

Isolation and identification of highly pathogenic avian influenza virus subtype H5N1 in Peafowl (*Pavo cristatus*)

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ABSTRACT

An outbreak of highly pathogenic avian influenza (HPAI) virus subtype H5N1 was first diagnosed in a “back-yard” flock of peafowl raised in a palace premises in the Kingdom of Saudi Arabia (KSA) in December 3, 2007. The flock consisted of 40 peafowl birds and their ages ranged from 3 to 5 years old. Affected birds suffered from depression, anorexia and white diarrhea. Four dead birds were submitted for HPAI diagnosis at the Central Veterinary Diagnostic Laboratory (CVDL) in Riyadh. Brain and liver tissues, tracheal and cloacal swabs were taken from the dead birds and processed for real-time RT-PCR test and virus isolation in specific-pathogen-free (SPF) embryonating chicken eggs (ECE). H5N1 subtype of avian influenza virus (AIV) was isolated from the 4 dead birds and identified by real-time RT-PCR before and after egg inoculation. The virus isolates were characterized as HPAI H5N1 virus by sequencing analysis. Phylogenetic comparisons revealed that the H5N1 viruses isolated from peafowl belong to the genetic clade 2.2 according to the WHO nomenclature. The peafowl H5N1 virus falls into 2.2.2 sublineage and cluster with the H5N1 viruses isolated from poultry in Saudi Arabia in 2007 – 2008.

MATERIALS AND METHODS

Case history. During the HPAI outbreaks in KSA during 2007- 2008, a small flock of peafowl, that was raised in the “backyards” of a palace premises, suffered major clinical signs of depression, anorexia and white diarrhea. The case was at once reported to the avian influenza emergency office under the Ministry of Agriculture. The flock consisted of 40 peafowl and their ages ranged from 3 to 5 years old. Clinical examination was performed at the site by state veterinarians. Four fresh dead birds were submitted for HPAI diagnosis at the Central Veterinary Diagnostic Laboratory (CVDL) in Riyadh.

Sample collection and processing. Pooled cloacal and tracheal swabs, liver and brain tissues were collected from the submitted dead birds. The swab samples and tissue homogenates were clarified at 3000g for 30 min and then supernatants were treated with multi-antibiotic mixture for AIV detection by virus isolation and real-time RT-PCR.

Virus isolation. A volume of 0.2 ml each specimen supernatant was inoculated in five 11-day-old specific pathogen free (SPF) embryonating chicken eggs (ECE) (Venky's (India) Ltd, Pune) via the chorioallantoic sac route. Virus-containing chorioallantoic fluids (CAF) were collected 24 – 48 hours post-inoculation or after embryo death. The harvested CAF was tested for the presence of haemagglutinating (HA) activity and AIV serotyped by haemagglutination-inhibition (HI) test according to standard methods (12) using subtype specific antisera obtained from VLA, Weybridge, UK. The Neuramidase Inhibition (NI) test was carried out in an OIE Reference Laboratory in IZSVe, Padova, Italy.

rRT-PCR. The clinical samples and harvested CAF were tested by the two step rRT-PCR following the same protocol described in a recent published paper (9).

Gene sequencing. The gene sequencing was conducted at the OIE/FAO Reference Laboratory for avian influenza in Italy (IZSVe). Briefly, viral RNA was extracted from the CAF sample using the Nucleospin RNA II Kit (Macherey-Nagel Düren, Germany). Amplification of the HA and NA gene segments were carried out by RT-PCR using gene-specific primers. The purified (ExoSAP-IT; USB, Cleveland, Ohio, USA) PCR products were sequenced using an ABI PRISM BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and corresponding primers for each gene. The products of the reaction sequence were purified with an AutoseqTM G-50 Dye Terminator Removal kit (GE Healthcare, Little Chalfont, UK) and run on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Genetic comparison was carried out using the ClustalW software and the N-J algorithm in the MEGA 3 programme (10).

RESULTS AND DISCUSSION

Peafowl H5N1 infection. Peafowl birds experienced mainly whitish diarrhea, depression, anorexia, disorder, and convulsions prior to death. Paled head skin and nasal discharge were also observed. Feathers around the vent were wet and soiled. Initial onset of the H5N1 HPAI infection in peafowl caused 10% mortality (4 deaths out of 40 birds) in one day. Actual mortality remains unknown because the other 36 live birds were euthanized and disposed by standard procedures once the diagnosis of H5N1 was confirmed. Necropsy examination of the dead birds revealed organ hemorrhagic lesions on proventricular mucosa membrane and splenomegaly.

Virus isolation and rRT-PCR. The inoculated ECE were examined and embryo mortality was recorded (Table 1). Embryo deaths occurred as early as 18 hours post-inoculation. This early embryo death was proven to be “specific” by HA and HI testing. The harvested CAF was tested for HA activity and characterized for AIV subtypes by means of HI and NI tests. Results confirmed that the isolate was H5N1 AIV positive. This peafowl H5N1 isolate sequencing data was submitted to GenBank with the accession number identifies as A/peacock/Saudi Arabia/3489-74VIR08/2007 (Lab Id No.1201) (H5N1). By rRT-PCR, the peafowl brain tissue yielded a strong positive reaction (ct-values of 15.05 for type A matrix and 14.85 for H5). The early embryo mortality and the low rRT-PCR cycles prompted us to conduct HA test on original tissue homogenates. As a result, supernatant of brain homogenate was tested HA positive at 1:16 end point titer. This HA activity was confirmed to be AIV and specific H5 subtype by HI test using both type A and subtype H5N1 specific antisera. These findings suggest that brain is one of important target organs of AIV infection in peafowl.

Gene sequencing. The amino acid sequence of peacock H5N1 virus revealed a cleavage site characteristic of HPAI (PQGERRRKR*GLF) in the HA molecule. In the NA molecule, mutations related to antiviral drugs resistance were not detected. Phylogenetic analysis showed that the virus belongs to the genetic clade 2.2 according to the WHO nomenclature. The analyzed isolates fall into 2.2.2 sublineage and cluster together and with the H5N1 viruses isolated from domestic poultry in KSA in 2007 (11) (Fig 1). The similarity ranged from 99.3% to 100% for HA gene and from 99.4% to 100% for NA gene. In a previous study, the nucleotide (nt) sequences of the entire genome of the viruses isolated from domestic poultry (chickens, ostriches, turkeys, duck) were closely related to each other for all eight gene segments and were placed in sublineage (2.2.2). The high similarity was seen with H5N1 viruses of the same sublineage isolated in Nigeria during 2006 (the nt identity for the HA gene ranged between 98.9 and 99.6% with the A/ck/Nigeria/ FA6/2006 strain). On another HPAI cases, two H5N1 isolates from the Houbara bustard and falcon clearly clustered in sublineage 2.2.3. (9). Interestingly, these two isolates had the highest nt similarity in the HA gene with H5N1 viruses isolated in Kuwait from domestic birds and a falcon (99.8% similarity with strain A/falcon/Kuwait/1019-7/2007) (11), which suggested that the H5N1 isolates obtained in KSA and the near east countries were from a same source of origin.

Type of Sample (pooled of 4 birds)	Embryo mortality (dead/total)	Virus isolation in ECE	rRT-PCR on original sample	rRT-PCR on allantoic fluid	HA test on original sample
Brain homogenate	5/5	+	+	+	+
Tracheal swabs	5/5	+	+	+	-
Cloacal swabs	3/5	+	+	+	-
Liver homogenate	not done	not done	+	not done	not done

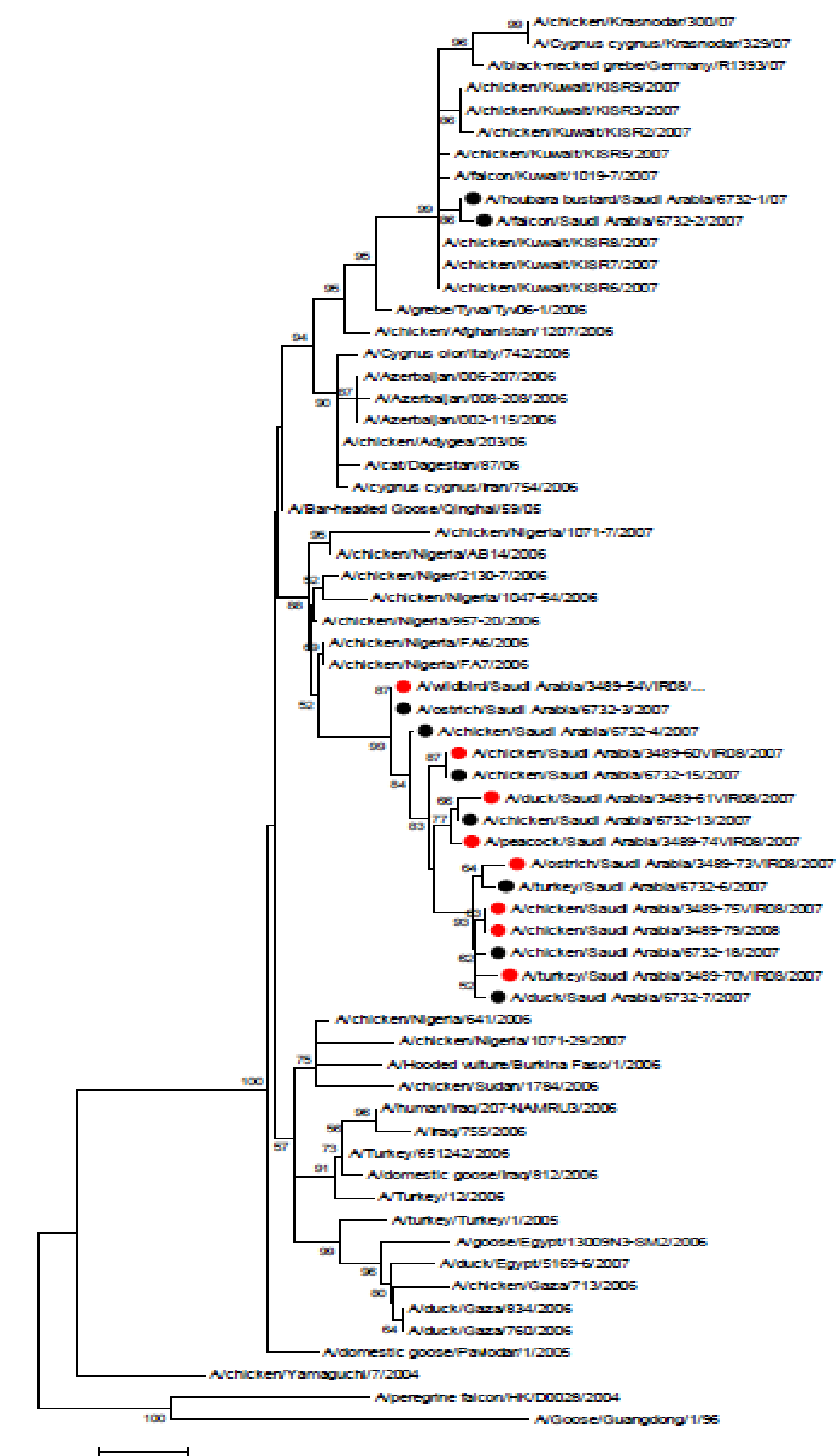


Fig 1: Phylogenetic tree for the HA gene segment. The analysed viruses are labelled with a red circle. The isolates from Saudi Arabia, analysed in the previous study (Monne et al., 2008), are marked with a black circle.

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