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**Isolation and characterisation of an H10N7 avian influenza virus from poultry
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Maria Serena Beato¹, Calogero Terregino¹, Giovanni Cattoli¹ & Ilaria Capua^{*}

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¹*OIE, FAO and National Reference Laboratory for Avian Influenza and Newcastle Disease*

*Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, 10, 35020, Legnaro,
Padova, Italy*

Running title: H10N7 avian influenza virus from poultry carcasses smuggled from China into Italy

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^{*}*Corresponding author: Ilaria Capua. Tel.: 0039-049-8084371, Fax: 0039-049-8084360; e-mail:
icapua@izsvenezie.it*

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Isolation and characterisation of an H10N7 avian influenza virus from poultry carcasses smuggled from China into Italy

Maria Serena Beato¹, Calogero Terregino¹, Giovanni Cattoli¹ & Ilaria Capua*

Abstract

Intensified official veterinary controls on warehouses supplying Chinese retailers, resulted in the seizure of smuggled poultry products. Frozen and frozen vacuum-packed raw duck and chicken carcasses and anatomic parts (legs) of these species, were collected and processed for laboratory investigations aiming at the detection of avian influenza virus. RRT-PCR targeting the M gene, performed on lung and trachea from a duck and chicken carcass yielded positive results. Virus isolation attempts in SPF embryonated eggs yielded an H10N7 virus from the duck carcass. The isolate had an IVPI of 0.0, and phylogenetic analysis revealed a nucleotide homology of 97% and 96% with virus A/duck/Zhejiang/52/2000 (H5N1) for the PB2 and NS genes respectively.

The results of this study indicate that LPAI viruses may be introduced into a country through trade of carcasses regardless of the non systemic nature of this disease. The export ban applied to countries affected by the H5N1 epidemic may result in increased smuggling of poultry products, which remains one of the means by which influenza viruses can cross national boundaries. Increased surveillance at borders and at a national level is recommended.

Introduction

Avian Influenza (AI) infections have increased in relevance from both the animal and human health perspective. Wild waterfowl are known to be the reservoir of AI viruses, representing a continuous source of virus for domestic birds. Domestic ducks are frequently infected with several subtypes of AI viruses of low pathogenicity and are known to harbour these viruses (Hinshaw *et al.*, 1980).

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Following increasing evidence that Highly Pathogenicity Avian Influenza (HPAI) viruses emerge from Low Pathogenicity Avian Influenza (LPAI) progenitors of the H5 and H7 subtype (Garcia *et al.*, 1996; Perdue *et al.*, 1998; Suarez *et al.*, 2004), the OIE (International Organisation for Animal Health) has revised its position with regard to the notification of avian influenza infections for the purposes of international trade. The new OIE regulation includes a new definition of Notifiable Avian Influenza, that describes avian influenza in its notifiable form (NAI) as an infection of poultry caused by any influenza A virus of the H5 or H7 subtypes or by any AI virus with an intravenous pathogenicity index (IVPI) greater than 1.2 (or as an alternative of at least 75% mortality) (OIE, 2005). NAI viruses can therefore now be divided into highly pathogenic notifiable avian influenza (HPNAI) and low pathogenicity notifiable avian influenza (LPNAI). This modified definition is a logical consequence of the evidence that HPAI viruses emerge in domestic poultry from LPAI precursors of the H5 and H7 subtypes. Therefore, there is a case that not only HPAI viruses but also their LPAI progenitors should be controlled in domestic poultry (Capua & Marangon, 2000; Alexander, 2003).

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The term NAI excludes all other AI viruses of low pathogenicity, defined low pathogenicity avian influenza, which are viruses of the H1-H4, H6, H8-H16 subtypes, from any trade requirement as there are no provisions mentioned for trade related issues in case of infection with any of the LPAI viruses.

Chapter 2.7.12 of the OIE Terrestrial Code, lists requirements for the trade of commodities on the basis of the presence of HPNAI or LPNAI in a given country/zone/compartiment. The

different provisions for trade in case of HPNAI and LPNAI are based on the marked difference there is between viruses that become systemic and those that, based on current knowledge, are unlikely to do so. When addressing the trade implications of commodities with reference to AI status, current knowledge on the pathogenesis of this disease has been used as an indicator of the risk posed by certain commodities. That is to say that the pathogenesis of the disease in the infected host will determine in which organs or products these viruses are present during the acute phase of infection and will be highly influenced by the type of virus (LPAI or HPAI), by the strain of virus, by the animal species involved and possibly, within a species, by exacerbating factors and age.

Infections with HPAI viruses, particularly in chickens and turkeys, follow a generalised course with extensive viraemia. Virus may be detected not only in the respiratory and enteric tracts but also in internal organs such as spleen, pancreas, heart, liver, kidney, nervous system as well as muscle and skin (Starick & Werner, 2003, Swayne & Beck, 2005).

Theoretically, LPAI viruses are restricted to replication in the respiratory and intestinal tracts and infections should not result in infective material outside these areas. However, experimental evidence (Swayne & Beck, 2005) indicates that although viraemia was not established with an H7N2 LPAI virus, this was recovered from internal body wash fluids and therefore LPAI viruses could contaminate the carcass. Furthermore, under exacerbating conditions more generalised LPAI virus infections have been reported, especially in turkeys (Capua *et al.*, 2000) and therefore the theoretical absence of LPAI viruses in some poultry products cannot be guaranteed. This can also be caused by incomplete evisceration or carcass contamination due to the extensive replication of LPAI viruses in the intestinal tract and the large amounts of virus excreted in the faeces. Several occurrences therefore represent a means by which LPAI may be introduced into a given country and therefore may pose a potential risk to susceptible birds if adequate mitigation measures are not practised.

The following report describes the means by which a LPAI virus of the H10N7 subtype of Asian origin entered the EU through smuggled poultry carcasses.

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Case History

Following the provisions of the Italian Ministry of Health for additional measures to prevent the introduction of the Asian HPAI H5N1 into the national territory, an intensification of controls in establishments at risk was implemented (Anonymous, 2005). This led to investigations in storage deposits owned by Chinese citizens, currently supplying retail shops specialised in Chinese food. One of these deposits contained goods that had not been registered, and among these, was a crate containing poultry products. Following inspection by the veterinary services, one vacuum-packed frozen duck carcass, two frozen chicken and one frozen duck carcasses, four frozen duck legs and four frozen chicken legs were seized. The carcasses were plucked and partially eviscerated (lung and trachea were still in place). The seized products were contained in cardboard boxes, with a few Chinese ideograms and there was no information concerning when or how the products had arrived.

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No documents were available for inspection.

The aim of this investigation was to establish whether any viable avian influenza viruses were present in the carcass. The reason for this is that the destiny of meat scraps resulting from illegally imported carcasses can easily enter the animal food chain as swill feeding. This occurrence could represent the first step to the initiation of an infectious cycle in EU poultry. This assumes greater significance with respect to trade, as there are currently no trade regulations concerning infection with Low Pathogenicity Avian Influenza viruses (LPAI) (H1-4, H6, H8-16).

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Materials and Methods

Samples of lung and trachea and of pectoral and thigh muscle were collected from the carcasses and fragments of available muscle tissue were collected from the frozen legs. All samples were processed individually except for the lung and trachea belonging to the same animal that were pooled together. Tissues were homogenised and RNA was extracted as described (Cattoli *et al.*, 2004). Real time PCR (RRT-PCR) was performed on all samples with primers and probe targeting

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the M gene (Spackman *et al.*, 2002) and subsequently positive samples were processed by RRT-PCR with primers sets and probes specific for the H5 and H7 genes.

Samples were also processed for virus isolation in fowl's SPF eggs. Briefly one gram of sample was weighed and homogenised with PBS (Phosphate Buffer Solution) containing antibiotics in a 1:10 w/v suspension. The intravenous pathogenicity index (IVPI) was performed as described (CEC, 1992). Allantoic fluids from embryos showing mortality 24-48hrs after inoculation were tested for bacterial contamination by routine methods. The haemagglutinin (H) and neuraminidase (N) subtypes were determined using polyclonal chicken antisera as described by Alexander and Spackman (1981).

Sequences of RT-PCR products were performed directly using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) in a 3100-Avant genetic analyser (Applied Biosystems). For sequence analysis and phylogeny the Tajima-Nei and Neighbour-joining algorithm were used.

Results and Discussion

The lung and tracheal homogenates originating from both the frozen duck and chicken carcasses tested positive by RRT-PCR targeting the M gene. The same samples were negative for the H5 and H7 PCR assay. All other samples yielded negative results. A haemagglutinating agent was isolated on first passage from the duck sample that yielded positive results in the Real-time PCR assay. The sample originating from the chicken carcass resulted negative. This isolate was characterised as a virus of the H10N7 subtype. The IVPI of the isolate was 0.00.

By using the set of primers indicated in table 1, RT-PCR and sequence analysis of the HA and NA gene segments confirmed the identity of the isolate. A BLAST search in the GenBank database revealed an homology at the nucleotide level of 91 % for the HA gene with A/mink/Sweden/84 (H10N4) and of 96% for the NA gene with A/chicken/Germany/R28/03 (H7N7); A/Netherlands/33/03(H7N7) and A/duck/Mongolia/736/02 (H7N7).

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In order to evaluate whether the H10N7 isolate shared internal genes with the H5N1 HPAI currently circulating in China, the six internal genes of the virus were partially amplified using primers listed in table 1. Sequencing of the H10N7 M, NP, PA and PB1 genes indicate a low homology at the nucleotide level (<94%) with the corresponding genes of the H5N1 viruses circulating in the Far East. For the PB2 and NS gene, a 97% and 96% homology at the nucleotide level was revealed with virus A/duck/Zhejiang/52/2000 (H5N1) respectively. Sequences have been deposited in GenBank (Accession numbers DQ374396-374403).

The results reported herein indicate that a viable LPAI virus was introduced into the EU through poultry carcasses smuggled from China. Although HPAI of the H5N1 subtype has been isolated from frozen duck meat imported from China into South Korea (Tumpey *et al.*, 2002) and from China into Japan (Mase *et al.*, 2005), this appears to be the first report of isolation of a LPAI virus from an imported poultry product. These findings are in keeping with the experimental findings of Swayne & Beck (2005), who report of the isolation of LPAI H7N2 from the respiratory tract, gut and body washing but not from meat of experimentally infected chickens.

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Notwithstanding a presumably long journey, and not ideal storage conditions, the virus was isolated on first passage from the pooled lung and trachea of a partially eviscerated frozen duck carcass. The virus was not isolated from any muscle sample collected from the carcasses or leg samples collected from the seized products.

The virological data indicate that viable virus that correlated with viruses that are currently circulating in Asia was present in the seized products. Very little is known about the destiny of these smuggled products in ethnic communities. In particular it is not known whether scraps of meat and viscera could have entered the animal food chain uncooked. Swill-feeding is considered the main means by which certain pathogens are able to generate major epidemics in free countries, and for this reason it is forbidden in the EU (EC, 2002).

Smuggling of live birds has been shown to be a means of introducing avian influenza viruses into the EU. In December 2004 two Crested-Hawk live eagles were seized at Brussels airport and

found positive for the H5N1 HPAI Asian virus. Smuggling of poultry and poultry products has been identified as a means of introducing AI into the EU (EC, 2003), and probably as a consequence of the ban on imports of poultry products from Asian countries, smuggling of commodities could become an attractive business, resulting in increased numbers of commodities smuggled per unit of time. This situation should be kept in mind by public veterinary authorities, and should result in a strengthening of the inspection service at borders.

Currently there are no trade regulations concerning LPAI viruses (H1-4, H6, H8-16) (Capua & Alexander, 2006). The results of this investigation indicate that even under non-optimal storage conditions viscera contained in frozen duck carcasses may contain viable LPAI virus. LPAI viruses infect ducks frequently, although for trade purposes it would be unreasonable to expect freedom from LPAI infections in birds belonging to the Order *Anseriformes*. However, it appears reasonable to recommend that in poultry abattoirs destined for export, special attention should be paid to the complete evisceration of duck carcasses.

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