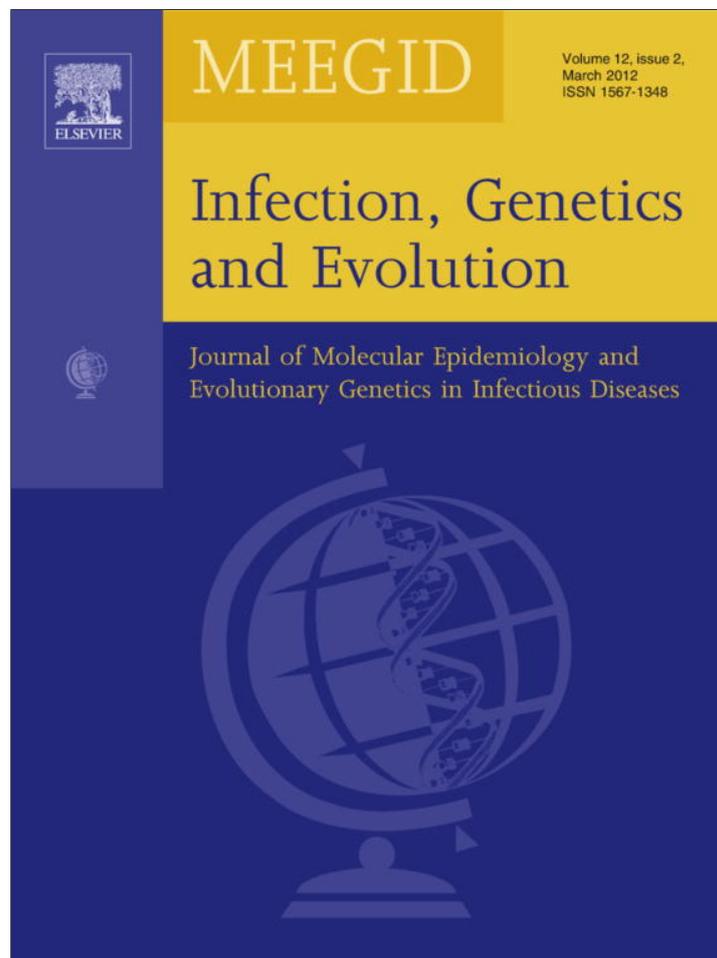


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## Short communication

## Study of influenza A virus in wild boars living in a major duck wintering site

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## ABSTRACT

Wild birds, which are reservoirs of influenza viruses, are believed to be the original source of new influenza viruses—including highly pathogenic ones—that can be transmitted to domestic animals as well as humans and represent a potential epizootic and/or pandemic threat. Despite increasing knowledge on influenza A virus dynamics in wild birds, the viral circulation in wild boars remains largely unknown. This is of particular interest since pigs can be infected with both human and avian viruses; upon co-infection, they can act as a mixing vessel through reassortment, a mechanism that resulted in the emergence of the pandemic H1N1 virus in 2009. The Camargue (Southern France) appears as an ideal study area to investigate inter-species transmission of influenza A viruses from wild birds and possibly humans to wild boars. Indeed, the important local wild boar population shares wetland use with humans and the largest concentration of wintering ducks in France, that are both susceptible to infection by influenza A viruses. Additionally, wild boars occasionally prey on ducks. We conducted a virological and serological survey on wild boars in the Camargue (Southern France) between September 2009 and November 2010. No influenza A virus was detected in the collected nasal swabs ( $n = 315$ ) and no influenza specific antibodies were observed in the serological samples ( $n = 20$ ). As the study was mainly focused on viral excretion, which is limited in time, we cannot exclude that low or occasional influenza A virus circulation took place during the study period. Although, wild boars did not seem to be a key element in the dynamics of influenza A virus circulation in the Camargue, wild boar influenza A virus infections should be more widely studied to determine if the pattern observed here represents the normal situation or an exceptional one.

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## 1. Introduction

Influenza A viruses (IAV) are segmented RNA viruses belonging to the *Orthomyxoviridae* family. They are widespread in the animal kingdom, occurring mainly in birds, humans, pigs, horses, dogs, and sometimes in cetaceans, mustelids, cats, and minks. Wild waterfowl, especially Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and waders), play a central role in their ecology (Alexander, 2000). Waterfowl are the reservoir of all known IAV subtypes from which IAVs can be transmitted to other avian or mammalian species and eventually establish new lineages in a new host.

IAVs are highly variable and undergo continuous genetic evolution via two mechanisms related to their RNA and the segmented nature of their genome. These mechanisms consist in the accumulation of point mutations at each replication cycle and reassortment events involving gene segment exchange that occur when a cell is co-infected by different viruses (Webster, 1998). These mechanisms contribute to the emergence of new variants with the ability to transmit to new hosts and/or with epidemic or even pandemic potential. Analysis of the viruses from past pandemics indicates a key role of pigs in the emergence of IAVs with the potential to initiate human-to-human transmission. Indeed, although the virus responsible for the Spanish influenza pandemic in 1918–1919 was not isolated at the time, its genomic sequence has been determined and revealed an avian-like H1N1 virus that contains human-like signature amino acids in several proteins (Taubenberger et al., 1997) that may, according to recent phylogenetic analyses, have been acquired through replication and reassortment events in pigs (e.g., Dos Reis et al., 2009). Similarly, the human H1N1 virus that caused

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the 2009 pandemic (H1N1pdm09) resulted from a reassortment between a triple (avian/human/swine) reassortant swine influenza virus originating from North America and an Eurasian avian-like swine virus (Neumann et al., 2009). The fact that pigs can be naturally and experimentally infected with human and avian viruses (see Brown, 2000 for a review) further supports the concept of their role as a mixing vessel allowing human and avian strains to reassort.

A better understanding of the epidemiology of IAV in avian, human, and swine compartments and their interplay appears essential. In particular, there is much to learn about the dynamics of IAV in wildlife populations. Since the beginning of the ongoing H5N1 epizootic, wild bird surveillance programmes have been launched worldwide, leading to a rapid increase in our understanding of the epidemiology of avian IAVs (Germundsson et al., 2010; Lebarbenchon et al., 2010a; Siembieda et al., 2010). Despite this increasing knowledge of IAV dynamics in wild birds, very little is known about their circulation in feral pigs and wild boars (both belonging to the *Sus scrofa* species like domestic pigs; Kuntz-Simon and Madec, 2009). Influenza A virus infections in wild boars have rarely been reported. Serological studies performed in several European countries such as in Poland (Markowska-Daniel, 2003,  $n = 746$ ), Spain (Vicente et al., 2002,  $n = 78$ ), Slovenia (Vengust et al., 2006,  $n = 178$ ), and Germany (Kaden et al., 2008,  $n = 1221$ ) emphasize the strong variability of swine influenza A virus (SIV) seroprevalence, which varies from 0% (e.g. Slovenia) to 26% (e.g. Southern Germany) depending on the region. Antibodies directed against the three European subtypes of swine influenza virus (SIV), H1N1, H3N2, and H1N2, have been detected in the different wild boar populations studied, but H1N1 seems to be the most prevalent subtype (e.g., Kaden et al., 2008; Vicente et al., 2002). Interestingly, H1N1 is also the most prevalent subtype in domestic pig populations in Europe (Kyriakis et al., 2011; van Reeth, 2007). In the USA, the few studies that have focused on SIV antibodies in feral swine populations also reported highly variable seroprevalence (0–91%) depending on the region and year (Corn et al., 2009,  $n = 118$ ; Gipson et al., 1999,  $n = 20$ ; Hall et al., 2008,  $n = 215$ ; Salihi et al., 1998,  $n = 120$ ). Moreover, in a recent study performed in South and North Carolina (USA) high seroprevalence was found in feral pigs living near high-density commercial swine production sites while the seroprevalence was null in areas with low-density domestic pig farms (Corn et al., 2009). Nevertheless, disease transmission between domestic and feral pigs or wild boars is poorly understood (Corn et al., 2009), and transmission between birds and wild boars appears to be undocumented.

In this context, studying IAV in populations of feral pigs and wild boars is necessary to understand all components of influenza virus epidemiology. The Camargue situated in the Rhône delta (Southern France) appears as an ideal study area to investigate inter-species transmission of influenza A viruses from wild birds and possibly humans to wild boars. Indeed, although there has been no recent large population study, the total number of wild boars in the Camargue is estimated to range from 2000 to 3000 individuals (Olivier pers. com.). This important local population shares wetlands use with the largest concentration of wintering ducks in France (e.g., Deceuninck and Fouque, 2010), which are commonly infected by IAV (Lebarbenchon et al., 2007a,b). Interestingly, wild boars occasionally prey on dead ducks. During the winter of 2010, up to 160 150 ducks were observed in the region (Gauthier-Clerc unpubl. data). In addition, studies indicate that IAV levels are high in these ducks during early fall and winter (mean prevalence in winter 2006–2007 = 2.1%,  $n = 1119$ , Lebarbenchon et al., 2007b). This raises the concern of potential transmission and establishment of avian IAVs in wild boars, as has been shown to happen with domestic pigs (Pensaert et al., 1981). Furthermore, human populations have increased continuously in the region of focus over the last decade, which has led to increased contact be-

tween humans and wildlife and a large number of humans (e.g., hunters, farmers, naturalists) frequent the habitats used by wild boars. Humans are commonly infected by IAVs especially during winter and could potentially be a source of IAV infection in wild boars. Indeed, human IAVs have been shown to transmit back from humans to domestic pigs and to establish in the pig population. Considering these informations, the Camargue area provides unique opportunities for influenza A virus transmission from waterfowl, and possibly humans, to wild boars. Therefore, to gain insights into the circulation of IAVs in the wild boar population, we conducted a virological and serological survey in the Camargue between September 2009 and November 2010.

## 2. Materials and methods

### 2.1. Animals and sample collections

Because our aims were to test if wild boars are commonly infected by IAVs and to investigate the origin of circulating strains, the analysis was based on nasal swabs with a target set to be able to detect a 1% prevalence with a 95% confidence interval. In case of a positive detection, contrary to serological samples, nasal swabs would also allow isolation of IAV strains for analysis of phylogenetic and molecular characteristics. Samples were taken from wild boars supplied by a network of hunters throughout the Camargue during the 2009–2010 hunting season (September 2009 to January 2010) and at the beginning of the 2010–2011 season (August 2010 to November 2010). Nasal swabs were collected and placed in universal transport media (Copan Diagnostics Inc.) following Offlu recommendations (OIE/FAO, 2009), usually within 1 h of death. In addition, samples were taken from animals caught in the Tour du Valat estate using walk-in drop door traps from December 2009 to November 2010. All traps were baited with soured corn and used a hook attached to a string to trigger the door. Wild boars were marked before a nasal swab and, when possible, a blood sample ( $\approx 8$  ml collected with a sterile needle) were taken. The animal was then released. Nasal swabs were kept at 4 °C during transport to the laboratory at the Tour du Valat research centre and then frozen at  $-80$  °C. Serum ( $\approx 2$  ml) was obtained from whole blood by centrifugation and stored frozen at  $-80$  °C.

### 2.2. Viral analysis

All nasal swabs were analysed for the presence of IAVs at the Molecular Genetics of RNA Viruses unit (Pasteur Institute, Paris). RNA extraction was carried out on 140  $\mu$ l sample using the Nucleospin 96 Virus Core Kit (Macherey–Nagel) following the manufacturer's instructions with minor modifications, and RNA was eluted in 60  $\mu$ l of elution buffer. The presence of IAVs was detected by a one-step TaqMan real-time reverse transcription polymerase chain reaction (RT-PCR) targeting the matrix gene segment using a Light-Cycler 480 (Roche). We chose this method since it is one of those that have been validated and recommended by the FAO to detect H1N1pdm09 and other influenza viruses in swine (FAO, 2010). Amplification was performed on 2.5  $\mu$ l of RNA combined with the Superscript III Platinum kit reagents (Invitrogen) in a total reaction volume of 10  $\mu$ l. The primers and probe were previously described by Duval et al., 2010. The following cycling conditions were used: 15 min at 45 °C, 3 min at 95 °C, 50 cycles of 10 s at 95 °C, 10 s at 55 °C, 20 s at 72 °C, and finally 30 s at 40 °C.

### 2.3. Serological examination

All sera were analysed at Anses (Ploufragan), the national reference laboratory for swine influenza. Sera were tested for antibodies

against the nucleoprotein of influenza A viruses using the ID Screen® Antibody Influenza A Competition ELISA kit (reference FLUACA) from ID-Vet (Montpellier, France) according to the manufacturer's instructions.

### 3. Results and discussion

During the study, a total of 315 nasal swabs were collected, of which 176 were from hunted wild boars and 139 were collected from 105 trapped individuals (34 were captured twice). Samples tested using real-time RT-PCR targeting the M gene gave negative results, indicating that no IAV could be detected in these animals. Our sampling for virological examination extended over the whole year and covered a significant portion of the targeted population (approximately 9–14%, depending on the population estimates). Our results should reflect the epidemiological situation in the Camargue since our sample size would permit to detect a 1% prevalence with 95% confidence. However, it is important to note that the swine viral excretion pattern is variable. Pigs typically excrete influenza A viruses for 7–10 days (Brown, 2000). Also, excretion can be intermittent as shown by Lange et al. (2009) in a study implying pigs experimentally infected with H1N1 swine virus (A/Regensburg/D6/09/H1N1). Moreover excretion timing and duration may differ between domestic pigs and wild boars. Therefore, we cannot exclude that a low and/or occasional IAV circulation took place in the population of focus during the study period. As a complementary analysis, serological tests were performed. A total of 20 sera samples were obtained from 19 trapped animals (one recapture). All ELISA tests for IAV-specific antibodies as markers of past infection were negative. These results, although limited in number, support the idea that few, if any, IAV infections occur in wild boars in the Camargue.

Wild boars could have been infected by water contaminated by viruses shed by infected birds (contact or ingestion) or through direct ingestion of dead infected ducks. Indeed, during the study period, wild waterfowl had IAV infection rates varying from 4% to 15%, depending on the month (Vittecoq et al. unpubl. data). In addition, mallards (*Anas platyrhynchos*) reared and released for hunting in the Camargue during the study period (>30,000 each year; Champagnon et al. unpubl. data) have also been shown to be infected by avian IAVs at variable rates before their release (Vittecoq et al. in preparation). Avian-like viruses have frequently been detected in domestic pigs but to our knowledge have never been reported in wild boars or in feral swine. This absence could reflect a higher probability of avian-to-swine viral transmission in high-density farming conditions: industrial rearing practices create new ecosystems where pathogen transmission is different compared to what is observed in natural habitats (e.g., Lebarbenchon et al., 2010b). Furthermore, as is the case in other mammals including humans, transmission in swine usually implies either animal-to-animal contact via the nasopharyngeal route, with the virus being shed in nasal secretions, or air-borne infection through disseminated droplets or aerosols (Brown, 2000). Such a transmission route is greatly affected by population density and further limits the possibilities of establishing new IAVs in wild boars.

Influenza A virus transmission to wild boars could also originate from humans that share with them the use of wetlands and were infected by the H1N1pdm09 virus throughout our sampling period in this area (GROG, 2010; INVS, 2010). The ability of the H1N1pdm09 virus to transmit back from humans to pigs has been documented and the H1N1pdm09 virus is believed to have become endemic in the pig population in many regions (e.g., Forgie et al., 2011; Hofshagen et al., 2009). However, no human-like virus was observed in wild boars during our study. To our knowledge, no human-like influenza viruses have been observed in other wild boar

populations. This likely illustrates that closer and more frequent contact between humans and swine as in intensive pig farming conditions is necessary for virus transmission. Indeed, antibodies specific to swine influenza are frequently detected in people who work in close contact with domestic pigs (e.g., Krumbholz et al., 2010; Kuntz-Simon and Madec, 2009; Terebuh et al., 2010).

### 4. Conclusions

No influenza A virus was detected in the wild boar population of the Camargue between September 2009 and November 2010, suggesting a low or null viral circulation in this animal population. This finding does not contradict previous serological studies led on wild boars in France and other European countries. Indeed, Payne et al. (2011) recently highlighted very variable seroprevalence in French wild boars populations (0/109 to 31/351 positive individuals) with very low IAV seroprevalence in the two departments from Southern France, Pyrénées-Atlantiques and Aveyron with 0/109 and 1/110 positive individuals, respectively. In the same way, a large serological study underlined very variable seroprevalences in Germany (0/120 to 21/81 positive individuals; Kaden et al., 2009). However, our results are in contrast to the fact that a substantial proportion of the human and waterfowl populations, that share habitats with the wild boars population in the Camargue, were infected by IAVs during the study period. Thus, viral transmission from both waterfowl and humans to wild boars appears to be rare, if any, and wild boars did not seem to be a key element in the dispersion dynamics of IAVs in the Camargue during our study. More studies on feral swine and wild boar infections are needed to determine whether our observations reflect the normal situation or an exception. In the context of the continued circulation of highly pathogenic IAVs such as H5N1 and of the worldwide circulation of the new H1N1pdm09 virus in both humans and domestic pigs, such studies should be integrated into multidisciplinary projects involving research on both the avian, swine and human compartments.

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