

Pandemic influenza A/H1N1 virus in a swine farm house in Sicily, Italy

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Abstract

This report describes a pandemic A/H1N1 (H1N1 pdm) virus outbreak occurred in December, 2009 in a swine farm used as research facility (Istituto Mediterraneo Trapianti e Terapie ad Alta Specializzazione) for preclinical studies, located in Sicily, Italy. All the 13 pigs of the farm, showed cough, fever, inappetence and weakness. At the same time, an unvaccinated worker of the stabling showed influenza-like symptoms. RNAv extracted from two swabs collected from infected pigs resulted positive by Real Time RT-PCR for Influenza A virus. Furthermore, after growth on embryonated eggs, viral isolates were identified by Real Time RT-PCR specific for H1N1 pdm virus and characterized antigenically. Sequencing of the whole genome was also performed. All sera taken from animals and from the worker were tested by a competitive Influenza A ELISA and by the haemoagglutination inhibition test. Serological findings confirmed the circulation of influenza virus H1N1 pdm in pigs and the presence of specific antibodies against H1N1 pdm in human serum. The results of this study seem to support a H1N1 pdm transmission from man to animals showing the importance of serological and virological investigation to control the pig farms and the importance of close cooperation between the different authorities like veterinarian and human public.

Publication Data

Paper received:
18 August 2010

Revised received:
05 January 2011

Accepted:
07 March 2011

Key words

Swine, Pandemic influenza, A/H1N1 virus, Sequencing, Zoonosis

Introduction

The first cases of H1N1 pdm infection in human were reported in March-April, 2009 in Mexico and California (proMED-mail, 2009a). The rapid spread of the virus, reported in more than 200 countries, has led in June, 2009 the World Health Organization (WHO, 2009a,b) to declare the level of the sixth phase of pandemic alert.

The H1N1 pdm virus can be defined as a chimeric virus with gene segments derived from quadruple reassortment of influenza viruses of avian, porcine and human origin (Ma *et al.*, 2010; Vincent *et al.*, 2008). The virus transmission from man to animals (turkey, dog, cat) has been demonstrated (Hofshagen *et al.*, 2009; WHO 2009a). The first outbreak of H1N1 pdm infection in pigs was

reported in a herd in Canada in May, 2009 (proMED-mail, 2009b). In Italy, the first isolation of H1N1 in pigs has been identified in Nerviano (Lombardia), in a herd of 1,250 animals, of which 375 subjects had symptoms of influenza (proMED-mail, 2009c). In this circumstance any restrictive control measure was applied and the outbreak ended with the spontaneous healing of all animals; it hasn't been possible to trace the source of infection.

The aim of this study was to describe the second outbreak of H1N1 pdm in pigs in Italy, which occurred in a research facility in Sicily, following a probable transmission from an infected individual.

Materials and Methods

Animals: The survey was carried out on 13 female hybrid pigs Pietrain x Pic breed at an Italian herd, located in Sicily (Latitude 37°49' 51" N; Longitude 13°25' 51" E), Italy. The farm is being used as a research facility (Istituto Mediterraneo Trapianti e Terapie ad Alta Specializzazione) for preclinical experimental studies.

On 9th December 2009, the presence of flu syndrome in the farm animals was reported. All the pigs, with different age (ranging between 2 and 4 months) and body weight (21-25 kg), showed cough, fever (+38/+39°C), sneezing, nasal discharge, eye discharge, lack of appetite, lethargy, dullness, breathlessness and weakness. No other clinical signs were observed and no animals died. At the same time, a worker of the stabling, not subjected to influenza vaccination, showed influenza-like symptoms.

Experimental design: At the beginning of the study, nasopharyngeal and oropharyngeal swabs were made in all pigs. After 7 days of the onset of the disease, nasopharyngeal and oropharyngeal swabs were made again, from all pigs housed in the farm. At the same time, blood samples were collected by means of a jugular or mammary venipuncture. One of the infected subject (n. 13) was sacrificed and 17 days after onset of the disease, more diagnostic tests and trials in pigs were performed and on man assigned to the enclosure blood sample was collected.

Laboratory assays: The viral RNA (RNA_v) extracted from swabs was examined by real time RT-PCR for identification of influenza viruses type A (Spackman *et al.*, 2002) and by Real Time RT-PCR specific to H1N1 pdm virus, according to the CDC Atlanta (USA) protocol (WHO, 2009b). The samples were also inoculated into *Gallus gallus* Specific Pathogen Free (SPF) eggs and in MDCK (Madin-Darby canine kidney) and Caco-2 (human colon adenocarcinoma) cells. Sera were tested using a competitive ELISA for the detection of antibodies against the nucleoprotein (NP) of influenza viruses type A as screening test (De Boer *et al.*, 1990). The haemagglutination inhibition test (HI) was also performed according to OIE Manual (2008) using the reference strains: A/Sw/Fin/2899/82 H1N1, A/Sw/CA/3633/84 H3N2 and swine field strains: A/Sw/290271/09 (H1N1) pdm, A/Sw/It/1521/98 H1N2 (Marozin *et al.*, 2002). For the complete sequencing of the strains the RNA_v was extracted from of 1 allantoic passage in embryonated eggs, using the kit QIAamp[®] Viral RNA Mini Kit. N° 46 one-step RT-PCR for amplification of the entire viral genome with a specific primers set (Applied Biosystems, Foster City, CA, USA) were then held. Sequencing reactions were prepared after marking each amplicon. Sequence analysis was performed using the software Lasergene-DNAStar.

Results and Discussion

The swabs taken during the first inspection in the farm from 2 pigs (n. 10 and n. 13) with acute symptoms resulted positive for H1N1 pdm virus by real time RT-PCR. Two viruses were isolated at first passage in embryonated eggs: A/Swine/Italy/85437/2009 and A/Swine/Italy/85429/2009 (HI titer = 64). Only A/Swine/Italy/85437/2009 strain grew in Caco-2. Viruses isolated were identified by Real Time RT-PCR specific to H1N1 pdm virus. Subsequently, they were sequenced. The sequences obtained were deposited in Gen Bank (accession numbers: IZSPA-85429.1 CY057075-82 and IZSPA-85437 CY061544-51). The sequences of the 2 isolates showed a high percentage of homology, up to 100%, for all gene segments analyzed. Comparing these sequences with similar sequences available in Gen Bank, it is possible to highlight some unique aminoacidic changes (PB2: V227I; PA: I14V e Q256K; M1:

Table - 1: Serological results of ELISA and HI tests in pigs

Pig no.	ELISA	A/Sw/Fin/2899/82 H1N1	A/Sw/290271/09 H1N1 pdm	A/Sw/It/1521/98 H1N2	A/Sw/CA/3633/84 H3N2
1	+	-	≥1280	-	-
2	+	-	≥1280	-	-
3	+	-	320	-	-
4	+	10	40	-	-
5	+	-	40	-	-
6	+	-	-	-	-
7	+	-	-	-	-
8	-	-	-	-	-
9	+	-	10	-	-
10	+	-	-	-	-
11	+	-	20	-	-
12	+	20	20	-	-

+ = Positive, - = Negative

M43L; NS1:E71K) hitherto not associated with markers of virulence of influenza viruses.

At the second sampling all swabs were negative at real time RT-PCR for the detection of Influenza-A. The serological results are reported in Table 1. Sera with titres < 10 were considered negative. It is noteworthy that sera from pig 1, 2 and 3, resulted high positive to H1N1 pdm virus, did not cross-react in HI tests against other antigens. Low HI titres against A/Sw/2899/82 H1N1 observed in pig 4 and 12 could be due to prior exposition to circulating H1N1 swine influenza viruses. Pig 10 was positive only by ELISA. No more information on pig n. 13 could be collected (slaughtered).

After 17 days of the onset of the disease, the animals showed no clinical symptoms. Only the ill swine (n. 10) showed a very slow recovery with decrease in weight. From swabs taken on that date, was not detected the virus by real time RT-PCR. The serological titres in HI have confirmed the results obtained in previous tests, except the pig 10, which showed specific seroconversion (HI titer = 40) to the influenza virus (H1N1 pdm). The serum of the enclosure officer presented a titre of 128 in HI for H1N1 pdm, as confirming the suspected prior infection.

During the current pandemic, contact with infected people seems to be the most likely route of H1N1 pdm transmission from man to animals. Epidemiological data of the described outbreak and the results of serological positivity to H1N1 pdm found in the person in contact with animals, seems to support this hypothesis.

In analogy to that reported in previous outbreaks of natural infection by H1N1 pdm in pigs, the disease has not caused severe clinical symptoms, mainly it is observed weight loss (Hofshagen *et al.*, 2009; proMED-mail, 2009b). However, if it spread at high levels, the infection could become a problem for the pig industry, because it would cause major economic losses.

The transmission of influenza viruses from man to animals is not only a problem confined to the livestock sector, but it is a public health issue in term of zoonotic potential represented by these viral agents. Swines have not played an important role in the epidemiology of H1N1 pdm infection (WHO, 2009a). This does not mean that, as a result of viral evolution, the swine could not become a more efficient agent of viral spreading, becoming responsible for transmission of influenza viruses to humans. Furthermore, the coinfection of the same animal with different influenza viruses, might lead to the genesis of new variants derived from viral reassortment which could be more virulent and dangerous for animals and/or humans (Ma *et al.*, 2009; Ma *et al.*, 2010; Vincent *et al.*, 2008).

Therefore, the results of this study confirm the importance of serological and virological investigation to control the pig farms. Furthermore, the reported data point out the importance the monitoring of influenza viruses circulation for animals in research facilities. The identification of pandemic virus infection in animals demonstrates again the importance of close cooperation between the different authorities like veterinarian and human public health in order to fight emerging zoonotic infections in a timely and coordinated way.

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