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Phylogeny of Spanish swine influenza viruses isolated from respiratory disease outbreaks and evolution of swine influenza virus within an endemically infected farm

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ABSTRACT

In the present study, outbreaks of respiratory disease were investigated for the presence of swine influenza virus (SIV). In 14 cases the circulating SIV strains were isolated, fully sequenced and compared with other known SIVs. The viruses causing the outbreaks belonged to the H1N1 (including human pandemic H1N1), H3N2 and H1N2 subtypes. In 11/14 cases the phylogenetic analyses indicated the occurrence of probable reassortment events. In the second part of the study, the genetic evolution of H1N1 SIV was assessed in a longitudinal study in closed groups of pigs over six months. Sequencing of the 22 isolates indicated co-circulation of two different variants for the same virus, as well as the emergence of SIV reassortants at certain time-points. These results indicate that reassortment events in SIV are common, and point towards the need for a better understanding of the epidemiology of SIV, particularly in endemic farms.

1. Introduction

Swine influenza viruses (SIV) belong to the genus *Influenzavirus A* within the *Orthomyxoviridae* family. Influenza viruses are enveloped single stranded negative sense RNA viruses in which the genome is organized in 8 different segments encoding 12 different proteins. One of the key characteristics of influenza viruses is their potential for rapid evolution. On the one hand, evolution of influenza viruses is based on the lack of proof-reading activity of the viral RNA-polymerase, allowing the continuous generation of mutations that are responsible for the genetic and antigenic drift (Olsen et al., 2006). In pigs, antigenic drift phenomena have been said to play a minor role in SIV evolution when compared with human viruses (de Jong et al., 2007; Noble et al., 1993). This has been attributed to the high replacement rate in pig herds that implies a high and constant flow of susceptible animals, resulting thus in a low selective pressure for the virus. On the other hand, antigenic shift, that is the arising, by genetic reassortment, of new influenza viruses containing genes of different subtypes and/or genotypes of a same subtype, is considered

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to be a major force in the generation of influenza pandemics. For example, the reassortant human-like H3N2 SIV and the reassortant human-like H1N2 SIV circulating in Europe are the product of the reassortment between European avian-like H1N1 SIV and human (seasonal) H3N2, and between reassortant human-like H3N2 SIV and human H1N1, respectively (Campitelli et al., 1997).

It is also known that pigs can be infected with avian, swine and human influenza A viruses, and for that reason, pigs have been classically proposed to be the mixing vessel where reassortant "humanized" influenza strains can arise (Brown et al., 1998; Castrucci et al., 1993; Scholtissek et al., 1985). The reason behind this concept is related to the fact that avian influenza viruses have a high affinity for α-2,3 sialic acid receptors while mammalian influenza viruses usually bind to α-2,6 receptors. Pigs have both types of receptors (Ito et al., 1998). It is now known that avian viruses can infect humans without previous adaptation in pigs, as is the case with the highly virulent avian H5N1 (de Jong et al., 1997). Nevertheless, the recent emergence of a human pandemic influenza A H1N1 virus (pH1N1) harbouring SIV genes and the arising of new reassortants between H3N2 SIV, H1N2 SIV and the pH1N1 isolated from pigs and minks are evidence favouring the notion that reassortment occurs frequently in SIV (Moreno et al., 2011; Tremblay et al., 2011).

The entry of a new influenza virus into a swine herd is classically considered to cause a clinical outbreak with common flu signs: fever, lethargy, conjunctivitis, nasal discharge, coughing, laboured breathing and eventually abortions (OIE, 2008). However, increasing evidence indicates that SIV infections are often endemic and may remain as a subclinical or insidious problem (OIE, 2008; Simon-Grifé et al., 2012). Thus, endemic situations where viral circulation keeps on going for prolonged periods are optimal for studying drift and shift phenomena. The objective of the present study was to characterize influenza A virus isolated in clinical outbreaks in Spanish swine herds and to establish their genetic relationship with other SIV, as well as to assess the evolutionary events in SIV circulating in an endemically infected pig farm.

2. Material studied, area descriptions, methods, techniques

2.1. Ethics statement

The present study was carried out in accordance with the guidelines of the Good Experimental Practices (GEP) standard adopted by the European Union, and with the recommendations approved by the Animal and Human Ethics experimentation Committee (CEEAH) of the Universitat Autònoma de Barcelona, that ensures the protection and welfare of the animals used in research, in agreement with the current European Union legislation.

2.2. Sampling

2.2.1. Outbreaks of respiratory disease suspected to be swine influenza

This study was conducted from January 2010 to August 2011 in a NE Spain area that accounts for more than 40% (>10 million pigs) of the Spanish pig production, with the collaboration of swine veterinarians who reported the cases. Twenty-two reports of respiratory disease outbreaks compatible with SIV were followed-up. For each suspected case, clinical data and the age group of affected animals were recorded. In each case, nasal swabs from 20 animals showing clinical signs were collected. These swabs were immediately suspended in 1 ml of transport medium (PBS, 0.15 M, pH 7.2 70%; glycerol 20% and 10% of penicillin/streptomycin solution) and sent to the laboratory at 4 °C where they were processed (<24 h after collection). When possible, lungs from dead or euthanized pigs were also sent to the laboratory. Two additional cases with no clear signs of influenza were included in the study because of in-field positive influenza results (Flu Detect Swine test; Synbiotics, Lyon, France) reported by the veterinarian.

2.3. Longitudinal study carried out in a farrow-to-finish farm

For the purpose of the present study, a whole batch of 3-week-old piglets ($n = 121$; 11 litters) was followed up during the whole productive period until pigs were sent to the slaughterhouse. The vaccination plan of the farm did not include SIV vaccination. Animals were ear-tagged at the beginning of the study in order to follow them individually, and were sampled (nasal swabs) weekly fr0m 3 to 13 weeks of age. The pigs were then sampled at 15, 17, 20 and 24 weeks of age. From these samples, 22 isolates were selected to be analyzed corresponding to pigs of weeks 3 of age (eight isolates), 4 weeks (four isolates), 7 weeks (five isolates), 13 weeks (three isolates), 15 weeks (one isolate) and 20 weeks of age (one isolate). Infection dynamics, clinical outcome and subtype characterization (H1N1) of influenzavirus infection in the farm are described in Simon-Grifé et al. (2012) and correspond to Farm 1 in the cited work.

2.4. Processing of samples

Viral RNA was extracted with a commercial kit (Qiamp, Qiagen) according to the instructions of the manufacturer, and detection of SIV was performed by means of a Taq-Man real time reverse transcriptase/polymerase chain reaction (RRT-PCR) aimed at detecting the *M* gene of influenza A viruses (Busquets et al., 2010). Samples yielding RRT-PCR positive results were inoculated into specific pathogen free (SPF) embryonated chicken eggs (ECE) in an attempt to isolate SIV. Briefly, nasal swab suspensions were centrifuged, and 100 μl of the supernatant were inoculated into the allantoic cavity of 9–11-day-old ECE. Allantoic fluid was harvested 3 days after inoculation and viral growth was detected by the haemagglutination assay using chicken red blood cells. Negative allantoic fluids in the first passage were inoculated in ECE again before being discarded. At the same time, nasal swab suspensions were inoculated into Madin-Darby Canine Kidney (MDCK) cells cultured with added trypsin (2 μg/ml) (Sigma–Aldrich). Cell culture supernatants were collected at approximately 75% cytopathic effect, centrifuged and later tested as above. Samples were discarded if negative after the second passage in cell culture. ECE allantoic fluid was only selected