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RESEARCH

# Isolation of H5 Highly Pathogenic Avian Influenza Virus from Cattle Egret *(Bubulcus ibis)* near Affected Broiler Chicken Flocks in Egypt

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## ABSTRACT

**Background*:*** The role of free living birds in the spread and epidemiology of H5N1 Highly pathogenic avian influenza is often neglected. Cattle egret is one of the free-living birds that is often present near commercial chicken farms with a possibility of catching avian viral infections specially H5N1 AIV; this is augmented by broken biosecurity which is often in small and medium sized poultry production facilities.

**Objectives:** This study investigates the possible isolation of H5N1 AIV from cattle egrets and its role in the spread and epidemiology of AIV in Egypt.

**Methods:** rapid test strip for detecting avian influenza antigen was used to test 15 tracheal pool samples collected from 15 commercial broiler flocks showing rapid increase in mortality, respiratory and enteric manifestation in Desouk and Qallin, Kafr-elshiekh Province, Egypt from 2014 to 2015. Another 60 swab samples (oropharyngeal as well as cloacal swabs) were collected from 30 cattle egrets near these flocks. Trials for isolation of AIV was conducted on collected samples via allantoic sac inoculation in 10 days old specific pathogen free (SPF) embryonated chicken eggs (ECEs) followed by hemagglutination (HA) and hemagglutination inhibition (HI) testing of allantoic fluids using specific polyclonal antisera against AIV H5 and H9 and confirmation by RT-PCR.

**Results:** The rapid field test revealed detection of AIV in one broiler flock (6.67 %). Isolation revealed 2/15 (13.3 %) AIV H5 isolates from broiler chicken flock samples, whereas 1/60 (1.67 %) AIV H5 isolate from cattle egret samples. Two positive AIV H5 (cattle egret isolate and one broiler chicken isolate) were selected for partial sequencing of HA. Phylogenetic analysis, that revealed clustering with Egyptian highly pathogenic avian influenza virus isolates from 2011-2015 in clade 2.2.1.2. Sequences were submitted to NCBI GenBank with accession numbers KT454807 for chicken H5 sequence and KT454808 for cattle egret H5 sequence.

**Conclusion*:*** Isolation of HPAIV H5 from cattle egret shed the light on the possible epidemiological role of this resident wild bird in transmission of AIV to susceptible chicken. Further detailed analysis is required to detect the complete antigenic/ genetic characteristics of cattle egret H5 influenza isolate.

**Keywords:** broiler chickens, cattle egret, Egypt, Highly Pathogenic Avian influenza virus, HI, RT-PCR, Egypt.

## BACKGROUND

Egypt is home to more than 480 species of resident and migrant bird species (Crewe *et al.*, 2016). Wild birds are usually regarded as visible indicators of diverse and healthy environments. From a public health perspective, this positive view is not always valid (Jones, 2005) as they can carry a wide range of viral, bacterial, fungal and protozoan pathogens harmful to poultry or other vertebrates including human. These wild birds either being themselves diseased or being seemingly healthy carriers, or hosts of infected vectors (Hubálek, 2004).

Infection of domestic poultry by avian influenza viruses (AIVs) typically produces syndromes ranging from asymptomatic infection to respiratory disease and drops in egg production to severe systemic disease with up to 100% mortality, in addition to economic impact

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that can occur as an outcome of the restrictions and embargoes placed on infected countries (Easterday *et al.,* 1997; Swayne and Suarez, 2000).

Avian Influenza (AI) virus is the only species in the genus influenza virus A, which is single-stranded, anti-sense, segmented RNA virus belonging to the family *Orthomyxoviridae* (Cox *et al.* 2000 and ICTV, 2012). Avian influenza virus genome encodes for at least 10 known proteins from which only HA (segment 4 encoded protein, Hemagglutinin) and NA (segment 6 encoded protein, Neuraminidase) are exposed on the outer viral surface (Webster *et al.,* 1992). AIVs have 18 different hemagglutinins (H1 to H18) and 11 different neuraminidases (N1 to N11) (Tong *et al*., 2012 and 2013). Over 118 combinations of AIVs can be isolated from wild birds except H17N10 and H18N11 which were recently isolated from New World bats (Tong *et al.,* 2012; Tong *et al.,* 2013; and Swayne *et al.,* 2013).

Standard diagnosis of AI is carried out by isolation and characterization of the virus (OIE, 2015). During recent years, the outbreak of Asian H5N1 avian influenza virus that has subsequently spread to Russia, Middle East, Europe and Africa, has put an increased focus on the role of wild birds as reservoir of HPAI viruses (Normile, 2006; Olsen *et al.,* 2006; Capua and Alexander, 2007). Prevalence of AIV in wild birds has been investigated in Egypt by many studies; Saad *et al.,* (2007); Soliman *et al*. (2012) and El-Zoghby *et al.,* (2013). Their studies focused on migratory wild birds rather than resident wild birds. The aim of the present work was to investigate the role of cattle egrets in H5 AIV epidemiology as one of the most common resident wild birds specially those which may live in vicinity of AI infected chicken farms.

## MATERIALS AND METHODS

**Rapid field detection of avian influenza type A In broiler chicken flocks:**

Immuno-chromatography kits (Synbiotics, FLU DETECT$^{TM}$) were used according to the manufacturer's instructions for detection of AI antigen in 15 oropharyngeal swab pools collected from 15 broiler chicken flocks belonging to Desouk and Qallin (Kafr el-Sheikh Governorate), Egypt during the period from 2014 to 2015. These flocks exhibited rapid increase in mortality rate and/or sharp decrease in feed consumption with respiratory, digestive and nervous manifestation, in addition to some birds in one flock exhibited subcutaneous hemorrhages and edema of the shanks and toes. From the fifteen flocks 12 flocks were vaccinated with inactivated AIV H5 vaccine.

**Samples:**

Aseptically 15 tracheal tissue pool samples were collected from freshly dead and euthanized moribund chickens from all AI field tested flocks in addition to 60 swab samples (30 oropharyngeal and 30 cloacal swabs) were collected from 30 apparently normal cattle egrets *(Bubulcus Ibis)* captured by nets in the area surrounding the affected farms. Tissue samples were grinded in antibiotic and antifungal 5X diluent, pH 7.2 (each 10ml contained 9.5 ml sterile phosphate buffered saline (PBS) + 0.5 ml antibiotic and antifungal 100X LONZA® (OIE, 2015). The swab samples were inoculated in cryovials containing viral transport media each 100ml contained: 89 ml PBS + 10 ml Tryptose soya broth + 0.5 ml antibiotic and antifungal 100X LONZA® + 0.5 ml Fetal bovine serum albumin with final pH 7.2 (Lennette, 1995). All samples were clarified by centrifugation at 3000 rpm for 20 minutes then the supernatants were collected and stored at -80 $^{o}$C until used (OIE, 2015).

**Virus isolation:**

Sample supernatants were inoculated into the allantoic cavity of 10 days old SPF ECEs, 0.2 ml/egg (OIE, 2015). Eggs were incubated at 37 $^{O}$C with daily candling for 4 days. Eggs showed embryonic deaths before 24 hours of inoculation were discarded and considered non-specific. Eggs showed embryonic death after 24 hours and/or remained alive up to 4 days were chilled. Allantoic fluid was harvested and tested for HA using 10% and 1 % (V/V) chicken RBCs (OIE, 2015). The HA negative samples were passaged one further time in SPF eggs, and the HA titer of the allantoic fluid was determined (OIE, 2015).

**Virus identification:**

**Haemagglutination inhibition (HI):**

Harvested allantoic fluids were tested by HI using specific polyclonal antisera against AIV (subtype H5 and H9), 96 well V-bottomed HA microtiter plate, 1 % (V/V) chicken RBCs, PBS, control HA-positive AIV H5 antigen and negative control (non-infected allantoic fluid) (OIE, 2015).

**RNA extraction and RT-PCR:**

Viral RNA was extracted from 140 µl positive HA-allantoic fluid samples as well as from positive control AIV H5 and negative control (non-infected allantoic fluid) using QIAamp Viral RNA Mini Kit (Qiagen, Germany) and the extract was subjected to one step RT-PCR using Verso™ 1-step RT-PCR Kit (Thremo Scientific, USA). Oligo nucleotide primers VH5F: 5'-GAT TGT AGT GTA GCY GGA TGG-3' and VH5R: 5'- CTT GTC TGC TCT KCM KCA TC -3' for amplification of 406 nt from HA gene (Hussein *et al*., 2015) were used. RT-PCR reaction mixture was adjusted to 25 µl as recommended by the kit manual instructions. Each tube was centrifuged for 10-20 seconds to remove any drops in the tubes lids and then transferred to thermal cycler adjusted as showed in table (1). Amplified PCR products were visualized in Ethidium bromide stained 1.2% agarose gel along with 100 bp molecular size marker (Vivantis ™) and documented using ultraviolet trans-illuminator.

Table (1): Thermo profile for amplification of HA gene fragment of AIV H5

| Step | Temperature | Time | Number of cycles |
|---|---|---|---|
| Reverse transcription | 50 ºC | 15 minutes | 1cycle |
| Initial denaturation | 94 ºC | 5 minutes | 1cycle |
| Denaturation | 94 ºC | 30 seconds | 40 cycles |
| Primer annealing | 51 ºC | 30 seconds | |
| Extension | 72 ºC | 45 seconds | |
| Final extension | 72 ºC | 10 minutes | 1cycle |
| Cooling | 4 ºC | ∞ | --------- |

**Sequencing:**

Purified PCR products were sequenced in both orientations by the di-deoxy chain-termination method using the same primers of amplification. Gene sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) at MACROGEN, Korea using 3730XL DNA sequencer. The obtained sequences were subjected to BLAST analysis then sequences were imported into BioEdit version 7.0.4.1. Comparative analysis of (HA) gene sequences of AIVs were carried out and compared with the available

sequences using the National Center for Biotechnology Information (NCBI). Sequence editing, correction, frame adjustment, Amino acid alignment and allocation of antigenic sites were also conducted using different options of BioEdit version 7.0.4.1 software. All finely adjusted sequences were exported from BioEdit version 7.0.4.1 as separate FASTA files. FASTA files were inserted into MEGA program version (7.0.18) DNA alignment tool and exported into MEGA format (*.meg). MEGA file was used as a base for phylogenetic analysis and the evolutionary history was inferred using the neighbor-joining method and the reliability of each tree branch was estimated by performing 1000 bootstrap replicates (Tamura *et al.*, 2007).

## RESULTS and DISCUSION

In this study, fifteen broiler chicken flocks were examined by immuno-chromatography tests in the field (Fig 1) revealed detection of AIV antigen in one broiler flock representing, 6.67 % (1/15) of the total tested flocks, while isolation and identification by HI and RT-PCR (Fig. 2) resulted in two isolates of AI H5 from two broiler chicken flocks 13.3 % (2/15). Trials for isolation of AIV H9 were not successful.

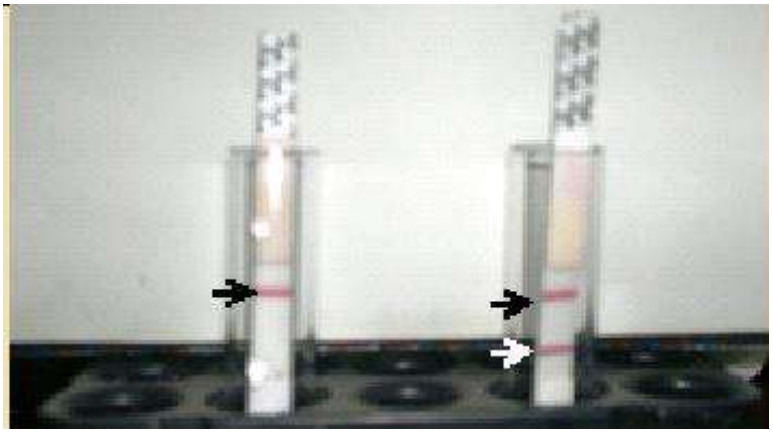

**Fig. 1:** Rapid immune-chromatography test strips. Positive AIV *(*right arrows refer to two pink bands control line and test line) and Negative AIV (arrow refers to one upper pink band control line).

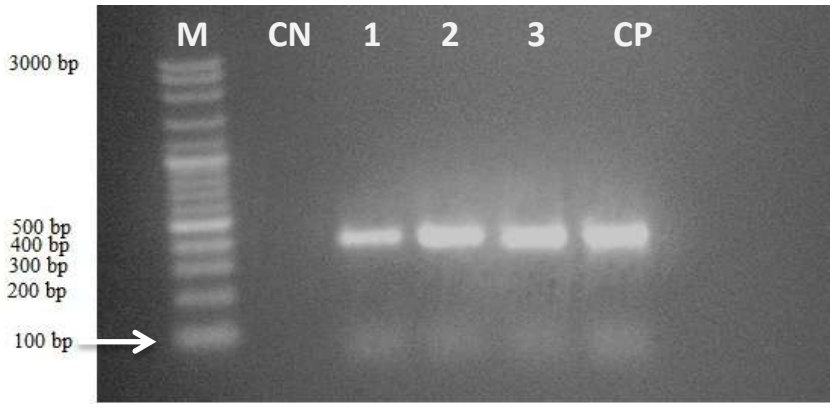


**Fig. 2:** Ethidium bromide stained agarose gel electrophoresis containing the RT-PCR products of internal part of HA gene of H5 AIV (406 bp). Lane M= DNA ladder (100-3000 bp), Lane CP= Positive control, Lane CN= Negative control, and Lane 1- 3= Positive H5 AIV samples.

These results showed that sensitivity of immuno- chromatography is 50% while specificity is 100% (calculated according to Wallach (2007)). These findings agreed with authors demonstrated that chromatographic immunoassay test kits were less sensitive than standard virus

isolation or transcriptase polymerase chain reaction (RT-PCR). Chromatographic immunoassays require high viral shedding titer, cannot detect samples lower than $10^{4.7}$ $EID_{50}$/ ml (50% egg-infective dose) and hasn't not been validated in several species particularly wild birds (Woolcock and Cardonna, 2005; Chua *et al*., 2007; Slomka *et al*., 2012).

One AIV H5 isolate from cattle egret *(Bubulcus ibis)* oropharyngeal swab was detected with incidence 1.67 % (1/60) of total cattle egret's samples. Trials for isolation of AIV H9 were not successful. It could be suggested that cattle egret may acquire AIV infection through scavenger feeding habits on infected poultry farm wastes and carcasses. According to available literature; no authors reported isolation of AIV H5 from cattle egrets although, Siembidea *et al.* (2010) recorded qRT-PCR detection of AIV Matrix gene from nestling cattle egrets sampled in California from October 2005 to January 2008, but subsequent virus isolation attempts gone unsuccessful. However, isolation of AIV subtype H5N1 was reported from closely related species such as little egret *(Egretta garzetta)* in Hong Kong by Ellis *et al.* (2004) and Great egret *(Ardea alba)* in Egypt by Soliman *et al.* (2012).

Due to multiple genetic lineages of H5N1 influenza viruses are circulating in the Egyptian poultry, RT-PCR and sequencing of partial or complete HA sequence is a must to detect relatedness to other sequences available from global database repositories and to detect changes in the newly identified antigenicity related sites (Cai *et al*., 2012). Oligonucleotide primer design was based on HA gene criteria and amplicon selection included the 130-loop region and receptor binding domain of hemagglutinin gene (Hussein *et al.*, 2015). Multiple nucleotide sequence alignment revealed both the two isolates shared the three-nucleotide deletion mutation characteristic for classical clade 2.2.1.2. The deduced amino acids sequence identity of two selected AIV H5 isolates was 96.1 % and amino acids alignment revealed the presence of characteristic deletion of receptor binding domain (Δ129S) (Fig 3) according to H5 numbering (Duvvuri, *et al.* 2009). Sequences were submitted to NCBI GenBank database with accession numbers KT454807 for egret H5 sequence and KT454808 for chicken H5 sequence.


**Fig. 3:** Amino acid alignment for the 2 AIV subtype H5 (A/Chicken/Desouk/Egypt/MS/3/2015 and A/Cattle Egret /DesouK/Egypt/MS/12/2014) isolates showing characteristic deletion of receptor binding domain (Δ129S) in comparison with other representative strains of H5N1 AIVs circulating among Egyptian chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence.

Phylogenetic analysis clustered the two isolates in the same subclade 2.2.1/ C with Egyptian HAIV H5N1 isolates from 2011-2013 (Fig 4). These findings agreed with Helal *et al.* (2014) who reported continuous circulation of classical group 2.2.1/C of H5N1 avian influenza viruses in the Egyptian poultry sectors during 2014, and Hussein *et al*. (2015) who revealed genetic diversity of avian influenza H5N1 subclade 2.2.1/C in commercial poultry in Egypt during 2013.

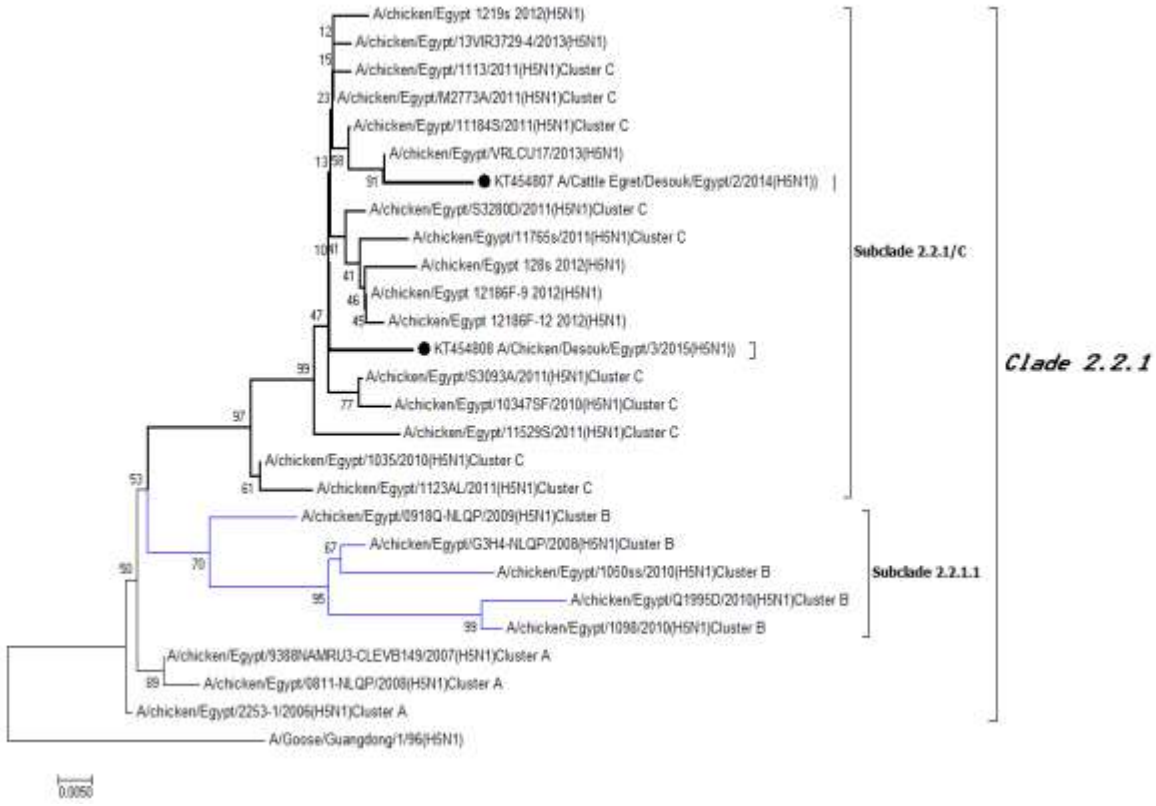


**Fig. 4:** Phylogenetic rooted tree (A/Goose/1/96(H5N1) of HA gene based on nucleotide sequence showing the clustering of [(A/Chicken/Desouk/Egypt/MS/3/2015 and A/Cattle Egret /DesouK/Egypt/MS/12/2014) spotted by black dots] isolates with other representative H5N1 AIV genetic groups. Scale bar represents the number of substitutions per site. The phylogenetic tree was generated by the neighbor-joining method with the MEGA program (version 4.0).

In contrast to Phuong *et al.* (2011) who considered cattle egrets are highly susceptible to the infection and not considered significant reservoir hosts for H5N1 AI virus, isolation of HAIV H5 from apparently normal cattle egret may suggest that cattle egret may play an important role in the spreading of the virus. HPAI viruses are generally thought to arise in poultry after infection of domestic birds by LPAI H5 virus from wild bird reservoir (Alexander, 2000; OIE, 2015). The study observed 19 remaining positive HA allantoic fluid samples: 13 from broiler chicken flocks and 6 from cattle egrets not inhibited by antisera AI (H5 and H9) and confirmed negative to these viruses by RT-PCR. HA activity in these allantoic fluids may be due to universal use of Newcastle disease live vaccines (chicken samples), other paramyxoviruses, other AIV subtypes, avian adenoviruses or avian reoviruses (Cowen, 1988; OIE, 2012 and 2015). Finally, additional deep molecular and *in vivo* studies are required to understand the possibility and the route of HPAI viruses' transmission between cattle egrets and chicken flocks.

## CONCLUSION

Apparently healthy resident cattle egret can play a reservoir role for circulation of AIV H5 subclade 2.2.1/ C that prevalent in Egyptian chicken flocks. Extensive surveillance of free living birds must be important routine strategy in Egypt for prevention and control of new waves and clusters of HPAIV.

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