Polish J. of Environ. Stud. Vol. 19, No. 2 (2010), 379-385

Original Research

The Epidemiological Environment of Avian Influenza H5N1 Outbreaks in Wild Birds in Croatia

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Received: 26 June 2009
Accepted: 12 November 2009

Abstract

Following the spread from its origins in China, Asian lineage highly pathogenic avian influenza H5N1 was first recorded in Europe in Turkish poultry and in poultry and wild birds in Romania in early October 2005. On 19 October 2005 Croatia became the second European country to record an outbreak in wild birds, involving Mute Swans *Cygnus olor*. Subsequent surveillance in Croatia revealed further instances of H5N1 in dead and sick Mute Swans in 2005, and in 2006 in more dead Mute Swans and in living, apparently healthy, Black-headed Gulls *Larus ridibundus*, but not in poultry. The observations presented here suggest that Croatia experienced two independent incursions of Asian lineage HPAI H5N1, but genetic confirmation is not available. Potential routes of introduction by wild birds, the poultry industry and fish-farming practices are discussed, but the evidence for all of these remains circumstantial.

Keywords: wild birds, avian influenza, H5N1, poultry, fish farms, Croatia

Introduction

Asian lineage highly pathogenic avian influenza H5N1 (HPAI H5N1), first discovered in domestic geese in China in 1996 [1], remained confined to south-east Asia (mainly in poultry), until 2005. In April-May 2005, the virus was recorded from dead and sick wild aquatic birds at Lake Qinghai, northern China, and later during July-August it spread westwards across southern Russia, mainly in poultry, but was also isolated from wild birds in some regions where there had been poultry outbreaks [2, 3]. In Europe, HPAI H5N1 was first reported from Turkey and Romania, with outbreaks in poultry, and also in wild birds in Romania, in early October 2005 [4]. Shortly afterward, four Mute swans (*Cygnus olor*) were found sick and dying at Grudnjak fish farm in Slavonia, eastern Croatia (Table 1). These events stimulated surveillance for infected wild birds and poultry within Croatia and throughout Europe.

Our aim is to review the infections discovered in Croatia and place them in the wider Eurasian context, and to examine possible routes of virus incursion into Croatia.

Experimental Procedure: Surveillance

Following the initial discovery of the virus in Croatia a surveillance programme was initiated, targeting primarily water birds in the country's 19 largest wetlands [5].

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Table 1. Occurrence of HPAI H5N1 virus in Croatia in 2005 and 2006.

Year	Location	Coordinates	Onset of event	Last positive sample	Species infected	Health status of infected birds
2005	Fish-farm Grudnjak, Slavonia	45°38'20.22"N 18° 2'22.53"E	a) 19 – 25 October b) 19 November	19 November	a) *C. olor* b) *C. olor*	a) sick b) sick
2005	Fish-farms Našička Breznica and Ribnjak 1905, Slavonia	45°34'25.36"N 18°10'14.23"E	24 October	28 October	*C. olor*	sick and dead
2006	Slatina vllage on Island Čiovo, Dalmatia	43°29'58.15"N 16°20'16.91"E	14 February	14 February	*C. olor*	dead
2006	Wetland Pantana, Dalmatia	43°31'25.94"N 16°16'14.17"E	a) 20 February b) 28 Feb – 3 March	3 March	a) *C. olor,* b) *L.ridibundus*	a) Sick and dead b) clinically healthy
2006	Village Draž, backwater of the river Dunav, Slavonia	45°50'52.30"N 18°47'55.49"E	2 March	2 March	*C. olor*	dead
2006	Zagreb	45°46'5.91"N 15°53'59.76"E	28 March	28 March	*C. olor*	dead

These wetlands are important sites for many water bird species, especially Mallard *Anas platyrhynchos*, Bean Geese *Anser fabilis*, Great Cormorants *Phalacrocorax carbo*, Eurasian Coot *Fulica atra* and Black-headed Gulls *Larus ridibundus*. Three of the wetlands are close to the Adriatic coast; the remainder are further east on the mainland. Ten of the wetlands are man-made fish-rearing ponds, mainly 395-1,400 ha, but two are much bigger, at 23,000 ha and 41,000 ha. Carp are the main fish produced but other species, of no commercial value, live in the ponds. Fish production is extensive or semi-intensive and young fish are reared on-site. Fish farms attract water birds and most, including Grudnjak and Našička Breznica (see below), are "important bird areas" [6]. All fish farms, including Grudnjak and Našička Breznica, are used for recreational hunting in winter (October onwards, particularly by Italian hunters) mainly of Mallard and Coot. At some, Mallard are reared for hunting, but this does not occur at Grudnjak and Našička Breznica. The remaining wetlands are four smaller (280-1,660 ha) reservoirs whose outflow is used for electric power production, and larger (5,700-25,630 ha) natural water bodies that are nature parks, widely used for tourism and recreation.

Surveillance was co-ordinated by the hunting section of the Croatian Ministry of Agriculture, Fisheries and Rural Development and undertaken by the Poultry Centre of the Veterinary Institute, the Department of Ornithology of the Croatian Academy of Sciences and Arts, and by authorized veterinarians [7]. For wild birds, both passive surveillance (searches for dead and sick birds) and active surveillance (sampling of apparently healthy living and shot birds) were undertaken between March and December, to cover periods of spring migration (March-May), early autumn migration (August-September) and late autumn migration (October-December). In addition to the participating organizations, members of the public were asked to report birds found dead.

In 2005 and 2006, 7,646 living and dead wild birds sampled during the surveillance programme were tested for avian influenza at the Poultry Centre, Croatian Veterinary Institute, using the standard OIE protocols [8]. Birds from the same species and location were examined as pooled samples with a maximum of five individual birds per sample, according to OIE protocols. Isolated AI viruses were serotyped using reference antisera (Veterinary Laboratories Agency, Weybridge, UK). Pathogenicity assessment and phylogenetic analysis of the isolated H5 AI viruses were carried out using RT-PCR, with subsequent nucleotide sequencing and deduced amino acid sequence of the haemagglutinin cleavage site. The first H5N1 isolate, A/Cygnus olor/Croatia/1/2005, was sent for confirmation to the O I E/F A O Reference Laboratory for Avian Influenza and Newcastle Disease, Veterinary Laboratories Agency, Weybridge, UK. The gene sequences of subsequent samples of Croatian H5N1 viruses are not publicly available for comparison with A/Cygnus olor/Croatia/1/2005 or with other H5N1 viruses.

Samples from poultry from all flocks slaughtered in the 3 km buffer zone around confirmed outbreaks (apart from the single swans in Zagreb city and Draž village) were tested for Asian lineage HPAI H5N1. In 2005, structured and systematic sampling of poultry elsewhere in Croatia was not included in the surveillance programme, but since 2006 poultry have been sampled nationally for serological testing.

Results

During passive surveillance, the only species found to be infected were Mute Swans. The first case in Croatia, on 19 October, involved four Mute Swans found sick and dead at Grudnjak fish farm, Slavonia, eastern Croatia. Around 1,500 Mute swans had recently arrived and two days later a further 15 dying Mute swans were found there. Before death, the birds showed the characteristic signs of HPAI H5N1: neurological problems, torticollis, opisthotonus, rhinorrhea, and spinning, and autopsy revealed severe lung hyperaemia and oedema [9]. Five days later, 15 dead Mute swans, out of a flock of 244, were found at Našička

Breznica fish farm, 12 km from Grudnjak, showing the same symptoms before death and also infected with HPAI H5N1 [9]. At Grudnjak, sporadic swan mortality, preceded by behavioural signs, continued into early December; only one sick bird was tested, on 19 November, proving positive for HPAI H5N1 [9].

One of the infected dead Grudjnak swans, a first winter male, had been marked with a numbered neck collar at Balatonfured, Veszprem (46.58 N; 17.53 E) 149 km away in neighbouring Hungary on 9 September 2005. It was still at the place of marking on 22 September, showing no behavioural abnormalities [2]. On 25 October it died at Grudjnak (Bird ringing archive at Department of Ornithology, Croatian Academy of Sciences and Arts). In Europe, the only other 2005 case in wild birds involved > 500 swans in November near Astrakhan in the Volga Delta, Caspian Sea, but in Turkey and Romania outbreaks in poultry continued throughout the winter, as they did in the Crimea (Ukraine) from December onwards.

In February and March 2006 four more Mute swans were found dead, infected with Asian lineage HPAI H5N1, but this time at separate localitions in Croatia (Table 1). In addition, during active surveillance at one of these sites, the Pantan wetland in Dalmatia, 30 apparently healthy Black-headed gulls, were captured in mist nets and banded and released. Tracheal and cloacal swabs were taken and the samples were found to be positive for Asian lineage HPAI H5N1 [10].These gulls were caught 8-12 days after the discovery of a dead Mute swan at the Pantan and about 20 days after a dead Mute swan had been found on nearby Čiovo island.

All samples taken from poultry within the buffer zones around wild bird outbreaks, and in the subsequent national surveillance scheme, have tested negative for Asian lineage HPAI H5N1.

Discussion

In autumn 2005, shortly after the outbreak in Mute Swans in Romania, c. 850 km to the east, the swan deaths in Croatia were highly localized, being confined to Grudnjak and nearby Našička Breznica fish farms. There was no indication of the spread of the virus to other sites within Croatia or to neighbouring countries.

In spring 2006, the deaths of four Mute Swans at scattered locations in Croatia (Table 1) were contemporaneous with and proved to be part of a much wider viral dispersal through eastern and central Europe, involving a small number of birds, mostly waterbirds and especially Mute Swans, in 20 European countries. This appeared to be the result of dispersal away from cold weather in Eastern Europe, particularly around the Black (c. 850 km east of Croatia) and Caspian (c. 2,350 km east of Croatia) seas, where persistent outbreaks of Asian lineage HPAI H5N1 in poultry occurred throughout the winter, and in swans in November 2005 and February-March 2006 [11]. The late winter movements of Mute Swans and other species northwestwards across Europe did not occur during a time, or along any recognized route, of long-distance migration. During the spring 2006 outbreaks in Croatia, no infected birds were found at the Grudnjak and Našička Breznica fish farms, suggesting that the 2006 incidents did not result from over-winter survival of the virus at the fish farms.

These observations suggest that in October 2005 and February-March 2006, Croatia in fact experienced two independent incursions of HPAI H5N1, the first during the arrival of Mute Swans from autumn migration and the second as part of a wide-scale cold-weather dispersal.

Genetic Analysis

Partial nucleotide sequence of the HA gene of the virus isolated from one of the first Mute swans to die at Grudnjak revealed high similarity with the Asian lineage HPAI H5N1 isolates from dead wild geese ducks and gulls during the outbreak at Qinghai Lake, China, in May 2005. For instance, the Croatian H5N1 virus shared 99.7% nucleotide identity with the virus isolated from a Great Black-headed gull (*Larus ichthyaëtus*) at Qinghai Lake, but shared only 99.3% and 99.1% homology with the H5N1 viruses isolated in October 2005 from poultry in Turkey and Romania, respectively [9]. A subsequent analysis of complete genomes of selected recent Asian lineage HPAI H5N1 isolates showed the Croatian H5N1 virus to be closely related to a group of isolates from Mute Swans in Astrakhan (Volga delta, Russian Federation). These isolates formed a separate lineage [12], now termed clade 2.2.2 [13], of which the Croatian swan was the only wild bird representative, but which in 2006 was isolated from poultry in Nigeria and Niger [12] and wild birds in an outbreak centred on Rügen Island, Germany [14]. Viruses from Mute Swans in Italy in the spring 2006 outbreak fell into two different groupings (clades 2.2.1. and 2.2.3) [12] and multiple incursions of virus strains have been confirmed in France [15] and Germany [14, 16].

These genetic data do not permit the elucidation of clear and reliable pathways of viral strain spread, its mechanisms or its origins, except confirming that all of the European HPAI H5N1 viruses are clade 2.2 Qinghai-like viruses. The possible roles of wild birds and poultry or poultry products in virus spread are discussed below.

Asymptomatic Wild Birds

If wild birds are involved in HPAI H5N1 spread they must be able to migrate or disperse while infected and shedding virions. There is continuing debate on the ability of free-living wild birds to carry Asian lineage HPAI H5N1 viruses asymptomatically over long distances [14, 17-19]. With regard to the Croatian outbreaks in wild birds, particular questions relate to (a) the ability of Mute Swans to carry the virus asymptomatically from areas where it was known to be infecting wild birds and/or poultry and (b) the likelihood that other wild bird species might have been capable of introducing the virus through asymptomatic carriage.

(a). Experimentally-infected captive Mute Swans excreted virus asymptomatically for 5-7 days but survived less than 24 h after the onset of clinical signs [20, 21]. Mute Swans do not normally migrate or disperse over long distances [22]. When stimulated to do so by adverse winter weather, the distances they fly, their requirement for rest at stopover sites and the speed at which they travel are unknown. Importantly, there is also no information on effects on behaviour during the period of pre-clinical infection with HPAI H5N1. However, low pathogenic avian influenza (LPAI) viruses were found to impair foraging and migratory behaviour of free-living wild Bewick's swans (*Cygnus columbianus* Ord 1815) [23], and body mass and increased stopover time in some Mallard [24]. These subtle changes in behaviour resulting from LPAI infection raise questions about the ability of apparently asymptomatically-infected free-living waterfowl to undertake normal activities while incubating HPAI H5N1, a virus that has demonstrably proven to be highly lethal to infected wild individuals [2, 16, 25].

Mute swans that winter in Croatia normally breed in Poland, Germany and Hungary [7], not in the Black Sea and Caspian regions, where HPAI H5N1 virus outbreaks occurred in 2005-06. In October 2005, infected Mute swans would have needed to migrate while infected in order to carry the infection to Croatia. In February 2006, on the other hand, they would need to have been capable of undertaking cold weather movements stimulated by thermal stress and food shortage when their feeding sites froze over.

At the fish farms, only small proportions of mute swan flocks became infected, suggesting inefficient bird-to-bird transmission. This may be related to the greater propensity for HPAI H5N1 viruses to be spread through excretion via the oropharyngeal tract [16, 26, 27], suggesting spread by aerosol, rather than the faecal-oral transmission in water that is more typical of LPAI viruses.

Restriction of the October 2005 Croatian outbreaks to two nearby fish farms, together with the synchronous illness and death of the earliest infected birds at both farms, support the hypothesis that the Mute Swans contracted the virus locally, possibly at Grudnjak fish farm [2, 7]. Recurrent deaths at Grudnjak, but not at Našička Breznica, suggested a continuing source of infection at the former. This could have been through virus survival in the water or sustained virus passage in Mute Swans, or sustained passage in other asymptomatically infected water birds. In all of these cases it is difficult to see why similar persistence of infection did not occur at nearby Našička Breznica fish farm. Alternatively, the virus might have been introduced to Grudnjak fish farm by means of farm practices at that site (see below) or by infection carried asymptomatically by other wild birds in which infection was not detected during active surveillance.

(b). Arguably the most surprising aspect of the Croatian outbreaks was the finding of Asiatic lineage HPAI H5N1 in swabs from apparently healthy Black-headed Gulls, since this would represent the first convincing [17] demonstration of asymptomatic carriage of this virus by apparently healthy free-living wild birds. The gull virus is not publicly available and comparative studies with contemporary viruses in Croatia and elsewhere are highly desirable. Experiments on captive gulls have demonstrated differing responses to challenges by HPAI H5N1 [20, 28]. On immunological grounds, it appears unlikely that free-living wild birds could undertake long distance migration while infected with HPAI H5N1 [18].

When the gulls were caught, no assessment of their health (body mass and linear measurements, plumage condition, responsiveness to stimuli, orbital or nasal discharge, etc. [29] was made and it is unknown whether these birds behaved normally and survived after release. As swab samples were pooled, the number of birds that might have been infected is unknown. Broader ecological information on the numbers and identity of other water birds at the site or of poultry operations nearby [as advised by 29] was not collected at the time. However, small numbers of domestic ducks and geese are known to live on a small lake, from which a river feeds into the wetland. The Pantan is the only natural lagoon on the Adriatic coast of Croatia and supports a diversity of water birds [30]. It lies 6 km from Čiovo island, where the first infected Mute swan in 2006 was found.

If this finding of asymptomatic carriage of HPAI H5N1 in Black-headed Gulls is genuine, this warrants much more intensive surveillance of gulls as potential vectors. Gulls are known to carry H13 and H16 subtypes of avian influenza [3], but their potential to carry HPAI H5N1 would be of particular significance in the epidemiology of this virus subtype. Few gulls have been reported in HPAI H5N1 incidents and all have been sick or dead [2, 31]. Many gull species migrate over long distances, undertake dispersal movements in winter (especially in response to cold weather and changes in food availability), and they commute daily in winter from communal roosts to feeding areas tens of kilometres away. They feed commonly in association with human activities, especially agriculture and refuse disposal, and have been implicated in the possible dispersal of several bacterial pathogens, notably involving the contamination of water storage reservoirs [32].

The Poultry Industry in Croatia

There have been no reports of HPAI H5N1 in poultry in Croatia, either in commercial or in free-ranging poultry in villages. At Croatian commercial poultry farms access to wild birds is restricted, but some farms do not practice adequate biosecurity [33]. Following the outbreaks, all poultry nationally were ordered to be kept indoors.

In addition to companies registered for the rearing of poultry and production of poultry products, an informal market exists in Croatia, consisting of numerous owners of small incubators for the hatching of chicks that are sold without control and unrecorded. Croatia additonally imports and exports poultry. In the first eight months of 2005 Croatia exported 4,919 and imported 5,413 tons of poultry and poultry meat [34] but the source of imports was not given.

The history of Asian lineage HPAI H5N1 has illustrated the need for stringent national and international moni-

toring of movements of poultry and poultry products, along with an independent testing system at the source, in transit and at the destination. While some legal movements are subjected to health checks, they have nevertheless been implicated in viral spread, despite initial denials by the industry [35]. Illegal or unregulated movements, on the other hand, are never subjected to health checks and they appear to have been wildly involved in the spread of HPAI H5N1 in SE Asia, especially domestic ducks [36, 37]. The extent of such movements into Croatia is not known but could present a risk factor, especially if from countries experiencing concurrent HPAI H5N1 outbreaks, such as Romania.

The Fish Farms

Grudnjak and Našička Breznica fish farms, where the 2005 outbreaks occurred, are situated in a rural area where farming and animal husbandry are the main means of livelihood, and poultry are mostly raised for domestic consumption. In addition to Mute Swans, these two farms support a variety of other water birds, including gulls, geese and ducks [6, 7, www.ptice.net/print.php?sid=27]. The use of poultry manure in aquaculture is well developed in Asia, Russia and Eastern Europe [38]. Croatian Ministry of Agriculture, Fisheries and Rural Development epidemiological studies revealed that Grudnjak fish food did not contain animal matter or poultry manure and that it was not imported (A. Labrović *pers. comm*.). However, Grudnjak fish farm staff reported that fish foods for young fish are occasionally imported from Bečej (Serbia) (J. Radaković *pers. comm*.), raising doubts over the provenance of fish foods and their constituents.

Introducing Asiatic lineage HPAI H5N1 to fish farms presents a risk of further spread, particularly if domestic poultry, and especially ducks that are known to be susceptible to asymptomatic infection [39], are allowed access to the water. These ducks could act as intermediaries in spreading the virus to terrestrial poultry if taken back to farmsteads, and also to wild birds at the fish farms. Where Mallard are reared on fish farms for subsequent release for hunting, infection could be distributed more widely to poultry and wild birds. Aquaculture practices that risk introducing the virus to fish farms must therefore be strictly controlled.

Conclusions

It appears that Croatia experienced two independent outbreaks of Asian lineage HPAI H5N1 in 2005 and 2006, but this evidence is from the timing and scale of outbreaks; genetic sequencing of the viruses is needed to confirm this hypothesis. The source of the virus for the October 2005 outbreak is unknown. Introduction by asymptomatically-infected wild birds offers a plausible hypothesis, but evidence for it is entirely circumstantial. The outbreaks in late winter 2006 were part of a larger scale spread across eastern and central Europe, in which at least two virus subclades were involved. But the genotypes of the Croatian viruses are as yet unknown. Croatian surveillance in 2006 revealed virus shedding in apparently healthy Black-headed Gulls. This indicates a need for investigations of the ability of wild water birds to migrate long distances or to disperse while shedding the virus asymptomatically under stresses experienced by free-flying birds. While more comprehensive ornithological data and virus sequencing might have provided clues regarding virus origin, structured sampling of poultry and poultry products for virus and comprehensive monitoring of movements of poultry and poultry products, including when used in other agricultural and aquacultural contexts, are prerequisites for a fuller understanding of Asian lineage HPAI H5N1 epidemiology.

Acknowledgements

The results presented in this paper come from research projects on *Birds and human well-being: Ornithological models of protection*, funded by the Croatian Ministry of Science, Education and Sports. We thank dr. sc. V. Savić for helpful discussion and comments on the manuscript, along with two anonymous referees who made helpful comments.

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