic tree on the basis of the nucleotide sequences of 4b gene with corresponding reference sequences showed that our FPV isolate clustered in the same branch with other FPVs and APV from other species as pheasant, domestic turkey and parrot.

PCR was the most sensitive technique than histopathological and EM findings for detection of both FPV and ILTV viruses in the examined samples. FPV particles usually present within fat inclusion which be easily observed by either histopathology or with electron microscopy. While ILTV was barely detected even through ultrastructure section, consistent with that reported by Williams et al. (1994) who isolated ILT virus only from 19 samples of total 58 PCR positive samples. On the other hand, PCR results are usually available within few hours than routine virus isolation and histopathological techniques.

Even so avian pox is endemic in the Egypt particularly among chickens, turkey and pigeons (Abdallah and Hassanin, 2013), but there is no available literature describing this severe dual infection of wet FPV and ILTV.

Conclusions: We report the diagnosis of a naturally occurring dual FPV and ILTV infection in chickens in the Delta of Egypt. Also, mixed infections of both viruses should be taken together especially in diphtheritic tracheal lesions.

Authors contribution: Walied Abdo, designed the study, performed histopathological and EM examination, wrote the paper, Asmaa Magouz, designed the study, performed virus isolation, PCR, phylogenetic analysis, and wrote the paper. Fares El Khayat, designed the study, collected the Tissue samples, performed virus isolation and wrote the paper. Tarek Kamal designed the study, collected the tissue samples, performed PCR and wrote the paper. All authors revised the manuscript, and approved the final version to be published.

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