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Source: Journal of Wildlife Diseases, 43(4) : 789-793

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-43.4.789

Journal of Wildlife Diseases, 43(4), 2007, pp. 789–793

Influenza A Virus in Birds during Spring Migration in the Camargue, France

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ABSTRACT: Wild aquatic birds are considered to be the natural reservoir for influenza A viruses, and previous studies have focused mainly on species in the orders Anseriformes and Charadriiformes. In this study, we surveyed a larger spectrum of potential hosts belonging to 10 avian orders. Cloacal swabs (n=1,044) from 72 free-living bird species, were analysed by reverse transcription-polymerase chain reaction for the presence of avian influenza virus. Only two Mediterranean Gulls (*Larus melanocephalus*) tested positive; one of these viruses was identified as an H9N2 subtype. The absence of infection among passerine birds supports the idea that the prevalence of avian influenza virus infection in terrestrial species is low.

Key words: Avian influenza, H9N2, *Larus melanocephalus*, Mediterranean Gulls, passerine birds.

Wild aquatic birds in the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and waders) are traditionally considered as natural hosts for most avian influenza viruses (AIV; Webster et al., 1992). Although AIV have been isolated from other wild bird species (Olsen et al., 2006), less attention has been devoted to species in other avian orders. Consequently, the role of many avian species as potential hosts for AIV is unknown.

The Camargue area is an alluvial wetland covering some 140,000 ha in the Rhône delta in the south of France, and it is at the crossroads of numerous migratory routes of Palaearctic birds (Blondel and Isenmann, 1981; Berthold, 2001). Therefore, the Camargue is considered as a high-risk area for the introduction and transmission of diseases transmitted by wild avian species, such as West Nile virus (Jourdain et al., 2007b) and AIV. The risk of virus introduction by birds from sub-Saharan Africa into Western Europe is highest during spring migration between March and June (Jourdain et al., 2007a). The aim of this study was to investigate the prevalence of AIV infection in a large diversity of avian hosts during spring migration.

From mid-March to late June 2006, different bird species were trapped using mist nets. Nets were placed in bushes located a few hundred meters behind the Piémanson beach, southeast of Salins de Giraud (Arles, France), in an attempt to capture migratory birds immediately after their crossing of the Mediterranean Sea. Cloacal swabs were used to collect fecal samples, except for passerines; due to their small size, we collected samples of fresh droppings to avoid injury. For Ciconiiformes (herons and egrets) and Phoenicopteriformes (greater flamingo [*Phoenicopterus ruber*]), cloacal swabs were collected from chicks in breeding colonies. For gulls, fresh dropping samples were collected near nests.

Cloacal swabs were collected using the Viral Pack kit (Biomedics, S.L., Madrid, Spain) and kept at 4 C until they were transported back to the laboratory and kept at –80 C until RNA extraction was performed. Automatic RNA extraction was performed using the BioRobot MDx workstation and QIAamp Virus BioRobot MDX kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The presence of AIV was first detected by reverse transcription-polymerase chain reaction (RT-PCR) targeting

TABLE 1. Bird orders, families, species, number of samples analyzed, and number of infected birds.

Order	Family	Species	Common name	*n*	NIB[a]
Accipitriformes	Accipitridae	*Accipiter nisus*	Eurasian Sparrowhawk	1	
		Buteo buteo	Common Buzzard	2	
Caprimulgiformes	Caprimulgidae	*Caprimulgus europaeus*	European Nightjar	2	
Charadriiformes	Laridae	*Larus melanocephalus*	Mediterranean Gull	67	2
		Larus michahellis	Yellow-legged Gull	29	
	Recurvirostridae	*Recurvirostra avosetta*	Pied Avocet	4	
	Scolopacidae	*Actitis hypoleucos*	Common Sandpiper	1	
	Sternidae	*Sterna albifrons*	Little Tern	1	
Ciconiiformes	Ardeidae	*Ardea cinerea*	Grey Heron	1	
		Ardeola ralloides	Squacco Heron	33	
		Bubulcus ibis	Cattle Egret	46	
		Egretta garzetta	Little Egret	31	
		Ixobrychus minutus	Little Bittern	3	
		Nycticorax nycticorax	Night Heron	12	
	Ciconiidae	*Ciconia ciconia*	White Stork	19	
	Threskiornithidae	*Plegadis falcinellus*	Glossy Ibis	30	
Columbiformes	Columbidae	*Streptopelia orientalis*	Oriental Turtle Dove	1	
		Streptopelia turtur	European Turtle Dove	2	
Coraciiformes	Meropidae	*Merops apiaster*	European Bee-eater	6	
	Upupidae	*Upupa epops*	Hoopoe	13	
Passeriformes	Corvidae	*Corvus monedula*	Jackdaw	8	
	Emberizidae	*Emberiza cirlus*	Cirl Bunting	2	
	Fringillidae	*Carduelis cannabina*	Linnet	5	
		Carduelis spinus	Siskin	2	
		Coccothraustes coccothraustes	Hawfinch	1	
		Fringilla coelebs	Chaffinch	4	
		Serinus serinus	European Serin	1	
	Hirundinidae	*Delichon urbica*	House Martin	10	
	Laniidae	*Lanius senator*	Woodchat Shrike	3	
	Motacillidae	*Anthus campestris*	Tawny Pipit	2	
		Anthus pratensis	Meadow Pipit	1	
		Motacilla flava	Yellow Wagtail	2	
	Muscicapidae	*Ficedula albicollis*	Collared Flycatcher	1	
		Ficedula hypoleuca	Pied Flycatcher	24	
		Muscicapa striata	Spotted Flycatcher	9	
	Oriolidae	*Oriolus oriolus*	Eurasian Golden oriole	1	
	Paridae	*Parus major*	Great Tit	2	
	Passeridae	*Passer domesticus*	House Sparrow	17	
		Passer montanus	Tree Sparrow	4	
	Prunellidae	*Prunella modularis*	Dunnock	2	
	Sturnidae	*Sturnus vulgaris*	Common Starling	1	
	Sylviidae	*Acrocephalus scirpaceus*	Eurasian Reed-warbler	2	
		Cettia cetti	Cetti's Warbler	1	
		Hippolais icterina	Icterine Warbler	4	
		Hippolais polyglotta	Melodious Warbler	3	
		Locustella naevia	Grasshopper Warbler	1	
		Phylloscopus bonelli	Bonelli's Warbler	3	
		Phylloscopus collybita	Chiffchaff	29	
		Phylloscopus sibilatrix	Wood Warbler	2	
		Phylloscopus trochilus	Willow Warbler	93	
		Phylloscopus trochilus acredula		5	
		Regulus regulus	Goldcrest	3	
		Sylvia atricapilla	Blackcap	31	

TABLE 1. Continued.

Order	Family	Species	Common name	*n*	NIB[a]
		Sylvia borin	Garden Warbler	18	
		Sylvia cantillans	Subalpine Warbler	26	
		Sylvia communis	Whitethroat	13	
		Sylvia conspicillata	Spectacled Warbler	3	
		Sylvia melanocephala	Sardinian Warbler	5	
		Sylvia undata	Dartford Warbler	1	
	Troglodytidae	*Troglodytes troglodytes*	Wren	3	
	Turdidae	*Erithacus rubecula*	Eurasian Robin	145	
		Luscinia megarhynchos	Common Nightingale	14	
		Oenanthe hispanica	Black-eared Wheatear	2	
		Oenanthe oenanthe	Northern Wheatear	1	
		Phoenicurus ochruros	Black Redstart	9	
		Phoenicurus phoenicurus	Common Redstart	65	
		Saxicola rubetra	Whinchat	1	
		Turdus merula	Blackbird	7	
		Turdus philomelos	Song Thrush	29	
Phoenicopteriformes	Phoenicopteridae	*Phoenicopterus ruber*	Greater Flamingo	113	
Piciformes	Picidae	*Jynx torquilla*	Wryneck	3	
Strigiformes	Strigidae	*Asio otus*	Long-eared Owl	1	
		Otus scops	Eurasian Scops Owl	2	

[a] NIB = number of infected birds.

the conserved matrix gene segment. Superscript II kit (Invitrogen, Carlsbad, California, USA) was used for RT-PCR in a final volume of 25 μl containing 5 μl total RNA in the presence of 0.4 μM final concentration of each primer: M52C, 5′-CTT CTA ACC GAG GTC GAA ACG-3′ and M253R, 5′-AGG GCA TTT TGG ACA AAK CGT CTA-3′ (Fouchier et al., 2000). Cycling conditions included a reverse transcription step for 30 min at 45 C, 15 min at 55 C, and 2 min at 94 C; PCR reaction was performed during five cycles, including 15 sec at 94 C, 30 sec at 45 C, and 30 sec at 72 C and during 35 cycles for 15 sec at 94 C, 30 sec at 55 C, and 30 sec (plus 2 sec per cycle) at 72 C. The RT-PCR amplification products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

To confirm RT-PCR results, positive and weakly positive samples were tested again by real-time quantitative RT–PCR (RT-qPCR) using the same primers as for RT-PCR with a specific hydrolysis probe, 5′-(Fam)GCT AAA GAC AAG ACC AAT CCT GTC ACC TCT G(Tamra)-3′ (Sigma-Proligo, Saint-Quentin, Fallavier, France), for the AIV matrix gene. LightCycler RT-qPCR was carried out using the LightCycler RNA amplification kit for probe hybridization (Roche Biochemicals, Basel, Switzerland). Amplification and detection were performed on a LightCycler 1.5 (Roche Biochemicals) after one cycle of reverse transcription (55 C, 15 min; 95 C, 2 min) and 45 cycles of amplification (95 C, 10 sec; 58 C, 30 sec). The RT-qPCR results were analyzed by LightCycler 3.0 software (Roche Biochemicals). Positive samples were subtyped at the Institut Pasteur (Paris, France), using a SYBR Green-based RT-qPCR technique. For the H5, an RT-qPCR was performed on a LightCycler 1.5 using primers H5/+/1544, 5′-CCG CAG TAT TCA GAA GAA GC-3′ and H5/–/1683, 5′-AGA CCA GCT ACC ATG ATT GC-3′ and the specific probe H5/probe/1638–1662, 5′-(Fam) AGT GCT AGG GAA CTC GCC ACT GTA G (Tamra)-3′ following reaction conditions mentioned above.

Fecal samples were collected and tested

from 1,044 free-living birds representing 72 species, 30 families, and 10 orders (Table 1). Passerine birds ($n=621$) represent 59.5% of our total sampling. Of the 25 samples found positive by the initial RT-PCR, only two fecal samples from Mediterranean Gulls (*Larus melanocephalus*) were confirmed positive by RT-qPCR. Prevalence for this species reached 3% ($n=67$); 2% when all Charadriiformes' samples ($n=102$) or gull samples ($n=96$) were considered.

The prevalence of AIV detected in gulls is comparable with recent data found in the literature for Eurasian species (Fouchier et al., 2003) and to prevalence estimates from numerous studies reporting AIV isolations from gulls and terns from 1974 to 1984 from Eurasia, North, America, and Australia (Stallknecht and Shane, 1988). Based on a summary from all published reports of AIV isolations from gulls worldwide, a prevalence of 1.4% was reported (Olsen et al., 2006). In gulls, AIVs are divided into American and Eurasian lineages, and some specific subtypes, such as the H13 and H16, are thought to be associated with a gull reservoir (Fouchier et al., 2005; Olsen et al., 2006).

Molecular subtyping of hemagglutinin and neuraminidase revealed that the virus from one of the two positive gull samples is H9N2, whereas the H5N1 and H5N2 subtypes were formally excluded for the other sample. The H9 AIV has been isolated in wild ducks throughout the world. However, for gulls, the only available report of H9 viruses is from the Americas (Obenauer et al., 2006), but these were not of the N2 subtype. Influenza A H9N2 viruses have been detected worldwide in poultry, and they currently are endemic in poultry in Asia (Li et al., 2005; Xu et al., 2007). Cases of H9N2 influenza virus infection in humans in this area have also been reported since 1999 (Peiris et al., 1999). Our results support a circulation of H9N2 AIV in Mediterranean Charadriiformes, although more investigations are required to better understand the AIV infection level for Palaearctic species.

Gulls breed in colonies located on small islands in salt marsh areas. Although it is possible that these habitats may be less favorable for environmental transmission via water than freshwater habitats (Stallknecht et al., 1990), this may be offset by a high contact rate. Like gulls, Ciconiiformes, and Phoenicopteriformes breed in colonies, with adults and juveniles crowded in small areas, this creates good opportunities for viral transmission. The absence of positive detection of AIV is of particular value, because very few data are available for these orders.

Prevalence of AIV infections in passerine birds is known to be particularly low (Fouchier et al., 2003; Morishita et al., 1999; Schnebel et al., 2005). Although our results support these observations, further data should be collected on a larger number of species and individuals, and at other periods of the year to more fully understand the ecology of AIV in Passeriformes.

We are grateful to Antoine Arnaud, Leire Paz, Philippe Perret, Christophe Pin, and Nicolas Vincent-Martin for help on birds sampling. We acknowledge the technical help of Frédérique Cuvelier, Patricia Jeannin, Vanessa Roca, and Philippe Thebault for analysis of the samples. Camille Lebarbenchon is supported by a "Tour du Valat/Région Languedoc-Roussillon" PhD grant. This work was funded by the French "Agence Nationale de la Recherche" (ANR) "Santé-Environnement."

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Received for publication 7 November 2006.