



Serological survey of influenza A viruses in domestic and wild *Suidae* in Corsica (France), a Mediterranean island environment

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ABSTRACT

Corsica is a mountainous French island in the north-western Mediterranean Sea. It is a rural area, where pig farming is a major economic activity. Although no acute respiratory outbreaks due to swine influenza A viruses (swIAVs) have ever been reported in this free-ranging pig breeding system, influenza A viruses (IAVs) could be circulating within this pig population. A serological study was conducted as a first approach to domestic pigs and wild boars. Serum samples from 543 pigs raised on 91 different farms were collected during the 2013–2014 slaughtering season, and 279 sera from wild boars were obtained over four hunting seasons (between 2009 and 2014). They were first analysed by ELISA and then IAV positive and doubtful sera were subjected to haemagglutination inhibition tests using antigens representative of the four major enzootic swIAV lineages in Europe, i.e. avian-like swine H1N1 (H1_{av}N1), pandemic-like swine H1N1 (H1N1pdm), H1N2 and H3N2. According to the ELISA results, 26.4% (CI95%: 17.7–36.7%) of herds had at least one positive animal (positive or doubtful by ELISA) and 12.4% (CI95%: 7.8–19.8%) of the pigs tested positive. Using the test characteristics (sensitivity and specificity), the true seroprevalence among Corsican pigs was estimated to be 16.4% (95% CI: 9.9–26.3). Antibodies directed against two different viral lineages were identified: H1N1pdm (in 66.2% and 45.8% of the IAV positive pigs and farms respectively) and H1_{av}N1 (15.0% and 20.8% respectively). Evidence of exposure to viruses from two distinct lineages were detected on a single farm but in two different animals. Among the wild boars, 1.4% (CI95%: 0.4–3.6%) tested positive by ELISA and antibodies against the same two viruses were detected. Altogether, these results suggest that swIAVs from at least two different lineages are circulating among Corsican pigs, i.e. the H1N1pdm virus, probably introduced during the 2009 pandemic, and the H1_{av}N1 virus, which is the most frequent swIAV in Europe. The low frequency of positive results observed in the Corsican wild boars hunted suggests that they would not play a major role in IAV dispersion dynamics on the island.

1. Introduction

Swine influenza is a respiratory disease of pigs very similar to human influenza. It is generally benign but can be exacerbated by co-infections or be maintained within domestic herds. Thus, it can cause substantial health problems and heavy economic losses for the porcine sector (Vincent et al., 2017). Swine influenza A viruses (swIAV) also concern public health in two ways. On the one hand, they are zoonotic by nature (Myers et al., 2007). On the other hand, pigs can potentially serve as mixing vessels, thus permitting the adaptation of avian viruses

to mammals, and the production of reassortants between influenza viruses originating from different animal reservoirs (Olsen et al., 2000). A new H1N1 reassortant containing genes previously adapted to the porcine species has emerged in humans in 2009 and was responsible for the last pandemic (H1N1pdm). It has confirmed the necessity, for human healthcare reasons, to study the viruses circulating in pigs. The H1N1pdm virus, which had never been detected in pigs before being identified in humans, was transmitted from humans to pigs during the pandemic, and has since become enzootic in many porcine populations around the world, and particularly throughout Europe (Watson et al.,

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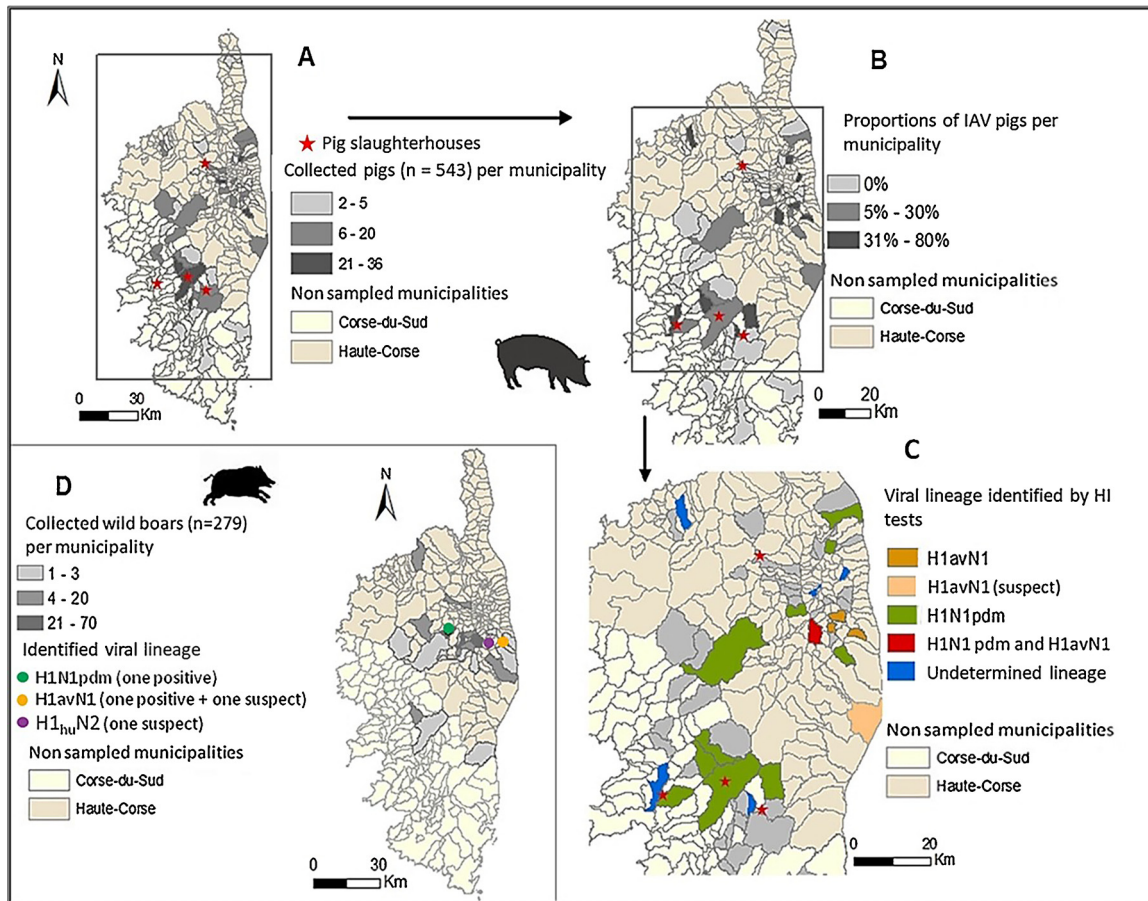


Fig. 1. Location of IAV-positive animals and virus lineages (A: pigs collected per municipality; B: IAV-positive pigs; C: viral lineages identified in the pigs; D: wild boars collected and virus lineages identified in the wild boars).

2015). Since then, four distinct virus lineages have been recognised as enzootic in European pigs: the “avian-like swine H1N1” (H1_{av}N1), the “human-like reassortant swine H3N2” (H3N2), the “human-like swine H1N2” (H1_{hu}N2) and the “pandemic-like swine H1N1” (H1N1pdm) (Simon et al., 2014).

Corsica is a French island in the north-western Mediterranean Sea, 15 km North of Sardinia and 90 km West of Tuscany in Italy. It is the fourth largest Mediterranean island (approximately 180 km long and 70 km wide), and also the most mountainous and most forested. There are two departments (Corse-du-Sud and Haute-Corse) and 365 municipalities (the smallest administrative unit in France) in Corsica (Fig. 1). The island is sparsely populated (36 inhabitants per km²), and while tourism is the leading economic activity, livestock farming (sheep, goats, pigs and cattle) is also an important one. Approximately 50,000 pigs (mainly local pigs) are bred in Corsica and among them 4,000 sows are used for reproduction. They are bred and raised free-range on almost 360 farms, but only 100 of these represent their owners' main income. A little more than 9,000 pigs are slaughtered every year from November to April in official slaughterhouses (ODARC, 2015) but much more are slaughtered illegally at homes. This seasonality in slaughter is linked to the traditional free-range Corsican pigs breeding. Indeed, pigs are used to be fed and fattened with acorns and chestnuts found on grassland during autumn and winter just before slaughter. The importation of live pigs is very limited and they are usually not mixed with local pigs. No respiratory outbreaks due to swine influenza A viruses have ever been reported in Corsica, probably due to the lack of health monitoring in swine. However, swIAVs could be circulating among Corsican pigs, especially since the 2009 pandemic, when many herds were contaminated worldwide due to reverse zoonosis (Simon et al.,

2014). No study on this issue has ever been carried out in Corsica to date. Moreover, as important interactions occur between this free-ranging pig population and wild boars (*Sus scrofa*), swIAVs may circulate from one species to the other, as ever shown for Hepatitis E virus (Jori et al., 2016).

The aim of this work was to assess through serological investigations whether IAVs are circulating among Corsican pigs and, if so, to identify the nature of the viral hemagglutinins (HA) towards whom antibodies are detected, leading to hypothesize the virus lineages that would be involved. Serum samples from wild boars killed by hunters were also analysed to evaluate the extent to which swIAVs may be circulating in the wild *Suidae* population.

2. Material and methods

2.1. Sampling

During the 2013–2014 slaughtering season (from November to April), blood samples from adult domestic pigs (most of them between 1.5 and 3 years old) bred in Corsica were obtained from the four Corsican slaughterhouses available for pigs (three in Corse-du-Sud and one in Haute-Corse, Fig. 1A). The sample size per slaughterhouse was proportional to the number of pigs slaughtered. The national identification system was used to trace the origin of pigs back to municipality level and to determine the owner. Pigs to be sampled were selected using systematic random sampling of each batch from same herd to be slaughtered the day of the sampling visit.

The sample size was first calculated assuming that expected prevalence would be the same as that in continental France, i.e. between

20 and 25% of animals (Hervé et al., 2011). Given the official number of pigs slaughtered every year in Corsica ($n = 9,000$), a relative precision of 20% and a confidence interval level set at 95%, an initial sample size of 281 pigs was thus obtained to estimate this expected prevalence. However, having no previous data about IAV circulation in Corsican pigs, it was decided to nearly double the calculated sample size to account for the large uncertainty. Thus, 543 pigs were sampled at the slaughterhouses. This sample number was sufficient for estimating a pig-level prevalence value of 15% minimum with a relative precision of 20% and a confidence level of 95%.

The animals were randomly selected from the slaughter line. As very small batches could be slaughtered in Corsican slaughterhouses, batches of less than two pigs were excluded. Consequently, the expected number of sampled farms was approximately 90, which was again sufficient for estimating a farm-level prevalence value of 45% minimum with a relative precision of 20% and a confidence interval of 95% for the overall population of 360 Corsican farms. The number of pig farms included in the study ($n = 91$) was not subjected to a strict sampling plan; indeed, it was directly related to the slaughtered pigs. The proportion of positive farms is therefore given as an observed frequency.

Samples from wild boars were obtained from one hunting team (10–20 hunters) over four hunting seasons (from August to February) between 2009 and 2014. These hunters hunt in different areas of the island (but mostly in the centre of Corsica) and they used to collect samples. As there is no reliable assessment of the number of wild boars in Corsica, the collected sera could only be used to report an observed frequency of positive results among the wild boars hunted by this team between 2009 and 2014. The hunters specified the municipality where the wild boars were killed.

2.2. Serological testing

Serum samples were first screened by ELISA to detect antibodies directed against the nucleoprotein of all influenza A viruses (IAV) using the ID Screen® Antibody Influenza A Competition kit (ID-Vet, Montpellier, France) according to the manufacturer's instructions. In a previous study conducted by the French National Reference Laboratory for Swine Influenza, this ELISA was shown to provide reasonably good diagnostic performance (unpublished data). The specificity (Sp) and the sensitivity (Se) of this kit were evaluated as $Sp = 100\%$ (95% CI: 98.7–100; $n = 294$ negative sera from SPF pigs) and $Se = 75.4\%$ (95% CI: 70.2–80; $n = 293$ positive sera from infected pigs). In the present study, positive animals (IAV+) were pigs or wild boars with a positive (S/N ratio > 50%) or doubtful (S/N ratio between 45 to 50%) serum. Other animals (S/N < 45%) were considered negative (IAV-). A pig herd (or a farm) was considered positive if at least one sampled animal tested IAV-positive.

Sera from IAV+ animals were then tested against antigens representative of the viral lineages currently enzootic in pigs throughout Europe, i.e. A/Sw/Cotes d'Armor/0388/2009 (H1_{av}N1), A/Sw/Sarthe/0255/2010 (H1N1pdm), A/Sw/Scotland/410440/94 (H1_{hu}N2) and A/Sw/Flanders/1/98 (H3N2), through haemagglutination inhibition (HI) assays performed according to standard procedures (OIE, 2008). Two-fold serum dilutions were tested starting from a dilution of 1:10. Titers equal to or above 20 were considered positive; those equal to 10 were considered doubtful and those below 10, negative. Given the high specificity of the HI test, as confirmed in low cross-reactions using reference antigens and homologous control sera (data not shown), cross-reactivity is expected to be limited, especially between subtypes. However, it may exist between some lineages within a subtype, such as H1_{av}N1 and H1N1pdm (Kyriakis et al., 2010). Thus, if antibodies against multiple lineages were found in one farm, antibody titers of individual animals were taken into account and interpretation rules were applied in order to discriminate between multiple infections and serological cross-reactions, as previously described (Kyriakis et al., 2013). Briefly, if the same animal had antibodies against several

antigens, it was interpreted as positive against the antigen with the highest titer, considering the other lower titers as cross-reactions in the HI test. An animal showing the same antibody titer level against several lineages was multivalent-positive. Thus, a farm was considered positive for a subtype when at least one pig was found positive.

2.3. Data analysis

The individual seroprevalence estimates of IAVs within the pig population were obtained using a generalised estimating equations (GEE) logistic model with a farm cluster effect (proc genmod, SAS 9.4) to take into account the correlated results expected among pigs sampled in a same farm. In the GEE model, an exchangeable correlation matrix structure was used. As the ELISA characteristics are known (sensitivity and specificity), the true seroprevalence could be estimated (Rogan and Gladen, 1978). Concerning IAV positive farms and positive wild boars, proportions were calculated with exact binomial confidence intervals (CI) of 95%. The proportions for each lineage were calculated among the positive animals (positive or doubtful by ELISA) or farms (with at least one positive animal). Differences in proportions were considered statistically significant if $P < 0.05$.

3. Results

3.1. Pigs

A total of 543 adult pigs from 91 different farms located in 56 municipalities were included in this study (Fig. 1A). Nearly 53% of the animals were from the department of Haute-Corse (Fig. 1A), and six animals per farm were collected on average (95% CI: 5.0–6.9). Among the 543 collected pigs, 80 were found IAV positive (68 clearly positive and 12 doubtful by ELISA test), leading to an estimated apparent pig-level seroprevalence of 12.4% (95% CI: 7.8–19.8) in Corsica (Table 1). The intra-herd correlation in the GEE logistic model was 0.43. Individual seroprevalence values in Haute-Corse and Corse-du-Sud were 14.5% and 10.1% respectively, but were not significantly different ($p = 0.45$). The true seroprevalence of IAV among Corsican pigs was estimated to be 16.4% (95% CI: 9.9–26.3). Positive animals were found in 20 out of the 56 municipalities from which the sampled pigs originated. Thirteen of them were located in Haute-Corse and seven in Corse-du-Sud (Fig. 1B).

Twenty-four out of the 91 sampled pig farms (26.4%; 95% CI: 17.7–36.7) had at least one positive pig. The within-herd frequency of positive animals for IAV-positive farms (adjusted with respect to the number of pigs sampled per farm) was 35.2% (95% CI: 24.7–45.7; Table 1).

After the HI tests performed using antigens bearing HAs from H1_{av}, H1pdm, H1_{hu} and H3 swIAV lineages, respectively, it appeared that none of the 80 IAV+ sera contained antibodies directed against the H3N2 virus. Only 2/80 sera, both issued from a single herd, gave low positive HI titers (20) to the H1_{hu}N2 antigen. As these sera concurrently obtained higher HI titers with the H1N1pdm (320 and 640, respectively) and the H1_{av}N1 (80 and 160, respectively) antigens, these sera were not interpreted H1_{hu}N2 positive. The stronger potential cross-reactivity within H1 subtype was observed for H1pdm and H1_{av} antigens.

Table 1
Proportion of IAV ELISA-positive pigs and pig farms in Corsica.

Analysis level	IAV + percentage	95% CI
Animal ($n = 543$)	12.4 ^a (True seroprevalence: 16.4%)	7.8 – 19.8 (9.9 – 26.3)
Farm ($n = 91$)	26.4	17.7 – 36.7
Within-herd ($n = 24$)	35.2	24.7 – 45.7

^a GEE logistic model estimate accounting for the farm cluster effect; CI = Confidence Interval.

Table 2

Proportion (and number) of the different virus lineages against which antibodies were identified in the sera of IAV-positive animals and farms.

Virus lineage	IAV + pigs (n = 80)	IAV + farms (n = 24)
H1N1pdm	66.2% (53)	45.8% (11)
H1 _{av} N1	15.0% (12)	20.8% (5)
Doubtful results for H1 _{av} N1	1.3% (1)	4.2% (1)
H1N1pdm and H1 _{av} N1	0.0% (0)	4.2% (1)
Negative for each of the 4 lineages (H1 _{av} N1, H1N1pdm, H1 _{hu} N2 and H3N2)	17.5% (14)	25.0% (6)

Positive or doubtful results towards the H1N1pdm virus were obtained in 56 and four sera, respectively. Positive HI titers ranged from 20 to 640, with a mean HI titer of 140. These 60/80 sera came from 15/24 IAV + farms. In two of them, the H1pdm-positive sera tested negative with the H1_{av}N1 antigen. By contrast, in the 13 other farms, most H1pdm-positive sera concurrently obtained doubtful or positive HI titers with the H1_{av}N1 virus. However, they were lower at the individual level in nine of them, leading to interpret these farms as H1N1pdm positive only. In the last four farms with positive or doubtful results with H1N1pdm and H1_{av}N1, one was interpreted positive towards both antigens and the three others were interpreted positive to the H1_{av}N1 only. Finally, two farms had sera that were found positive to the H1_{av}N1 virus only. Altogether, at the individual level, positive and doubtful results towards the H1_{av}N1 lineage were obtained in 39 and 13 sera, respectively. Positive HI titers ranged from 20 to 160, with a mean HI titer of 61.5.

Thus, according to the rules followed to interpret HI tests taking into account possible cross-reactivity within H1 subtype, antibodies against two different viral lineages were identified in the Corsican pig population, i.e. H1N1pdm and H1_{av}N1 (Fig. 1C and Table 2). Antibodies against H1N1pdm were considered in 66.2% of IAV positive animals (n = 80) and in almost 46% of the farms where IAV infection was evidenced (n = 24). Antibodies directed against this lineage was found both in Haute-Corse and Corse-du-Sud (Fig. 1C). Antibodies against the H1_{av}N1 lineage were detected in 15% of IAV-positive pigs and almost 21% of positive farms. An additional farm had a suspect result (highest HI titer was 10) for the H1_{av}N1 lineage. All H1_{av}N1-positive or doubtful farms were located in a limited area in the eastern part of Haute-Corse (Fig. 1C). Both H1N1pdm and H1_{av}N1 lineages were interpreted positive together on one farm located in Haute-Corse, (Fig. 1C). The nature of the IAV antibodies could not be determined by HI tests in six farms (Table 2); three of them had only one ELISA doubtful pig among the tested animals.

3.2. Wild boars

A total of 279 wild boars were collected over four hunting seasons (96 in 2009, 89 in 2010–2011, 86 in 2012–2013 and 8 in 2013–2014) in 25 municipalities but most of these animals (95.2%) were killed in the department of Haute-Corse (Fig. 1D). Four serum samples were found positive or doubtful, which indicated an observed proportion of positive/doubtful results among the hunted wild boars of 1.4% (95% CI: 0.4–3.6). Two of them were from wild boars hunted in 2009–2010. One was found positive to H1_{av}N1 with a HI titer of 20, while the other was doubtful towards H1_{hu}N2 (Fig. 1D). The two other sera were from wild boars hunted in 2012–2013. One was suspected of possessing antibodies to H1_{av}N1 and the other one contained antibodies mostly directed against the H1N1pdm virus, with a HI titer of 160 (against an HI titer of 20 with the H1_{av}N1 virus).

4. Discussion

This is the first study reporting the circulation of IAVs in domestic

and wild *Suidae* populations in Corsica. The seroprevalence of Corsican pigs bred in a free-range system and the apparent prevalence at Corsican pig farm level were respectively 16.4% and 26.4%. In Continental France and in Italy which are the two closer area to Corsica, no serological studies have been carried out since the 2009 pandemic. At animals' level, the seroprevalence of swIAV was 23.2% in France (Hervé et al., 2011) and 63% in Italy (Van Reeth et al., 2008), reaching 48.8% and 88% of positivity at the farm level, respectively. Even if the serological profiles of these pig populations have certainly evolved since the introduction of the H1N1pdm virus in 2009 (Simon et al., 2014), these results show that the pig populations are more exposed to swIAVs in Continental France and Italy than in Corsica Island.

Antibodies against two viral lineages were identified in samples from Corsican pigs: the H1N1pdm virus, which was predominant (66% of IAV-positive pigs) and detected in both Haute-Corse and Corse-du-Sud; and the H1_{av}N1 virus (15% of IAV-positive pigs), which was only found in a small area of Haute-Corse (Fig. 1C).

During the 2009 pandemic, samples from Corsican human patients (n = 54) with influenza-like illness were analysed to detect influenza viruses; H1N1pdm was the only influenza A virus identified and it was found in 63% of the patients (Falchi et al., 2011). Thus, H1N1pdm has clearly spread widely within the human Corsican population, and was certainly transmitted from humans to pigs, as it has all over the world (Nelson and Vincent, 2015). Indeed, pigs were shown to be highly susceptible to H1N1pdm virus infection due to its origins, and many pig populations that were swIAV-free (or almost free) before the pandemic became infected, as in Norway for example, probably because of a lack of immunity to IAVs and/or reduced viral competition (Er et al., 2016). The Corsican situation resembles that reported in another two French islands where pigs are also relatively raised free-range: New Caledonia (south-western Pacific Ocean) and Reunion Island (south-western Indian Ocean), where no acute respiratory outbreaks due to swine influenza A viruses were reported in pigs before the pandemic (Marchal et al., 2011; Cardinale et al., 2012). In these territories, IAV seroprevalence values among pigs were found to be higher than 80% in 2010–2011, H1N1pdm being the only lineage identified. Subsequent to human-to-pig transmission at the time of the pandemic and introduction into pig populations, it has also to be noted that Corsican pigs, as others, could get infected with the H1N1pdm during annual epidemics in humans, as this virus has become a seasonal virus in humans (Watson et al., 2015). No data is available about swIAV circulation in Corsica before 2009, but the fact that H1_{av}N1-positive pigs were found in addition to H1N1pdm-positive pigs in these samples from 2013 to 2014 indicates that H1_{av}N1 viruses might have been circulating among Corsican pigs for several years (perhaps even before the pandemic) as elsewhere in France and Europe, where it is still the most frequently identified swIAV (Simon et al., 2014). Even if the importation of live pigs from continental France or any other country is very limited in Corsica, the number of these animals is not precisely known and H1_{av}N1 viruses could have been introduced from imported animals.

For six IAV + farms, the nature of the serum antibodies was not determined in HI tests using reference antigens representative for the European enzootic swIAVs. This was probably due to a lower sensitivity of the HI test as compared to ELISA, and/or different amounts of hemagglutinin (HA) and nucleoprotein (NP) antibodies, as both methods do not detect the same type of antibodies. Indeed, among these six farms, three had only one IAV + animal which was doubtful by ELISA test, so the undetermined results in HI tests might not draw useful conclusions. However, it could not be excluded that IAV antibodies were directed against an IAV antigen that was not tested in this study, especially in the three other positive farms.

It appears that the frequent interactions between Corsican pigs and wild boars have not really impacted IAV transmission to the wild *Suidae* population. The observed proportion of positive sera from wild boars (1.4%) was very low, and similar to that found in continental France (between zero and 8.8%), suggesting that IAVs were not circulating to a

large extent in wild populations (Vittecoq et al., 2012; Payne et al., 2011). Among the Corsican wild boar population, H1_{av}N1 lineage (one positive and one doubtful result by HI test) was suggested in the same area where pigs were found positive for this lineage. Even if one wild boar had a doubtful result for the H1_{hu}N2 lineage, the lineages identified in Corsican wild boars matched those found in domestic pigs, confirming the hypothesis stated in other French studies that wild boars can become infected with the viruses shed by pigs, but that they do not play a major role in swIAV dispersion dynamics.

Further investigations are now necessary to refine the status of Corsica regarding IAV infections in pigs and to confirm the current circulation of H1N1pdm and H1_{av}N1 viruses. This serological study needs to be complemented by virological investigations into respiratory outbreaks and the isolation of IAVs from domestic Corsican pigs.

Competing interests

The authors declare that they have no competing interests.

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