

Isolation and identification of pandemic influenza virus H1N1/2009 S-OIV from commercial and backyard swine in Costa Rica.

Aislamiento e identificación del virus pandémico influenza H1N1/2009 S-OIV en cerdos de explotaciones comerciales y de traspatio en Costa Rica.

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Abstract: Influenza viruses are a threat not only to international trade of animal products but also to human health. The isolate H1N1/2009 S-OIV was diagnosed in mid-2009 in Mexico and the United States of America as a new variant of influenza in humans, and then was declared a pandemic by the World Health Organization (WHO). Costa Rica was the fourth country in the Americas to demonstrate the presence of this swine variant in humans. In 2010, the Food and Agriculture Organization of the United Nations (FAO) encouraged public awareness in some American countries like Costa Rica, where FAO sponsored and organized a program for detection and characterization of influenza H1N1 in pigs. To ascertain whether H1N1/2009 S-OIV was present or not in Costa Rican swine herds, a distributed sampling of 509 pigs from 25 farms was conducted from across all of the Costa Rican provinces. The results confirmed the presence of this subtype in at least 2 of the sampled farms. In one case, nasal swabs from 11 pigs tested positive in Real-time PCR (RRT-PCR) and were confirmed using virus isolation. The second case was found positive by RRT-PCR but was not confirmed by virus isolation. Phylogenetic studies based on HA, NA and M genes showed that this variant is closely related to previously reported H1N1/2009-S-OIV worldwide sequences from human origin. This finding should be brought to the attention of local and global human and animal health agencies (e.g. WHO, OIE). This report also shows the usefulness of conducting surveillance

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on farms where poultry, wild birds, pigs and humans interact, in order to improve early detection and prevent the spread of possible new variants that can become pandemic strains.

Keywords: Influenza Virus, PCR Real Time, H1N1/2009 S-OIV, swine, pigs, public health, Costa Rica.

Resumen: Los virus de influenza representan una amenaza no solo para el comercio internacional de bienes de origen animal sino también para la salud humana. A mediados del 2009 se diagnosticó una nueva variante de la influenza H1N1/2009 S-OIV en humanos en México y Estados Unidos, la cual culminó con una declaratoria de pandemia por parte de la Organización Mundial de la Salud (OMS). Costa Rica fue el cuarto país en América en el que se demostró la presencia de esta cepa de origen porcino en seres humanos. En el 2010 la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO, por sus siglas en inglés) promovió la toma de conciencia pública en algunos países de América como Costa Rica, donde patrocinó y organizó un programa para la detección y la caracterización de la influenza H1N1 en cerdos. Con el propósito de determinar si el subtipo H1N1/2009 S-OIV circulaba en los cerdos del área, se llevó a cabo un muestreo de 509 cerdos de 25 granjas distribuidas por todas las provincias de Costa Rica. Los resultados confirmaron la presencia de este subtipo en al menos dos de las granjas analizadas. En un caso, se detectó la presencia de este virus en hisopados nasales a través del RT-PCR de tiempo real en 11 cerdos, cuyo resultado posteriormente se confirmó en cultivo celular. El segundo caso se detectó por PCR mas no pudo ser confirmado por cultivo. Los estudios filogenéticos realizados en los genes HA, NA y M de este virus muestran que la cepa aislada está estrechamente relacionada en un mismo cluster dentro de las secuencias H1N1/2009 S-OIV analizadas a nivel mundial de origen humano. Este hallazgo debe darse a conocer a los servicios de salud humana y animal tanto a nivel local como global (por ejemplo, la OMS o la OIE). Este artículo igualmente muestra la utilidad de realizar estudios de vigilancia en granjas donde interactúen aves domésticas, aves silvestres, cerdos y seres humanos, con el objetivo de mejorar la detección temprana y prevenir el esparcimiento de nuevas variantes que puedan llegar a convertirse en cepas pandémicas.

Palabras claves: Virus de la influenza, PCR tiempo real, H1N1/2009 S-OIV, porcino, cerdos, salud pública, Costa Rica.

INTRODUCTION

Influenza viruses belong to the Orthomyxoviridae family, which are enveloped viruses with a segmented genome of single negative stranded RNA. It is well established that the lack of a polymerase proof-reading mechanism during RNA replication makes these viruses prone to high genetic variability. Under immunological pressure, those "minor antigenic changes" are known as "antigenic drift". Adding to the variation capabilities of RNA viruses, genetic mutations can be accompanied by recombinational events (gene swapping) from the segmented genome. This feature is essential in the pathogenesis of the influenza virus and the emergence of new pandemic variants because two different subtypes infecting the same cell may conduct big genome rearrangements or "major antigenic changes", also known as "antigenic shift" (Karasin, Carman, & Olsen, 2006). Through this variation mechanism, at least 17 Hemagglutinin (HA) gene subtypes of the influenza virus (Tong et al., 2012 recently described the HA 17 variant in bats in Guatemala) and 9 Neuraminidase (NA) gene varieties (Fouchier et al., 2005).

Although influenza viruses are typically host specific, interspecies transmission does occur (Zimmer & Burke, 2009). Aquatic birds (wild waterfowl) are considered the primary reservoir, and all these subtypes have been isolated in waterfowl (Tong et al., 2012) but occasional transmission between different host species has been well documented, raising the level of public health concerns about the continuous risk of creating novel strains with zoonotic potential. In mammals subtypes H1N1, H2N2 and H3N2, and less frequently the H5N1 and H3N8 subtypes, established themselves in humans (Di Trani et al., 2006). Subtypes H3N8 and H7N7 were isolated from equines (Guo et al., 1992; Murcia, Wood, & Holmes, 2011) and H1N1, H3N2 and H1N2 have been isolated from pigs (Gibbs, Armstrong, & Downie, 2009; Nalluswami et al., 2011; Zell et al., 2008). In the mid-70s, H3N2 strains that were circulating in pigs in Europe were antigenically related to H3N2 strains circulating in humans (Ottis et al., 1982). Since 1998, a triple gene rearrangement in H3N2 influenza viruses has given way to human, avian, and swine lineages that have been isolated in pigs in the United States and Canada. Swine are an important host because of the permissiveness of their tracheal epithelial cells to be infected by both bird and human influenza strains, favoring gene rearrangement (Ito et al., 1998). In the United States these strains were mixed with classical swine H1N1 strains to create a variant H1N2, which was isolated in humans around the world in the following years (Karasin et al., 2000; Lin, Gregory, Bennett, & Hay, 2004)

Such a concern was confirmed with the pandemic H1N1/2009 S-OIV declared by the World Health Organization (WHO) (for an extensive review of the worldwide distribution of H1N1/2009 S-OIV, initially denominated "swine flu", see Dotis & Roilides, 2009). As a result of this situation, an initiative was raised by the United Nations Food and Agriculture Organization (FAO) in the Americas with the purpose of answering at least one of following three questions:

- Are swine the source of human exposure?
- Can we demonstrate the absence of the Pandemic H1N1/2009 S-OIV virus?
- Is the Pandemic H1N1/2009 S-OIV virus being detected and collected from swine populations?

Therefore, the main purpose of this study was to determine whether variants of the Pandemic influenza A H1N1/2009 S-OIV and/or other influenza subtypes were circulating in Costa Rican swine herds during 2010. The results obtained confirmed the detection of this strain in pigs in at least two provinces of Costa Rica, although no human cases related to these findings have been reported so far. Genetic characterization of Costa Rican influenza strains showed that they are closely related to previously described H1N1/2009 S-OIV variants from humans. Therefore, this paper shows the need of conducting follow up surveillance studies in farms where poultry, wild birds, pigs and humans interact, in order to improve early detection and prevent spreading of possible new variants that can become pandemic strains.

Materials and Methods

Epidemiological Surveillance. A disease surveillance scheme was adapted for this study from the "FAO guidelines for surveillance of pandemic H1N1/2009 and other influenza



viruses in swine populations". The surveillance program targeted backyard, small producers' and high risk pig populations, particularly including farm pigs with respiratory diseases with signs consistent with the case definition of swine influenza. The case was defined as a cluster of clinical cases of pigs showing fever, sneezing, coughing, or with nasal discharge in at least one individual observation unit, developed during a one-week period and affecting at least 10% of the animals present in the unit. Examples included units with less than 10 backyard pigs, in which at least 2 respiratory cases were observed within the one-week period (Ferrari et al., n.d.).

The Observation Unit (OU) was defined as a group of pigs physically confined within the same space, which could be an entire shed or individual pens. Because no cases of H1N1 human influenza have ever been reported in Costa Rica linked to pigs, the case definition adopted in this program refers to general surveillance aimed at detecting and collecting swine flu viruses. Each individual unit was sampled according to the following criteria: samples were collected from no more than 20 animals for each OU. When the number of clinical cases was more than 20, all nasal swabs were collected from clinically affected animals; if the number of clinical cases was less than 20, samples were collected from other animals in contact to obtain the necessary 20 samples. If the overall number of pigs present in the unit of observation was less than 20, samples were taken from all those animals.

Under these conditions, a total of 509 samples from pigs distributed in 25 OU were taken and processed during the month of October 2010 and the first two weeks of November 2010. The OU was selected randomly, and at least 2 OU were picked in each province (Table 1).

Nasal swab samples were taken and immediately placed in separate tubes/containers with brain heart infusion broth (BHI). Samples were not pooled. All samples were labeled with a unique identification code, which was also used to record animal information on the sampling questionnaire (Supplementary Figure 1). Samples were sealed and maintained in refrigeration until tested.

Nucleic acid extraction. RNA was extracted from the samples using MagMAX™ magnetic beads for Viral RNA (Ambion, Austin Texas), following the manufacturer's instructions.

Real-Time Reverse Transcription RT-PCR (RRT-PCR) of type A influenza virus. Real-Time Reverse Transcription Polymerase Chain Reaction (RRT-PCR) assays designed for the detection of the 2009 H1N1 Influenza A virus included detection of the virus matrix (M) and neuraminidase (NA) genes using the standardized protocol provided by the National Veterinary Services Laboratories (NVSL) as described in Spackman et al., 2002. Samples were reverse transcribed and amplified using One step RT-PCR reagents, (Qiagen, Valencia, CA). For each 12.5 µl reaction tube a volume of 7.2 µl of extracted sample was used. Primers used for the matrix gene amplification (type A influenza) were 5' M+25, and 3' M-124, M-124 SIV, at a final concentration of 0.4 µM, plus the FAM probe M+64 at a final concentration of 0.12 µM.

Each RRT-PCR test included the following controls: ADV 204 Strain H5N9 (A/TY/WI/68) as extraction control; 305-IDV 0901 ADV Strain H1N1 (A/California/04/2009) and 203 ADV strain (CK/PA13552-1/98) as amplification controls. For quantification of the limit of detection (l.o.d.) of the matrix RRT-PCR, type A influenza, 1:10 serial dilutions of the ADV

203 Positive control stock (0.1 ng / µl concentration) were run in triplicate in RRT-PCR, using the test conditions described above.

RRT-PCR detection of influenza virus H1N1/2009 S-OIV. Samples that resulted positive for Matrix influenza A gene amplification were tested a second time using primers 5' N1-F210, and 3' N1-R330 (Suárez and Spackman, unpublished). The primers final concentration was 0.4 µM and 0.12 µM for the probe N1-F232, labeled with FAM. Reagents were from One step RT-PCR (Qiagen, Valencia, CA). with a final reaction volume of 12.5 µl and 7.2 µl of extracted sample.

Virus Isolation Assays. Those swabs with a positive RRT-PCR result were sent to the National Veterinary Services Laboratories (NVSL) for isolation and characterization of the virus. Virus isolation was performed using Madin Darby canine kidney cells (MDCK). For this purpose, swabs in transport medium (BHI) were stirred vigorously, then centrifuged at 1800 x g for 20 min at 4° C. The supernatant was diluted 1:2 or 1:3 in MEM and held at 4° C until processing. Confluent monolayers of MDCK cells were inoculated and incubated at 37° C for 5 to 7 days and during this period were observed daily to detect the presence of cytopathic effect (CPE). In samples where CPE was observed the pathogen was grown to reach highest possible titers (70-90% of the infected cells); confirmation by sequencing was performed covering the 100% of M, HA and NA genes using an automated system (ABI 3700) at the NVSL. The purified PCR products were sequenced bidirectionally using primers specific for these genes (Chan et al., 2006) (Table 1).

Table 1. Primers used for sequencing the Influenza A HA, M and NA genes.

Set	Gene Segment	Oligo	Sequence
a	4	HA+1Chan	AGCAAAGCAGGGGAAAATA
a	4	H1-703s	TTCCGGCTTGAACCTTCTTGC
b	4	H1+595	AAGAAGTCCTTGTGCTATGG
b	4	H1-1340s	TCGGCATTGTAAGTCCAAAT
c	4	H1+1099s	GGGATGGTAGATGGATGGTA
c	4	HA-1778Chan	AGTAGAAACAAGGGTGTTTT
A	6	N1-720S	CACATGCACATTCAGACTCT
A	6	NA+1Chan	AGCAAAGCAGGAGTGAAAA
B	6	N1+558	GTCGCTTGGTCAGCAAGTGC
B	6	N1-1103	TCTCCCTATCCAAACACCAT
C	6	N1+960	ATAGGATACATATGCAGTGG
C	6	N1rev	TCACTATAAGTAGAAACAAGGAG
a	7	M+1Chan	AGCAAAGCAGGTAGATATT
a	7	M-741s	GCCTGTAAATTTTCAAGAAG
b	7	M+605s	GGATCGAGTGAACAGRCAGC
b	7	M-1027Chan	AGTAGAAACAAGGTAGTTTTT



Phylogenetic analysis. The assembled sequences were aligned using guidance software (Penn et al., 2010). HA, NA, and M genes were analyzed with genes from viruses that were phylogenetically representative of other lineages and included genes from viruses from multiple sources (wild birds, poultry, and mammals) and from multiple geographical regions including North America, Europe, and Asia (National Center for Biotechnology Information, Influenza Virus Resource, <http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>). Codon-based alignment using MAFFT and 100 bootstrap replicates were selected for the multiple sequence alignment. All identical sequences were removed and all sequences were edited with previously published sequences from 2009 H1N1 SOV downloaded from GenBank / DDBJ / EMBL using MEGA software, version 5.05 (Tamura et al., 2011). Phylogenetic and molecular evolutionary analyses were conducted with MEGA, using the corresponding tree-building method in concordance with the selected substitution model, with 1000 bootstrap replicates. All the accession numbers for the sequences are listed in Appendix 1. Costa Rican sequences were deposited in GenBank / DDBJ / EMBL with accession numbers JF279885- JF279902.

Lineage of sequences and genotype of genes analyzed in this paper were determined as described above (Dong, Ying, & Yuan, 2011). The genotype of influenza viruses was established as recommended at <http://www.flugenome.org/grouping.php> (Lu, Rowley, Garten, & Donis, 2007)

RESULTS

Data collection and sampling. Using the FAO criteria described in the Materials and Methods section, the study consisted of 25 OUs analyzed and distributed across all seven provinces in the country and in 16 out of 81 counties, which covers 19.75% of the total number of counties in Costa Rica. The total pig population included in the study was 37,085, although only 509 (1.37%) were sampled (see FAO design of the sampling Materials and Methods). Table 2 shows the distribution of OU by province, number of OUs in the province, sampled OUs, grid number, county name, number of farms and number of animals in each farm. Of all 25 OUs sampled only 13 (52%) presented animals with symptoms associated with the respiratory system, such as dyspnea, cough, runny nose, and sneezing as described in the case definition (see Materials and Methods).

A significant variation in size and husbandry was observed in the sampled OUs. The OU with the fewest animals had 29 pigs and was located in San José, Santa Ana county, Escazú district. The largest OU was in Cartago, Central County, with 25,000 pigs. Taken together, 32% of the OU had 100 or fewer animals, 56% of OU had between 200 and 1,000 animals, and 12% of the OU had more than 1,000 pigs.

Table 2. OU distribution by province, county, grid number, and number of animals sampled. Province names in bold indicate the OU with animals diagnosed as positive.

Province	Total No. of OU	No. of OU with symptoms	Grid number	County	No. of farms per county	No. of animals in farm	
San José	5	2	1262	Santa Ana	2	330	
							29
			NI	Puriscal	3	93	
							35
Alajuela	6	2	919	Zarcelero	2	85	
							316
			644	Grecia	1	200	
			122	Los Chiles	1	574	
			233	Upala	1	200	
Cartago	6	6	388	San Carlos	1	200	
			1927	Alvarado	2	800	
			1270				300
			1323	Cartago	3	25000	
			1326				700
Heredia	2	1	1377			70	
			1427	Guarco	1	200	
			1093	Santa Bárbara	2	1000	
			1092				100
Guanacaste	2	2	462	Bagaces	1	3000	
			734	Cañas	1	640	
Puntarenas	2	0	1194	Esparza	2	2000	
Limón	2	0	876	Pococi	1	500	
			1647	Talamanca	1	32	

Detection Limit of RRT-PCR for type A influenza matrix gene. The limit of detection (l.o.d.) for 203 ADV RNA control determined from triplicate tenfold dilution series was 72.2 attograms per microlitre, which corresponded to a 10^{-7} dilution and resulted in a Ct value of 33. From this result a Ct lower than 33 was considered a positive sample and a Ct of 33 or higher was considered suspicious, while any undetermined sample was considered negative.

Similarly, the l.o.d. for H1N1/2009 S-OIV viral variant was defined as having a cut-off value over 35 Ct for suspicious samples, any sample with Ct values under 35 was considered positive, while any undetermined sample was considered negative (conditions previously optimized for the NVSL Ames, Iowa).

RRT-PCR results. All 509 samples were first tested using the (M)-RRT-PCR for the type A Influenza virus previously described (Spackman et al., 2002) results showed 411 negative

samples, 18 positive cases and 81 samples considered suspicious. All 18 positive samples were concentrated in just two of the 25 OUs, designated with the laboratory protocols D4361-10 OU and D4364-10. These OUs were located 60 Km from each other, in Cañas (Guanacaste) and Esparza (Puntarenas), respectively as is shown in Fig. 5 and Table 2.

Both suspicious and positive samples (n=99 samples) were subsequently tested using the H1N1/2009 S-OIV RRT-PCR as described in Materials and Methods (Suárez and Spackman, unpublished). Results confirmed that only 11 samples were also H1N1/2009 S-OIV positive; interestingly, all of them belonged to the small group of 18 clearly positives using (M)-RRT-PCR for type A Influenza virus. From the rest of the 18 (M)-RRT-PCR type A Influenza virus positive samples, 6 were suspicious for H1N1/2009 S-OIV, with Ct values above 35, and 1 was clearly negative (Table 3).

Meanwhile, none of the 81 (M)-RRT-PCR type A Influenza virus inconclusive samples were positive for H1N1 RRT-PCR. To further analyze the reason for those inconclusive (M)-RRT-PCR type A Influenza virus results, 10 suspicious samples, which had the closest Ct value to the detection limit of 35, were selected for virus isolation testing (Table 3). Swab supernatant from those original specimens was inoculated in MDCK cells and observed for CPE as described in Materials and Methods. No viruses could be isolated in these samples during three blind passages. In addition, another 10 samples were randomly selected out of the 81 inconclusive samples and re-tested using the (M)-RRT-PCR type A Influenza virus, and the products were visualized in 2% agarose gel stained with GelRed™ (Fig. 1).

Six samples, which corresponded to lanes 1, 2, 3, 4, 5 and 9 (Fig. 1), were confirmed negative for (M)-RRT-PCR type A Influenza virus, showing only an unspecific amplification product at the level of 50 bp similar to the one observed in the negative control (lane 13). Lanes 6, 7, and 8 (all 3 belonging to the OU D4634-10, sample numbers 2, 5, and 8), as well as lane 10 (sample number 2 of the protocol D4365-10), were confirmed positive for (M)-RRT-PCR type A Influenza virus after being run in agarose gel, since they all showed a second amplification product in the range of 100 bp, the same size of the positive controls (lanes 11 and 12 of Fig.1).

Figure 1. Agarose gel (2%) was used to visualize the RRT-PCR products from 10 samples considered suspicious. Samples were dispensed from lanes 1 to 10; lane 11 shows the ADV 203 positive control, lane 12 shows the 204 ADV positive control, and lane 13 shows the negative control. MM: molecular marker MM 50 bp of GeneRuler Fermentas SM0371 is observed at the sides.

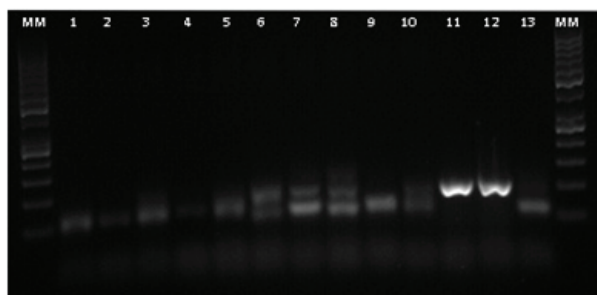


Table 3. Influenza A and H1N1 RRT-PCR Results.

ND: Not Done, NI: Not indicated, Pos: positive, Neg: negative, Susp: suspicious, Und: undetermined.

OU	Sample	Sex	Months of Age	Symptoms	Agarose	Virus Isolation	Ct Matrix PCR	Ct H1N1 PCR		
D4361-10	1	Female	2	None	ND	ND	30.9	Pos	36	Und
D4361-10	3	Male	2	None	ND	Pos	22.17	Pos	27.6	Pos
D4361-10	4	Male	2	Cough	ND	ND	29.56	Pos	34.8	Pos
D4361-10	5	Female	2	None	ND	ND	33.9	Pos	36	Und
D4361-10	6	Male	2	None	ND	ND	25.44	Pos	30.51	Pos
D4361-10	9	Male	2	None	ND	ND	29.79	Pos	34.75	Pos
D4361-10	11	Female	2	None	ND	ND	32.9	Pos	36	Und
D4361-10	13	Male	2	None	ND	ND	26.85	Pos	31.4	Pos
D4361-10	14	Male	2	None	ND	Pos	21.32	Pos	25	Pos
D4361-10	15	Male	2	None	ND	Pos	21.35	Pos	27.9	Pos
D4361-10	16	Male	2	None	ND	Pos	24.16	Pos	29.7	Pos
D4361-10	17	Male	1	None	ND	ND	24.0	Pos	Und	Neg
D4361-10	18	Male	1	Cough	ND	ND	26.09	Pos	31.5	Pos
D4361-10	19	Male	1	Cough	ND	Pos	17.6	Pos	24.8	Pos
D4361-10	20	Male	1	Cough	ND	Pos	14.8	Pos	20.4	Pos
D4364-10	2	Male	1	None	Pos	ND	27.3	Pos	36.7	Und
D4364-10	5	Male	1	None	Pos	ND	33	Pos	38	Und
D4364-10	8	Male	1	None	Pos	ND	33	Pos	41.3	Und
D4358-10	5	Female	2	None	ND	Neg	36.71	Susp	Und	Neg
D4353-10	13	Female	3	NI	ND	Neg	36.29	Susp	Und	Neg
D4358-10	3	Male	2	NI	ND	Neg	36.93	Susp	Und	Neg
D4353-10	19	Male	3	NI	ND	Neg	36.21	Susp	Und	Neg
D4353-10	5	Male	3	NI	ND	Neg	36.04	Susp	Und	Neg
D4318-10	8	Male	7	NI	ND	Neg	35.83	Susp	Und	Neg
D4352-10	2	Female	4	Cough	ND	Neg	35.72	Susp	Und	Neg
D4358-10	19	Male	2	NI	ND	Neg	36.84	Susp	Und	Neg
D4358-10	2	Female	2	NI	ND	Neg	36.17	Susp	Und	Neg
D4352-10	7	Male	4	NI	ND	Neg	36.94	Susp	Und	Neg



Phylogenetic analysis. Only six of the 11 positive samples had enough viral concentration for further genetic characterization by sequencing. Those samples were 3, 14, 15, 16, 19 and 20, all six from D4361-10 OU.

The complete sequence of HA, M and NA genes of those 6 isolates was obtained and analyzed using BLAST (Basic Local Alignment Search Tool) from GenBank. Results showed at least 99% nucleotide identity with strains isolated from human H1N1/2009 S-OIV worldwide for all three genes. Additionally, NA gene was 99% identical to the Chinese H1N1 isolate from swine A/swine/Shandong/N1/2009 (H1N1) previously published in GenBank (Acc. N. JF275950), and the M gene presented 100 % of identity with swine H1N1/2009 S-OIV from Thailand (Acc. N. CY062311). For phylogenetic analysis, multiple alignments including Costa Rica H1N1 and selected sequences from GenBank from pig, bird and human samples in different years and geographic locations (Appendix 1) were edited using Clustal W included in Bioedit (Hall, 1999). The best suited substitution models for each of the gene sequences were later determined, using MEGA version 5.05 package, (Tamura et al., 2011) we announce the release of Molecular Evolutionary Genetics Analysis version 5 (MEGA5, resulting in GTR+G+I, T92+G+I, and K2+G models, for genes HA, NA, and M, respectively. The corresponding phylogenetic trees are shown in Figs. 2 through 4.

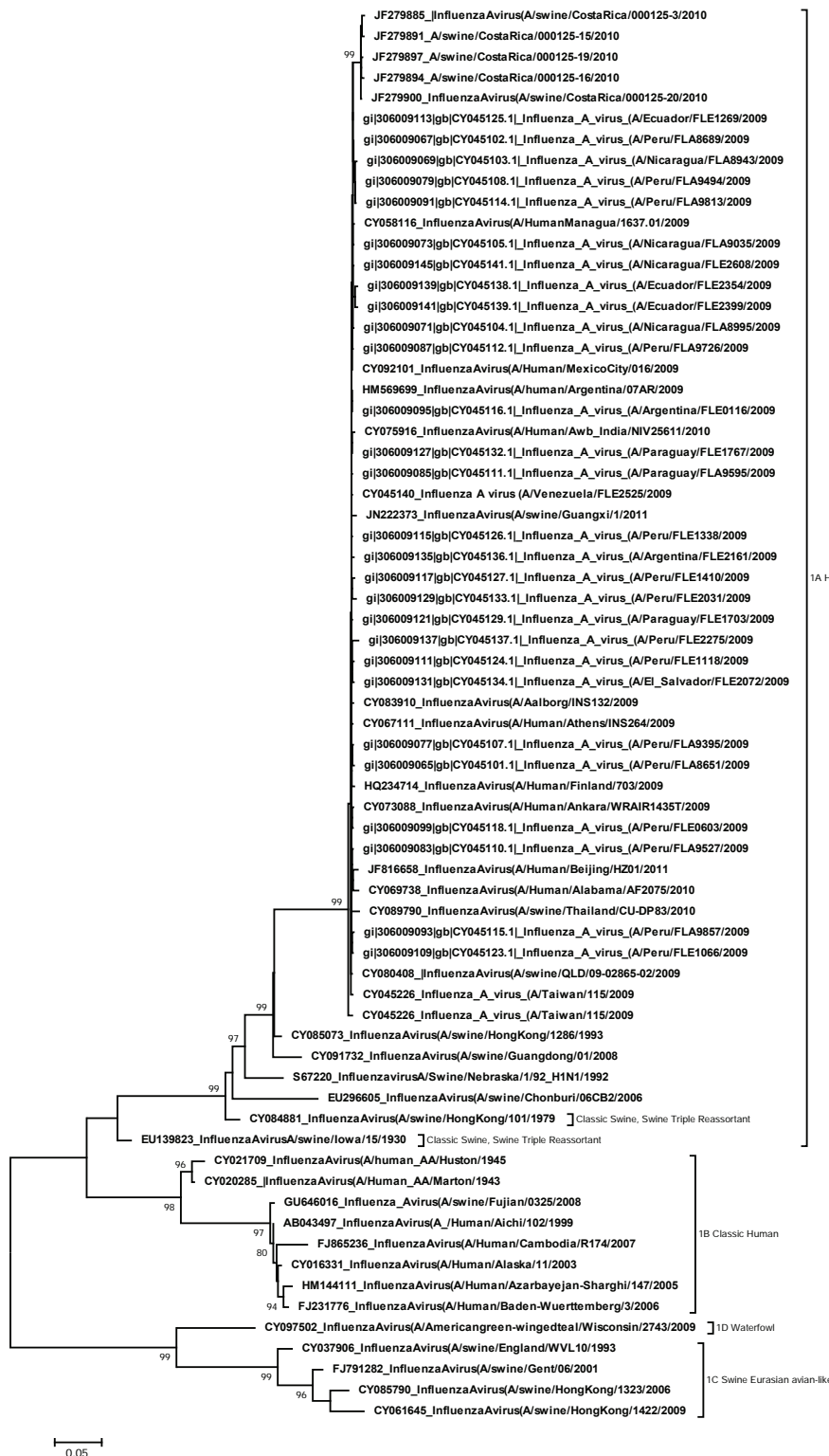


Figure. 2a. Phylogenetic tree of the H1N1 HA gene constructed using MEGA version 5.05 and the Maximum Likelihood method and the GTR+G+I substitution model, with 1000 bootstrap replicates. The analysis considered sequences obtained from GenBank taken from swabs from pigs, birds and humans in different years and geographic locations (Appendix 1). The figure also represents the H1N1 genotype and lineage.



All HA genes from the H1N1 Influenza virus were grouped as expected into four main clusters each for types 1A, 1B, 1C and 1D. All 2009 isolates constituted a single group within type 1A, except sequences CY061645 and CY097502, which were clustered into types 1C and 1D, respectively. Type 1A for this gene (HA) includes the lineage of 2009 S-OIV viruses, both Classic Swine and Swine triple reassortant. The divergence between types 1A and 1B is clearly shown in the figure, indicating that both lineages seem to share a common ancestor of classical human influenza. On the other hand, lineages 1C and 1D are genetically more distant; both belong to the group of waterfowl and the Eurasian swine influenza virus, respectively, which were also of avian origin.

As expected, all Costa Rican isolates were grouped within type 1A as a single cluster, indicating a greater genetic distance from the common ancestor. In both cases, bootstrap values were 99%, confirming the robustness of the analysis (Fig. 2b). Similar results were obtained for the NA (Fig. 3) and M (Fig. 4) genes. In summary, all the Costa Rican Influenza A isolates from pigs analyzed in this study are HA 1A, NA 1F, and MF1F, which confirms that these sequences belong to the 2009 H1N1 S-OIV genetic lineage. Tree phylogenetic topology confirms the 2009 H1N1 S-OIV origin of our isolates for all the three genes analyzed.

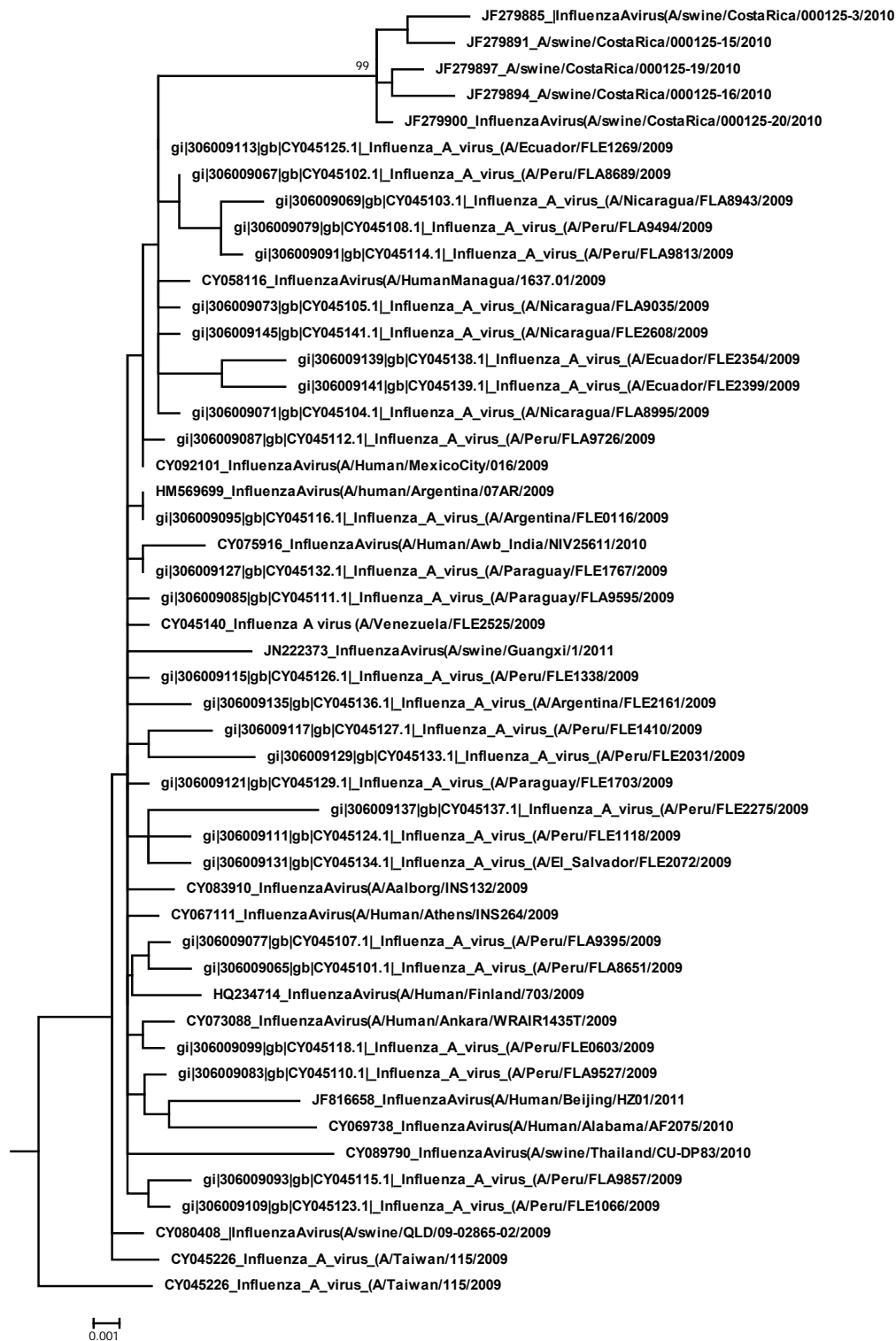


Figure. 2b. Subtree generated from the tree in Fig. 2a depicting the topological relationship of the H1N1/2009 sequences isolated from pigs in Costa Rica.

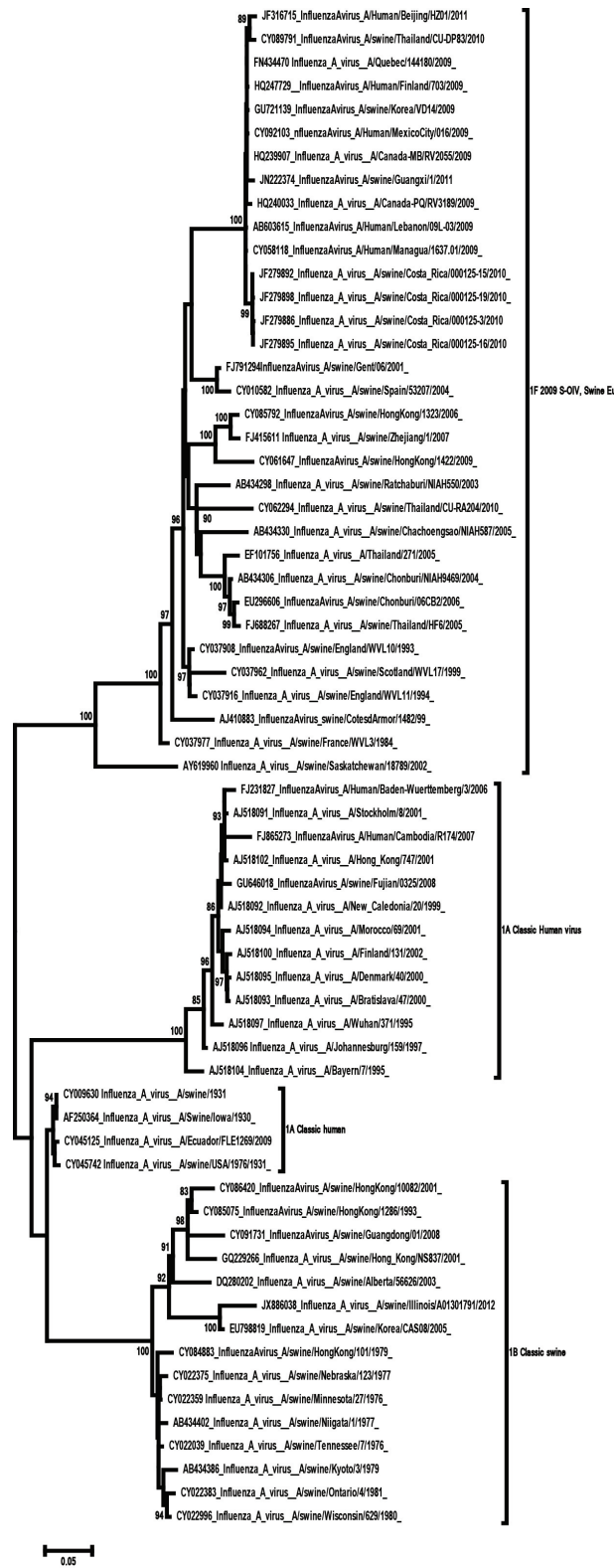


Figure 3. Phylogeny tree of H1N1 NA gene constructed using the Maximum Likelihood method with the T92+G+I model, 1000 bootstrap replicates according to MEGA version 5.05. The analysis considered sequences obtained from swabs from pigs, birds and humans in different years and geographic locations, both from GenBank and generated in Costa Rica. The figure also represents the respective genotype and lineage.

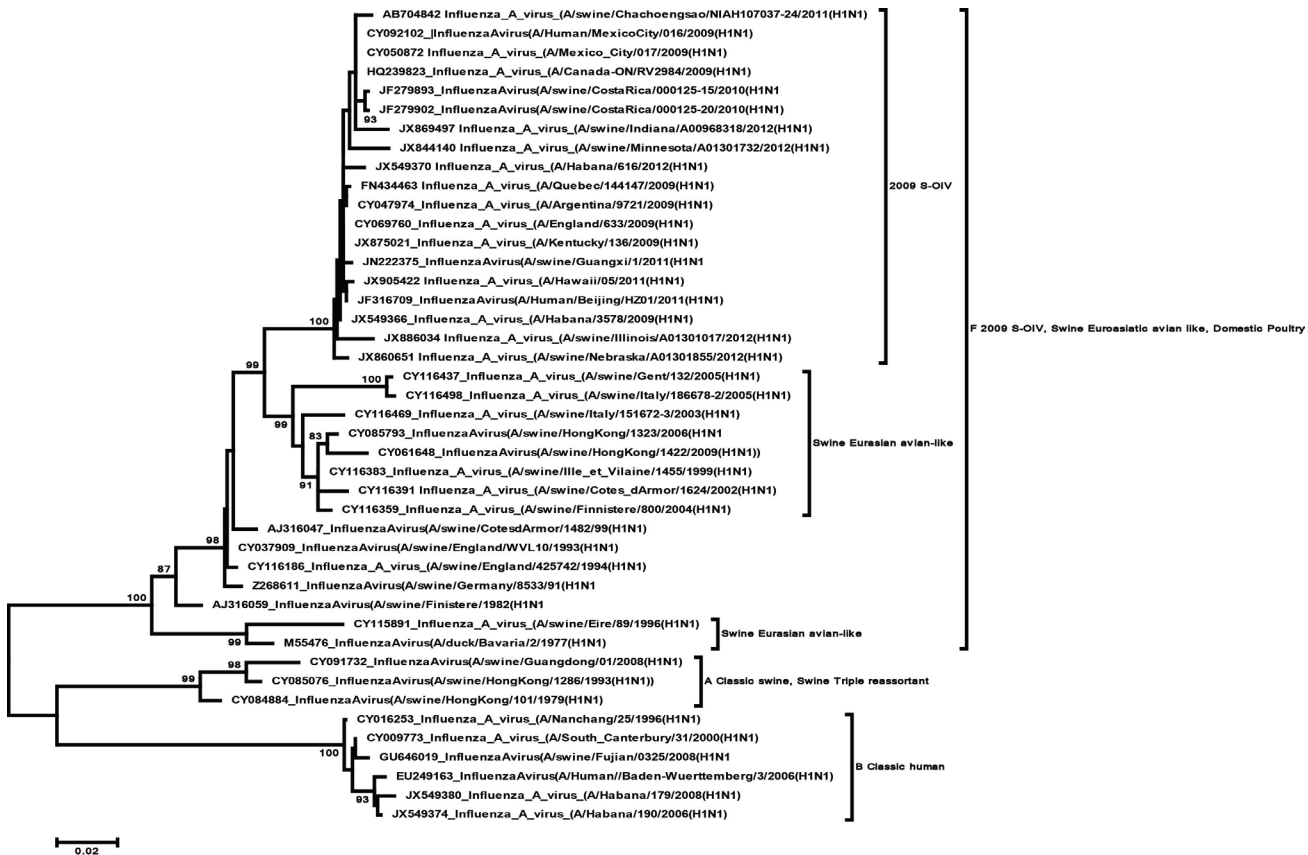


Figure 4. Phylogeny tree of H1N1 M gene constructed using the Neighbor Joining method with K2+G substitution model and 1000 bootstrap replicates. The analysis considered sequences obtained from swabs from pigs, birds and humans in different years and geographic locations. The figure also represents the respective genotype and lineage.

Discussion

We present the first evidence of the presence of Influenza H1N1/2009 S-OIV in swine in Costa Rica, a year and a half after the first cases appeared in humans in Mexico and the United States. In May 2009, for the first time the isolation of this new variant was reported in pigs in Alberta (Canada) (Howden et al., 2009); since then other countries such as Argentina and Brazil have detected this novel influenza virus in pigs (Pereda et al., 2010; Schaefer et al., 2011). It is important to note that the results presented here were not an epidemiological study of the incidence and distribution of H1N1/2009 S-OIV in Costa Rican swine, but just the search and confirmation of the circulation of such pandemic strain in pigs in the country.

The H1N1/2009 S-OIV influenza variant was declared a pandemic virus by WHO on June 11, 2009 (WHO, n.d.). Unlike previous pandemics originated from birds (1918 H1N1, 1957 H2N2, and 1968



H3N2), this new strain contains gene rearrangements that had not been previously reported for swine or human isolates of the influenza virus. Additionally, there is evidence that the virus may have been circulating in pigs for some time unnoticed, probably due to the scarce number of surveillance studies around the world (Garten et al., 2009). Therefore, given the role of swine species in recombination of bird and human viral subtypes (Karasin et al., 2006; Nagarajan et al., 2010), the global surveillance plan for detection of Influenza in this species should be advised especially in backyard or small non-commercial production units, where cohabitation and interaction of poultry, wild birds, pigs and humans take place, increasing the probabilities of producing new influenza variants with or without pandemic characteristics (Bi et al., 2010).

In that sense, the aim of this study is to alert and prevent the further spread of this variant to humans and other species, informing both the Costa Rican Department of Epidemiology of Animal Health Services (SENASA) and the Ministry of Health about the urgency of defining a strategy for monitoring backyard pig farms.

As previously explained, this study's sampling was directed toward the detection of the pandemic variant H1N1. Hence, it is no wonder that all positive animals came from OUs where previous or recent cases of respiratory distress have been reported, all were 2 month-old or younger pigs, and both premises were geographically very close to each other. It is possible that both premises have acquired the infection from other pig farming sources, what would indicate the existence in Costa Rica of other infected premises not included in this sampling. Unfortunately, there is no data in this study that would allow establishing management or sanitary links between the infected farms. The OU in Cañas is dedicated to finishing animals that were bought as weaned animals from other farms and could have received the virus from them. In the case of the OU in Esparza, despite the fact that the farm had a closed cycle of production, they mentioned having bought a boar for the only purpose of fathering grower/finisher pigs. The boar was on the farm during 2008-2010, which opens the option of having introduced this virus to the facility. Besides that, no other commercial or management relationships were found between the two farms.

Alternatively, since it is well documented that pigs and humans can infect each other (Song et al., 2010; Sreta et al., 2010; Hofshagen et al., 2009; Howden et al., 2009), it is also possible that pigs were infected from some H1N1 carrier visiting or working at the farm. Nevertheless, we corroborated that no human cases of influenza had been reported at the infected OUs, neither when samples were taken nor during the previous two years. In summary, with the data collected for this research project, there was no evidence of any link or relationship between the two farms that resulted positive for H1N1 presence in this study.

From the 81 suspect samples obtained in this study, two samples showed amplification for Influenza A RRT-PCR (D4361-10 sample 17 with a Ct of 24) or by agarose gel (D4365-10 sample 2, Fig. 1, lane 10) but not for H1N1/2009 S-OIV. It is possible that they contain an influenza A virus subtype other than H1N1/2009 for which our PCR was designed, or the H1N1/2009 S-OIV-PCR may have a lower sensitivity that did not allow detection of weak positives, as seems to be evident comparing the Ct values obtained with each RRT-PCR from the same sample (Table 3).

If this study had only considered positive cases confirmed by virus isolation, the prevalence of

influenza viruses in pigs sampled would have been extremely low (3.3%). In other words, although positive cases were found, this happened only in one single place out of the seven provinces sampled (Fig. 5, Table 2). The reasons for such low prevalence might reside in the low rate of infection nationwide during late 2010, if compared to the human H1N1/2009 S-OIV prevalence in Costa Rica during that time (INCIENSA, 2010). However, we are aware of other issues in the study that may have influenced this result. For instance, the possible influence of the sampling plan used cannot be discarded, which was prepared considering the possibility of detecting at least one positive animal, as it in fact occurred. In addition, it should be noted that our RRT-PCR did not have a PCR internal control to assess the effect of PCR inhibitors that may have been present on the swab and during the extraction process, which may have increased the number of false negative results.

Finally, the questionnaire provided to the OU for sampling purposes did not include a question regarding vaccination on the farm. Therefore, whether or not vaccination provided protection against the new strain cannot be evaluated in this study.

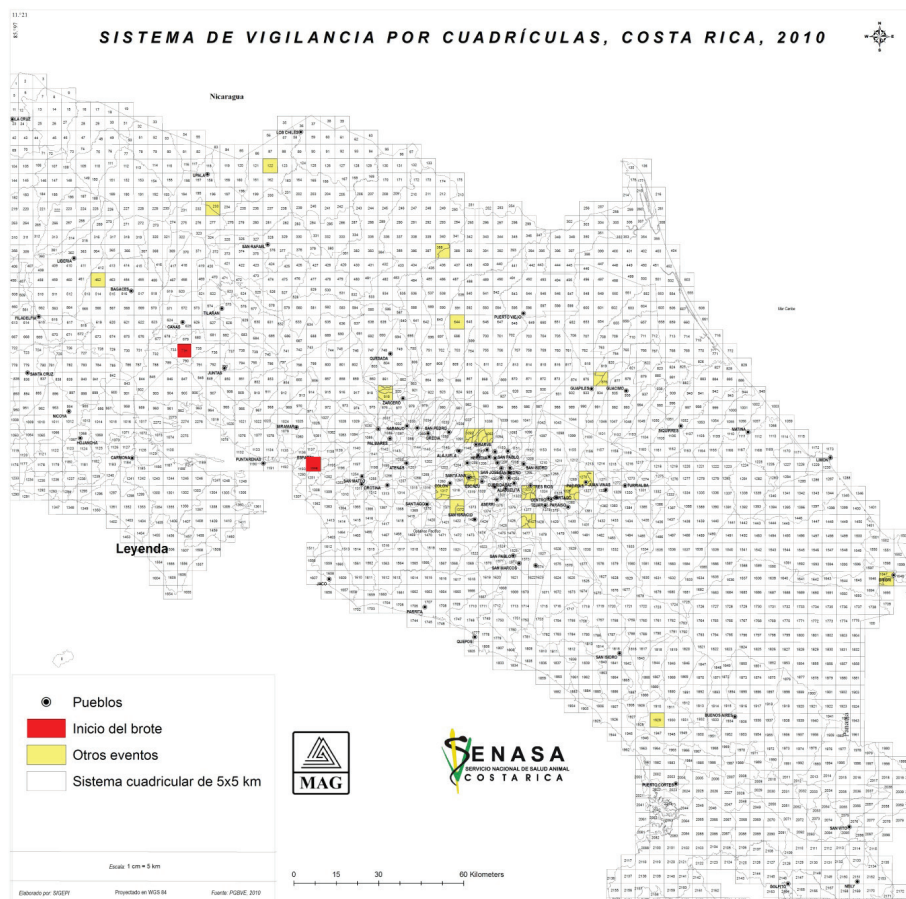


Figure 5. Costa Rican Surveillance System Map. The country is divided into a grid, each grid square representing 25 km². Yellow squares indicate sampled OU for this study, while red grids indicate OU where animals were H1N1/2009 S-OIV positive.

According to prior studies based on reassortment of Influenza genes that gave rise to the A/Thailand/271/2005 influenza virus (Komadina et al., 2007), it seems probable that this variant could have been previously circulating in pigs, without being clinically detected (Garten et al., 2009; Smith et al., 2009). Unfortunately, there is no information on which genotypes and subtypes of influenza could be circulating in Costa Rica in both pigs and/or humans, as this information would have allowed us to conclude whether the variant found in this study was circulating before the first cases of patients with H1N1 2009 S-OIV.

The classification made with the "Influenza A Virus Genotype Tool" (Lu et al., 2007) established that the HA gene belongs to lineage 1A, which comes from the classic swine flu virus, while the NA and M genes derived from swine influenza strains of Eurasian avian origin. This result is consistent with those previously reported by other authors (Garten et al., 2009; Nagarajan et al., 2010; Smith et al., 2009). Genetic analysis of isolates worldwide showed that the new influenza virus H1N1/2009 S-OIV kept six genes of the classic swine influenza viruses with the known triple rearrangement derived from the 1918 H1N1 and two genes, NA and M, from the swine Eurasian influenza virus H1N1 (Garten et al., 2009). One aspect that enhances the Eurasian origin in the M gene of the isolates in Costa Rica is a classical genetic marker of porcine strains arising from this lineage, which is evidenced by a change of a serine (S) to an asparagine (N) at position 31 of the M2 gene. This substitution of amino acid confers resistance to amantadine to these viruses (Nagarajan et al., 2010). All sequences obtained from pigs in Costa Rica in this study show that marker as it can be seen in Fig. 6; this result should be taken into consideration in these variants.

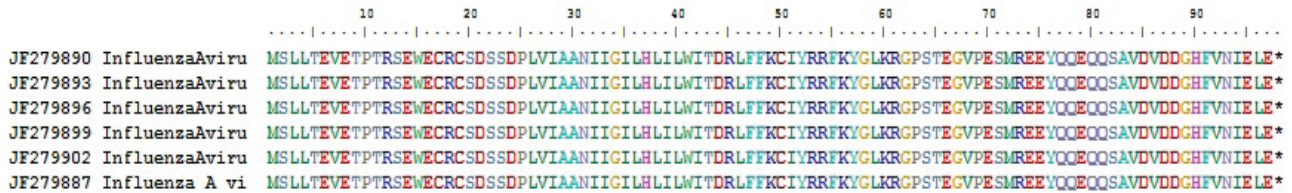


Figure 6. Amino acid sequence of the M2 gene of influenza virus isolates H1N1/2009 S-OIV in Costa Rica, according to the program Bioedit. For identification of the sequences see Appendix 1.

This study was intended to determine whether the pandemic H1N1/2009 S-OIV influenza was circulating among pig herds in Costa Rica in 2010, using diagnostic and strain characterization methods previously standardized by the NVSL in Ames, Iowa. The results obtained demonstrate beyond a doubt the presence of virus H1N1/2009 S-OIV in pigs' nasal swabs from Cañas and Esparza, indicating that there is a justified need for surveillance to detect the presence of new influenza virus strains, and point out the importance of molecular epidemiological studies to undoubtedly characterize variants that may have implications in human public health. Additionally, results indicated the need to strengthen Costa Rican surveillance systems in the livestock sector, enhance relationships with other institutions related to public health, and improve the methods to seek for information regarding influenza A viruses in general.

Further analysis using Bayes methods could determine if this virus variant was present in pigs before or after the H1N1/2009 S-OIV human outbreak in Costa Rica. However, another sampling analysis to follow up on surveillance studies and the evolution of this variant in pig herds would be the most help to understand the swine influenza epidemiological status in Costa Rica.

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Supplementary table 1. Identification of the sequences used in this study.

Matrix Gene	
1	EU249163 Influenza A virus (A/Human//Baden-Wuerttemberg/3/2006(H1N1))
2	JX886034 Influenza A virus (A/swine/Illinois/A01301017/2012(H1N1))
3	JX905422 Influenza A virus (A/Hawaii/05/2011(H1N1))
4	HQ239823 Influenza A virus (A/Canada-ON/RV2984/2009(H1N1))
5	JX875021 Influenza A virus (A/Kentucky/136/2009(H1N1))
6	JX860651 Influenza A virus (A/swine/Nebraska/A01301855/2012(H1N1))
7	JX869497 Influenza A virus (A/swine/Indiana/A00968318/2012(H1N1))
8	AB704842 Influenza A virus (A/swine/Chachoengsao/NAH107037-4/2011(H1N1))
9	JX844140 Influenza A virus (A/swine/Minnesota/A01301732/2012(H1N1))
10	JX549380 Influenza A virus (A/Habana/179/2008(H1N1))
11	JX549370 Influenza A virus (A/Habana/616/2012(H1N1))
12	JX549366 Influenza A virus (A/Habana/3578/2009(H1N1))
13	JX549374 Influenza A virus (A/Habana/190/2006(H1N1))
14	CY050872 Influenza A virus (A/Mexico City/017/2009(H1N1))
15	CY069760 Influenza A virus (A/England/633/2009(H1N1))
16	CY116469 Influenza A virus (A/swine/Italy/151672-3/2003(H1N1))
17	FN434463 Influenza A virus (A/Quebec/144147/2009(H1N1))

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16	CY116469 Influenza A virus (A/swine/Italy/151672-3/2003(H1N1))
17	FN434463 Influenza A virus (A/Quebec/144147/2009(H1N1))
18	CY116437 Influenza A virus (A/swine/Gent/132/2005(H1N1))
19	CY116383 Influenza A virus (A/swine/Ille et Vilaine/1455/1999(H1N1))
20	CY116391 Influenza A virus (A/swine/Cotes dArmor/1624/2002(H1N1))
21	CY116359 Influenza A virus (A/swine/Finistere/800/2004(H1N1))
22	CY115891 Influenza A virus (A/swine/Eire/89/1996(H1N1))
23	CY116498 Influenza A virus (A/swine/Italy/186678-2/2005(H1N1))
24	CY116186 Influenza A virus (A/swine/England/425742/1994(H1N1))
25	CY047974 Influenza A virus (A/Argentina/9721/2009(H1N1))
26	CY009773 Influenza A virus (A/South Canterbury/31/2000(H1N1))
27	CY016253 Influenza A virus (A/Nanchang/25/1996(H1N1))
28	JF316709 Influenza A virus (A/Human/Beijing/HZ01/2011(H1N1))
29	M55476 Influenza A virus (A/duck/Bavaria/2/1977(H1N1))
30	CY092102 Influenza A virus (A/Human/MexicoCity/016/2009(H1N1))
31	CY037909 Influenza A virus (A/swine/England/WVL10/1993(H1N1))
32	AJ316059 Influenza A virus (A/swine/Finistere/1982(H1N1))
33	GU646019 Influenza A virus (A/swine/Fujian/0325/2008(H1N1))
34	CY091732 Influenza A virus (A/swine/Guangdong/01/2008(H1N1))
35	Z268611 Influenza A virus (A/swine/Germany/8533/91(H1N1))
36	JN222375 Influenza A virus (A/swine/Guangxi/1/2011(H1N1))
37	CY084884 Influenza A virus (A/swine/HongKong/101/1979(H1N1))
38	CY085076 Influenza A virus (A/swine/HongKong/1286/1993(H1N1))

39	CY085793 Influenza A virus (A/swine/HongKong/1323/2006(H1N1
40	CY061648 Influenza A virus (A/swine/HongKong/1422/2009(H1N1))
41	JF279893 Influenza A virus (A/swine/CostaRica/000125-15/2010(H1N1
42	JF279902 Influenza A virus (A/swine/CostaRica/000125-20/2010(H1N1)
43	AJ316047 Influenza A virus (A/swine/CotesdArmor/1482/99(H1N1)
Hemaglutinine Gene	
1	JF279885 Influenza A virus (A/swine/CostaRica/000125-3/2010
2	JF279900 Influenza A virus (A/swine/CostaRica/000125-20/2010
3	JF279897 A/swine/CostaRica/000125-19/2010
4	JF279894 A/swine/CostaRica/000125-16/2010
5	JF279891 A/swine/CostaRica/000125-15/2010
6	CY092101 Influenza A virus (A/Human/MexicoCity/016/2009
7	CY073088 Influenza A virus (A/Human/Ankara/WRAIR1435T/2009
8	HM569699 Influenza A virus (A/human/Argentina/07AR/2009
9	CY067111 Influenza A virus (A/Human/Athens/INS264/2009
10	CY045226 Influenza A virus (A/Taiwan/115/2009
11	CY080408 Influenza A virus (A/swine/QLD/09-02865-02/2009
12	CY075916 Influenza A virus (A/Human/Awb India/NIV25611/2010
13	CY058116 Influenza A virus (A/HumanManagua/1637.01/2009
14	CY083910 Influenza A virus (A/Aalborg/INS132/2009
15	JN222373 Influenza A virus (A/swine/Guangxi/1/2011
16	HQ234714 Influenza A virus (A/Human/Finland/703/2009
17	AB704847 Influenza A virus (A/swine/Chachoengsao/NIAH107037-28/2011
18	JF816658 Influenza A virus (A/Human/Beijing/HZ01/2011
19	CY069738 Influenza A virus (A/Human/Alabama/AF2075/2010
20	CY089790 Influenza A virus (A/swine/Thailand/CU-DP83/2010
21	CY045125 Influenza A virus (A/Ecuador/FLE1269/2009
22	CY045141 Influenza A virus (A/Nicaragua/FLE2608/2009
23	CY045104 Influenza A virus (A/Nicaragua/FLA8995/2009
24	CY045103 Influenza A virus (A/Nicaragua/FLA8943/2009
25	CY045102 Influenza A virus (A/Peru/FLA8689/2009
26	CY045108 Influenza A virus (A/Peru/FLA9494/2009
27	CY045114 Influenza A virus (A/Peru/FLA9813/2009
28	CY045132 Influenza A virus (A/Paraguay/FLE1767/2009
29	CY045129 Influenza A virus (A/Paraguay/FLE1703/2009
30	CY045111 Influenza A virus (A/Paraguay/FLA9595/2009
31	CY045126 Influenza A virus (A/Peru/FLE1338/2009
32	CY045115 Influenza A virus (A/Peru/FLA9857/2009
33	CY045118 Influenza A virus (A/Peru/FLE0603/2009
34	AF091313 Influenza A virus (A/duck/Bavaria/2/1977

35	CY045123 Influenza A virus (A/Peru/FLE1066/2009
36	CY045110 Influenza A virus (A/Peru/FLA9527/2009
37	CY045116 Influenza A virus (A/Argentina/FLE0116/2009
38	CY045107 Influenza A virus (A/Peru/FLA9395/2009
39	CY045101 Influenza A virus (A/Peru/FLA8651/2009
40	CY045112 Influenza A virus (A/Peru/FLA9726/2009
41	CY045127 Influenza A virus (A/Peru/FLE1410/2009
42	CY045124 Influenza A virus (A/Peru/FLE1118/2009
43	CY045134 Influenza A virus (A/El Salvador/FLE2072/2009
44	CY045105 Influenza A virus (A/Nicaragua/FLA9035/2009
45	CY045133 Influenza A virus (A/Peru/FLE2031/2009
46	CY045136 Influenza A virus (A/Argentina/FLE2161/2009
47	CY045138 Influenza A virus (A/Ecuador/FLE2354/2009
48	CY045139 Influenza A virus (A/Ecuador/FLE2399/2009
49	CY045137 Influenza A virus (A/Peru/FLE2275/2009
50	S67220 Influenza A virus/Swine/Nebraska/1/92 H1N1/1992
51	CY091729 Influenza A virus (A/swine/Guangdong/01/2008
52	CY085073 Influenza A virus (A/swine/HongKong/1286/1993
53	CY084881 Influenza A virus (A/swine/HongKong/101/1979
54	EU296605 Influenza A virus (A/swine/Chonburi/06CB2/2006
55	EU139823 Influenza A virusA/swine/Iowa/15/1930
56	CY097502 Influenza A virus (A/Americangreen-wingedteal/Wisconsin/2743/2009
57	CY037906 Influenza A virus (A/swine/England/WVL10/1993
58	FJ791282 Influenza A virus (A/swine/Gent/06/2001
59	CY085790 Influenza A virus (A/swine/HongKong/1323/2006
60	CY061645 Influenza A virus (A/swine/HongKong/1422/2009
61	HM144111 Influenza A virus (A/Human/Azarbayejan-Sharghi/147/2005
62	FJ231776 Influenza A virus (A/Human/Baden-Wuerttemberg/3/2006
63	AB043497 Influenza A virus (A /Human/Aichi/102/1999
64	CY016331 Influenza A virus (A/Human/Alaska/11/2003
65	GU646016 Influenza A virus (A/swine/Fujian/0325/2008
66	FJ865236 Influenza A virus (A/Human/Cambodia/R174/2007
67	CY021709 Influenza A virus (A/human AA/Huston/1945
68	CY020285 Influenza A virus (A/Human AA/Marton/1943
Neuroaminidase Gene	
1	JX886038 Influenza A virus A/swine/Illinois/A01301791/2012
2	JF279898 Influenza A virus A/swine/Costa Rica/000125-19/2010
3	JF279892 Influenza A virus A/swine/Costa Rica/000125-15/2010
4	JF279886 Influenza A virus A/swine/Costa Rica/000125-3/2010
5	JF279895 Influenza A virus A/swine/Costa Rica/000125-16/2010

6	HQ240033 Influenza A virus A/Canada-PQ/RV3189/2009
7	FJ231827 Influenza A virus A/Human/Baden-Wuerttemberg/3/2006
8	JF316715 Influenza A virus A/Human/Beijing/HZ01/2011
9	AB603615 Influenza A virus A/Human/Lebanon/09L-03/2009
10	GU721139 Influenza A virus A/swine/Korea/VD14/2009
11	FJ865273 Influenza A virus A/Human/Cambodia/R174/2007
12	HQ247729 Influenza A virus A/Human/Finland/703/2009
13	CY058118 Influenza A virus A/Human/Managua/1637.01/2009
14	CY092103 Influenza A virus A/Human/MexicoCity/016/2009
15	CY089791 Influenza A virus A/swine/Thailand/CU-DP83/2010
16	EU296606 Influenza A virus A/swine/Chonburi/06CB2/2006
17	CY037908 Influenza A virus A/swine/England/WVL10/1993
18	GU646018 Influenza A virus A/swine/Fujian/0325/2008
19	FJ791294 Influenza A virus A/swine/Gent/06/2001
20	CY091731 Influenza A virus A/swine/Guangdong/01/2008
21	JN222374 Influenza A virus A/swine/Guangxi/1/2011
22	CY084883 Influenza A virus A/swine/HongKong/101/1979
23	CY086420 Influenza A virus A/swine/HongKong/10082/2001
24	CY085075 Influenza A virus A/swine/HongKong/1286/1993
25	CY085792 Influenza A virus A/swine/HongKong/1323/2006
26	CY061647 Influenza A virus A/swine/HongKong/1422/2009
27	AJ410883 Influenza A virus swine/CotesdArmor/1482/99
28	HQ239907 Influenza A virus A/Canada-MB/RV2055/2009
29	CY022039 Influenza A virus A/swine/Tennessee/7/1976
30	CY010582 Influenza A virus A/swine/Spain/53207/2004
31	CY022375 Influenza A virus A/swine/Nebraska/123/1977
32	CY022383 Influenza A virus A/swine/Ontario/4/1981
33	CY022359 Influenza A virus A/swine/Minnesota/27/1976
34	EF101756 Influenza A virus A/Thailand/271/2005
35	CY022996 Influenza A virus A/swine/Wisconsin/629/1980
36	AJ518095 Influenza A virus A/Denmark/40/2000
37	AJ518093 Influenza A virus A/Bratislava/47/2000
38	AJ518091 Influenza A virus A/Stockholm/8/2001
39	AJ518104 Influenza A virus A/Bayern/7/1995
40	AJ518102 Influenza A virus A/Hong Kong/747/2001
41	AJ518100 Influenza A virus A/Finland/131/2002
42	AJ518096 Influenza A virus A/Johannesburg/159/1997
43	AJ518092 Influenza A virus A/New Caledonia/20/1999
44	AJ518094 Influenza A virus A/Morocco/69/2001
45	CY009630 Influenza A virus A/swine/1931



46	CY045125 Influenza A virus A/Ecuador/FLE1269/2009
47	AJ518097 Influenza A virus A/Wuhan/371/1995
48	AB434298 Influenza A virus A/swine/Ratchaburi/NIAH550/2003
49	AB434306 Influenza A virus A/swine/Chonburi/NIAH9469/2004
50	AB434386 Influenza A virus A/swine/Kyoto/3/1979
51	EU798819 Influenza A virus A/swine/Korea/CAS08/2005
52	CY037977 Influenza A virus A/swine/France/WVL3/1984
53	CY037962 Influenza A virus A/swine/Scotland/WVL17/1999
54	CY037916 Influenza A virus A/swine/England/WVL11/1994
55	FJ688267 Influenza A virus A/swine/Thailand/HF6/2005
56	AB434402 Influenza A virus A/swine/Niigata/1/1977
57	AB434330 Influenza A virus A/swine/Chachoengsao/NIAH587/2005
58	FN434470 Influenza A virus A/Quebec/144180/2009
59	CY045742 Influenza A virus A/swine/USA/1976/1931
60	DQ280202 Influenza A virus A/swine/Alberta/56626/2003
61	AY619960 Influenza A virus A/swine/Saskatchewan/18789/2002
62	AF250364 Influenza A virus A/Swine/Iowa/1930
63	FJ415611 Influenza A virus A/swine/Zhejiang/1/2007
64	GQ229266 Influenza A virus A/swine/Hong Kong/NS837/2001
65	CY062294 Influenza A virus A/swine/Thailand/CU-RA204/2010

