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Short communication

Circulation of multiple genotypes of H1N2 viruses in a swine farm in Italy over a two-month period

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ABSTRACT

In August 2012 repeated respiratory outbreaks caused by swine influenza A virus (swIAV) were registered for a whole year in a breeding farm in northeast Italy that supplied piglets for fattening. The virus, initially characterized in the farm, was a reassortant Eurasian avian-like H1N1 ($H1_{av}N1$) genotype, containing a haemagglutinin segment derived from the pandemic H1N1 (A(H1N1)pdm09) lineage. To control infection, a vaccination program using vaccines against the A(H1N1)pdm09, human-like H1N2 ($H1_{hu}N2$), human-like H3N2 (H3N2), and $H1_{av}N1$ viruses was implemented in sows in November 2013. Vaccine efficacy was assessed by sampling nasal swabs for two months in 35–75 day-old piglets born from vaccinated sows. Complete genome sequencing of eight swIAV-positive nasal swabs collected longitudinally from piglets after the implementation of the vaccination program was conducted to investigate the virus characteristics.

Over the two-month period, two different genotypes involving multiple reassortment events were detected. The unexpected circulation of multiple reassortant genotypes in such a short time highlights the complexity of the genetic diversity of swIAV and the need for a better surveillance plan, based on the combination of clinical signs, epidemiological data and whole genome characterization.

1. Introduction

Influenza A virus (IAV) is a pathogen commonly found both in humans and in several animal species, including pigs. This species is susceptibility to human and avian IAV (Ma et al., 2009) and this characteristic confers them a crucial role in the ecology and epidemiology of influenza. This can lead to co-infection and genetic reassortment of viruses of swine, human or avian origin. Today, influenza is a common infection of pigs worldwide, which may cause severe respiratory diseases, while infection is maintained in endemic cycles without clear seasonality.

The "classical swine" H1N1 virus was the only lineage isolated from European swine herds until 1979, when a new H1N1 of avian origin was isolated in swine in Belgium and Germany, initially defined "avian-like swine H1N1" ($H1_{av}N1$) (Brown, 2000) and now referred to as "Eurasian avian-like H1N1" (EA) (Brown, 2013). This virus became the predominant circulating genotype in Europe, and subsequently reassorted with the human seasonal IAV that resulted in the co-circulation of three distinct subtypes in Europe: 1) avian-like H1N1 ($H1_{av}N1$), 2) human like-H1N2 ($H1_{hu}N2$), and 3) human-like H3N2 (H3N2) (Brown, 2013).

In April 2009 a novel influenza virus of the H1N1 subtype (A (H1N1)pdm09) was detected in humans in Mexico and the United States (Smith et al., 2009). Genetic studies showed that the virus was of swine origin, containing genes from both the North America triple reassortant (PB2, PB1, PA, HA, NS and NP) and the EA $H1_{av}N1$ (MP and NA) lineages (Neumann et al., 2009). Soon after its appearance in humans, the virus spread worldwide. As early as September 2009 this lineage was detected in European swine (Welsh et al., 2010), and has ever since established in European countries at varying frequencies, with the majority of cases registered in the UK (Watson et al., 2015).

Surveillance of swine farms across Europe between 2009 and 2013 revealed that, in addition to the four predominant genotypes described above (EA $H1_{av}N1$, human-like $H1_{hu}N2$, human-like

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H3N2, and A(H1N1)pdm09), 19 further reassortant genotypes were isolated (Watson et al., 2015). Eleven of these contained the internal genes from the EA $H1_{av}N1$ lineage, seven showed the internal genes from the A(H1N1)pdm09 lineage, while just three genotypes possessed internal gene segments from both the EA $H1_{av}N1$ and A(H1N1)pdm09 lineages (Watson et al., 2015). These 11 genotypes were detected at varying frequencies in European countries, with some circulating either in geographically restricted areas or in a specific country. One such genotype was a reassortant mainly found in Italy and containing the internal genes of the EA $H1_{av}N1$ lineage, while its HA derived from the A/swine/Scotland/410440/1994-like $H1_{hu}N2$ lineage and its neuraminidase (NA) from the A/swine/Italy/4675/2003-like N2 lineage (genotype F in Watson et al., 2015) (Moreno et al., 2013; Watson et al., 2015).

Between August 2012 and August 2013 repeated respiratory outbreaks caused by swIAV were registered in a breeding farm in northeast Italy that supplied piglets for fattening. Initially, the virus characterized during the outbreaks was a reassortant with the HA from the A(H1N1)pdm09 lineage and the NA and internal genes from the EA $H1_{av}N1$ lineage (Chiapponi et al., 2013). In November 2012 a vaccination schedule with the GripoVAC-3 vaccine (Merial) was initiated in sows prior to farrowing to confer high colostrum immunity (Mughini-Gras et al., 2015). However, no remission of clinical signs was observed; an additional vaccination program using a vaccine against the A(H1N1)pdm09 administered to control infection was used in sows in October 2013 (Mughini-Gras et al., 2015). The A(H1N1)pdm09 vaccine was imported and administered under derogation and finally its efficacy was assessed in the field (Mughini-Gras et al., 2015). The vaccine was injected in sows before farrowing, and piglets were monitored virologically over an eight-month period. Such vaccination plans provided the opportunity to implement a post-vaccination efficacy survey by sampling piglets longitudinally and then track the virus evolution at farm level.

We used a whole genome sequencing approach to characterize the genetic constellation of swIAV detected during this study on field vaccination efficacy.

2. Materials and methods

2.1. Study farm

The study location consisted of an intensive, closed-cycle swine breeding farm in the Treviso province, in northeastern Italy, housing approximately 1100 sows/gilts and 23,000 piglets. This farm supplied ~90 day-old piglets to fattening farms for the production of traditional Italian 'Parma' and 'San Daniele' dry-cured hams. Animals were kept under a continuous breeding cycle with production of weekly batches, without applying an all-in/all-out production system.

2.2. Sampling

Circulation of swIAV was assessed by collecting nasal swabs from 75 symptomatic piglets of 45–60 days of age randomly selected, soon after the implementation of the vaccination program (November 2013) against the A(H1N1)pdm09. Following vaccination, virus surveillance in the farm was conducted by collecting nasal swabs from 249 piglets of 35–75 days of age born from vaccinated sows. In total, eight sampling events were performed in: November and December 2013, January (3 sampling events), February, April and June 2014. Sampling was carried out on animals showing clinical signs, and temperature was registered. If animals appeared clinically healthy, random sampling was performed. Therefore, each sampling event had a different sample size. Individual swabs were immersed in MEM (Sigma Aldrich) supplemented with antibiotics, albumin (0.5%) and HEPES buffer and left at 37 °C overnight (WHO, 2002). Viral RNA was extracted from 200 μL of individual samples using a commercial kit (High Pure RNA Isolation Kit, Roche) and according to the manufacturer's instructions. Extracted RNA samples were tested for IAV using a Real Time RT-PCR protocol targeting the M gene (Hoffmann et al., 2010). Swabs positive to type A influenza were characterized by a multiplex RT-PCR (Chiapponi et al., 2012).

2.3. Sequencing and phylogenetic analysis

Nasal swabs collected between November 2013 and January 2014 which tested positive for IAV using real time RT-PCR were subjected to whole genome sequencing using an Illumina MiSeq platform, as previously described (Monne et al., 2014). Eight nasal swabs with the lowest cycle threshold (C_t) values were selected for such purpose: two had been collected in November 2013, two in December 2013, and two at the beginning and at the end of January 2014.

Maximum likelihood (ML) trees were estimated for all the eight gene segments using the best-fit general time reversible (GTR) model of nucleotide substitution with a gamma distribution of among-site rate variation (with four rate categories, Γ_4) and an SPR branch-swapping search procedure implemented in PhyML (Guindon and Gascuel, 2003). A bootstrap re-sampling process (100 replications) was used to assess the robustness of individual nodes of the phylogenies. Sequences are available in GISAID with the following accession numbers: EPI694843 to EPI694906.

3. Results

In the investigated farm, H1N2 was detected in piglets sampled between November 2013 and June 2014 (Table 1).

The phylogenetic analysis of the HA segment for eight selected Italian viruses showed that they form a single well-supported monophyletic group within the $H1_{hu}N2$ lineage, clustering with $H1_{hu}N2$ viruses previously isolated in Italy between 2009 and 2012 (Fig. 1; HA). In support of this finding the NA phylogeny shows that the viruses form a single well-supported group within the "recent human-like H3N2" viruses (A/swine/Italy/4675/2003-like N2 lineage in Watson et al., 2015) and are closely related to the 2009–2012 Italian $H1_{hu}N2$ isolates (Fig. 1; NA) (Moreno et al., 2012). However, the topologies constructed for the six internal genes revealed phylogenetic incongruities between the analyzed viruses, suggestive of multiple reassortment events involving the A(H1N1)pdm09 (red in Fig. 1) and the EA $H1_{av}N1$ (green in Fig. 1) swine influenza strains (Fig. 1; MP, NP, NS, PA, PB1, PB2).

Specifically, between November 2013 and January 2014 we identified two distinct genotypes in the investigated farm (Table 2). Viruses 50206-21, 11546-62 and 11546-65, detected during two different sampling events (December 2013 and end of January 2014), belonged to genotype F, which has been circulating in Italy since 2003 (Moreno et al., 2013; Watson et al., 2015). These viruses

Table 1
Number of positive H1N2 nasal swabs identified in the analyzed farm out of those sampled at each sampling event.

Sampling date	Nasal swabs
18-11-2013	6/75[a]
27-12-2013	6/13
08-01-2014	6/20
15-01-2014	2/11
29-01-2014	4/15
18-02-2014	0/10
01-04-2014	5/60
26-06-2014	5/16