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Genetic analysis of human and swine influenza A viruses isolated in Northern Italy during 2010–2015

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Summary

Influenza A virus (IAV) infection in swine plays an important role in the ecology of influenza viruses. The emergence of new IAVs comes through different mechanisms, with the genetic reassortment of genes between influenza viruses, also originating from different species, being common. We performed a genetic analysis on 179 IAV isolates from humans (n. 75) and pigs (n. 104) collected in Northern Italy between 2010 and 2015, to monitor the genetic exchange between human and swine IAVs. No cases of human infection with swine strains were noticed, but direct infections of swine with H1N1pdm09 strains were detected. Moreover, we pointed out a continuous circulation of H1N1pdm09 strains in swine populations evidenced by the introduction of internal genes of this subtype. These events contribute to generating new viral variants—possibly endowed with pandemic potential—and emphasize the importance of continuous surveillance at both animal and human level.

KEYWORDS

human, influenza A virus, swine

1 | INTRODUCTION

Swine influenza is regarded as one of the diseases that make up the porcine respiratory disease complex, causing significant economic losses for pig farming. Influenza A virus (IAV) infection in swine plays an important role in the ecology of influenza viruses, as this species is susceptible to infection by avian and human IAVs and is able to transmit them to other species (Kuntz-Simon & Madec, 2009; Olsen, Brown, Easterday, & Van Reeth, 2006; Zell, Scholtissek, & Ludwig, 2013; Zell et al., 2013).

The genome of IAV consists of eight segments of RNA encoding for internal and external proteins. The antigenic subtype is identified according to the characteristic of the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) (Cheung & Poon, 2007). The peculiar constitution of the influenza virus genome is the basis of its marked antigenic variability. The emergence of a new IAV can be achieved through different mechanisms: inter-species transmission, antigenic mutations (drift) and genetic reassortment due to the exchange of genes between two or more IAVs, also originating from different species. Over time, all these mechanisms have contributed to the evolution of swine IAV (swIAV) throughout the world, even though antigenic drift is observed less frequently in swIAVs than in human IAVs (Brown, 2013). Furthermore, although the enzootic IAV subtypes circulating in pig populations on all continents are only three (i.e. H1N1, H1N2 and H3N2), their origin (avian, swine, human) and their antigenic and genetic characteristics are variable in different geographical areas, resulting in an extremely complex and constantly evolving scenario (Lewis et al., 2016). The antigenic diversity of swIAVs represents a risk for the human population, as it could generate zoonotic strains able to spread in a non-immune, naïve and/or partly protected population.

The Italian pig population is characterized by circulation of the subtypes Eurasian avian-like swine H1N1 (EA H1avN1), H3N2 which originated from the reassortment of human and swine viruses representative of the H3N2 virus circulating in Europe, and H1huN2hu reassortant viruses of human and porcine origin (Moreno et al., 2013; Watson et al., 2015). The internal genes of the latter derive from EA swIAV, and the HA derives from A/swine/Scotland/410440/1994 H1huN2 (Scot/94); however, the NA originates from human-to-swine transmission. More precisely, H1huN2hu (i.e. A/swine/Italy/4675/2003) subtype circulating in Italian farms is characterized by a double deletion in the HA1 region (Moreno et al., 2013) and by an NA of human origin introduced in 2000 and has gradually replaced the European H1huN2 Scot/94-like virus, which instead showed an NA of an IAV circulating in pigs in the UK many years before the stabilization of the H3N2 subtype in the 1980s (Marozin et al., 2002; Zell, Bergmann, Krumbholz, Wutzler, & Durrwald, 2008; Zell et al., 2013;).

Since 2009, the circulation of the H1N1pdm09 IAV has been detected in Italy (Moreno et al., 2010) and this subtype currently appears to be circulating stably with a 10% incidence among the H1N1 isolates (Simon et al., 2014). The lack of longitudinal monitoring of the circulation of the IAVs among swine populations in the past has hindered attempts to reconstruct the origin of the recent pandemic caused by H1N1pdm09 IAV (Mena et al., 2016).

Impacts

- During the period 2010–2015 in Italy, we evidenced swine infections with human influenza A virus, subtype H1N1pdm09.
- A genetic analysis showed the circulation of different influenza A reassortant strains in pig populations.
- No significant zoonotic events were detected.

Recent whole-genome studies on swIAVs in USA, Asia and Europe have shown high levels of reassortment between enzootic lineages (Anderson et al., 2013; Nelson et al., 2012; Vijaykrishna et al., 2011; Watson et al., 2015). Moreover, the frequent introduction of human viruses to swine populations in the United States, Brazil, Mexico and Chile (Bowman et al., 2014; Nelson, Schaefer, Gava, Cantao, &

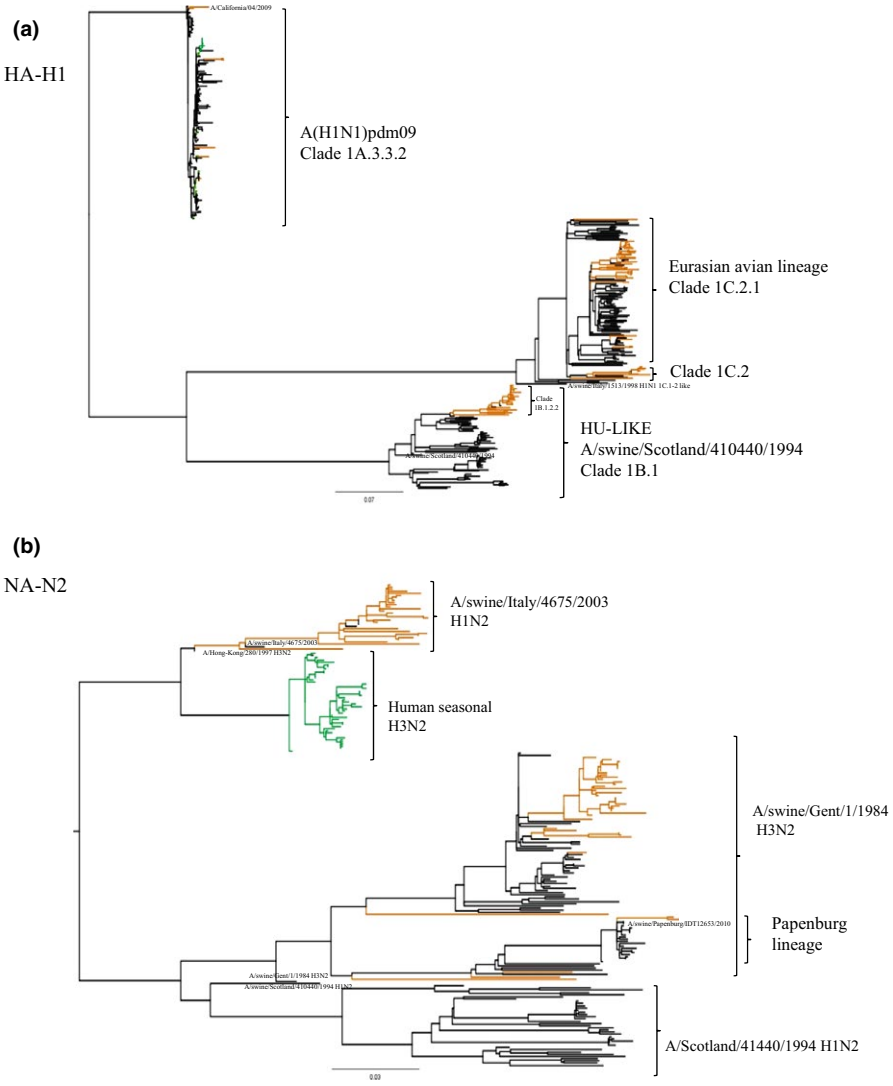


FIGURE 1 ML phylogenetic trees of full-length IAV genes (a) HA-H1, (b) NA-N2. Branches of IAV strains of this study are marked by the colour green (human) and orange (swine). H1 clade names were assigned using the nomenclature proposed by Anderson et al. (Anderson et al., 2016)

Ciacci-Zanella, 2015; Nelson, Stratton, Killian, Janas-Martindale, & Vincent, 2015; Nelson, Culhane, et al., 2015) has been described.

Human influenza is an acute respiratory disease that affects people of all ages even several times during their lifetime. The high degree of antigenic and genetic variation is responsible for the seasonal occurrence of epidemics and occasional pandemics. In Italy, the incidence and epidemiology of respiratory disease is monitored thanks to a dedicated network: InfluNet (http://www.iss.it/iflu/). The influenza sentinel surveillance system InfluNet is coordinated by the Ministry of Health in collaboration with the National Institute of Health (ISS), the Inter-University Centre for Research on Influenza and other transmissible diseases (CIRI-IT), the Regional Health Service, general practitioners and paediatricians and reference University laboratories. Human influenza infection affects 5%–10% of the EU population every year and causes a number of deaths estimated between 15,000 and 70,000 (Lopalco, 2016).

The IAV subtypes currently circulating in humans are H3N2 and, since 2009, H1N1pdm09, in addition to the circulating influenza virus type B.

Human-to-swine reverse zoonotic events have been frequently reported worldwide since 2009 (Nelson et al., 2014; Nelson, Schaefer, et al., 2015; Nelson, Stratton, et al., 2015), but also swine-to-human IAV transmission was detected. In the USA, many zoonotic infections of people by swine H3N2 subtype have occurred since 2011. The main risk factor for infection with H3N2 was identified as direct contact with swine, primarily during agricultural fairs (Bowman et al., 2014; Nelson et al., 2016).

Given the high variability of IAVs, it is crucial to monitor their circulation in different hosts and focus on the occurrence of transmission events of these viruses between animals and humans and vice versa. This study considers swine and human IAVs isolated in the period 2010–2015 in Northern Italy, more specifically in Emilia Romagna and Lombardy (accounting for nearly 15 million inhabitants—i.e. a quarter

Subtype	Genotype	HA	NA	PB1	PB2	PA	NP	M	NS	N. strains (%)	source
H1N1	1									29 (27.9%)	SWINE
H3N2	2									25 (24.0%)	
H1N2	3									21 (20.2%)	
H1N2	4									8 (7.7%)	
H1N1	5									6 (5.8%)	
H1N2	6									5 (4.8%)	
H1N2	7									3 (2.9%)	
H1N1	8									2 (1.9%)	
H1N2	9									2 (1.9%)	
H1N2	10									1 (1.0%)	
H1N1	11									1 (1.0%)	
H3N2	12									1 (1.0%)	
H1N1	5									32 (42%)	HUMAN
H3N2	13									44 (48%)	

EA avian H1-A/swine/Italy/1513/1998 H1N1
A(H1N1)pdm09-A/California/04/2009 H1N1
SW-H3N2-A/swine/Gent-1/1984 H3N2
HU H1-A/swine/Italy/4675/2003 H1N2
HU-H3N2 A/Hong/Kong/280/1997 H3N2
HU seasonal H3N2

FIGURE 2 Lineages and gene constellation of IAV strains of the study. The genotypes 1–13 were assigned to each genetic combination. The origin of each segment was assigned on the basis of genetic clustering with reference strains

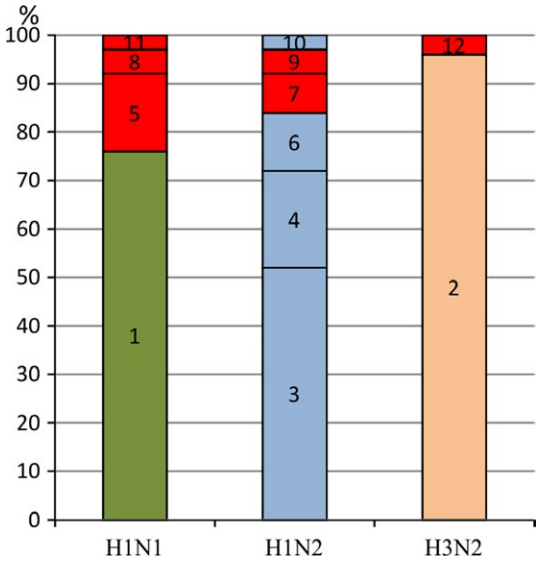


FIGURE 3 Percentages of genotypes 1–12 among H1N1, H1N2 and H3N2 subtypes of swIAVs. Genotypes with at least one A(H1N1) pdm09-derived gene are shown in red. Genotypes with endemic swIAV genes only are in green (H1N1), blue (H1N2) and orange (H3N2)

of the Italian population), an area with a high density of human population (~500 persons per square kilometre) and a high density of pig farms (~5,000,000 animals, which represent 60% of the Italian pig population). SwIAVs were collected in the framework of a passive surveillance programme connected to IZSLER routine diagnostic activities of respiratory disease outbreaks in pigs, while a selection of human IAVs came from the InfluNet network. The selected IAVs were examined genetically by whole-genome characterization to disclose the genetic exchange between human and swine IAVs.

2 | MATERIALS AND METHODS

2.1 | Sample collection and virus sequencing

Human respiratory samples were collected from outpatients with influenza-like illness (ILI) during the 2010–2015 seasonal epidemics by sentinel practitioners and were anonymously analysed at the reference laboratories in Milan and Parma, in the frame of the National Surveillance Plan (InfluNet). Informed consent was not necessary, as outpatients with ILI were included in a regional diagnostic and clinical management protocol. Human IAVs were amplified in MDCK cells after one passage (World Health Organization. 2014).

Swine respiratory samples were collected by IZSLER from respiratory outbreaks in pig farms.

SwIAVs isolated after two serial passages in MDCK or CACO-2 cell culture (Chiapponi, Zanni, Garbarino, Barigazzi, & Foni, 2010) and preliminarily subtyped at the time of collection (Chiapponi, Moreno, Barbieri, Merenda, & Foni, 2012; LeBlanc et al., 2009) were selected for genetic characterization. Swine-selected viruses came from independent respiratory outbreaks. When more than one isolate was obtained from the same respiratory outbreak, only one was chosen for further analysis.

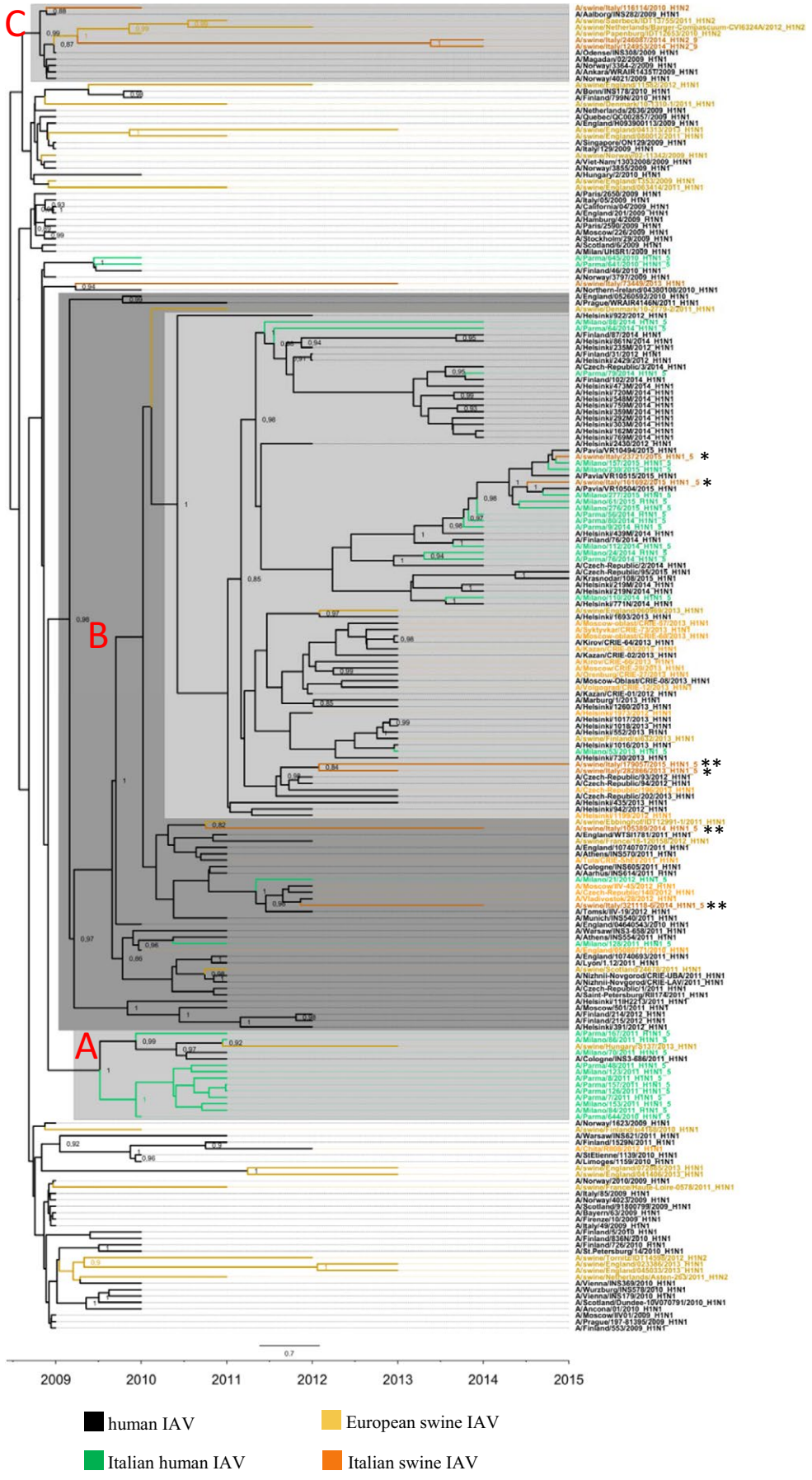
Viral RNA was extracted from samples using TRIzol (Thermo Fisher Scientific, MA, USA) or RNeasy Mini kits (Qiagen, Milan, Italy) according to the manufacturer's instructions. RT-PCR on all eight genome segments was performed as described by Lycett et al. (Lycett et al., 2012) using the SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity (Thermo Fisher Scientific, MA, USA). RT-PCR products were purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co., Düren, Germany). DNA libraries were made with a NEXTERA-XT kit (Illumina Inc. San Diego, CA, USA) according to the manufacturer's instructions. Pooled libraries were sequenced on a MiSeq Instrument (Illumina Inc. San Diego, CA, USA) using a Miseq Reagent Kit v2 in a 250-cycle paired-end run. Data were assembled de novo by the NextGen DNASTAR application and were analysed using Lasergene Package software (Ver 12.0). Obtained sequences were submitted to the GenBank database (Accession identifiers: KU321893-KU323320).

2.2 | Genetic analysis

We analysed all eight gene segments, performing a preliminary step by querying every nucleotide sequence in the GenBank database (http://blast.ncbi.nlm.nih.gov/BLAST) and identifying closely related sequences. Other European human and swine IAV reference sequences were retrieved from the GenBank Influenza virus resource database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database). Gene sequences were then aligned with ClustalW using MEGA5 (Tamura et al., 2011). For the purposes of lineage assignment, phylogenetic trees of the individual segments were inferred with the maximum-likelihood (ML) method implemented in IQ-TREE package 0.9.6 (Minh, Nguyen, & von Haeseler, 2013). The robustness of the ML trees was statically evaluated by bootstrap analysis with 1,000 bootstrap samples. The origin of each segment was named by its clustering with reference strains. In addition, swine H1 sequences were analysed and named using the recently developed tool available on the Influenza Research Database (IRD) at http://www.fludb.org (Anderson et al., 2016; Squires et al., 2012; Zhang et al., 2017).

Due to the segmented genome, every virus had to be described with a specific gene combination, and viruses were further classified into numbered genotypes.

FIGURE 4 Time-scaled Bayesian MCC tree inferred for the HA segment of A(H1N1)pdm09 IAV lineage. Posterior probabilities >0.8 are included. Human and swine viruses are represented by branch colours. The strains cited in the text are marked with asterisks (*, **, ***). Clade A: 2011 human Italian IAVs, clade B: 2011–2015 Italian and European viruses. Clade C: 2009–2010 H1N1pdm09 human viruses, H1N2 swine strains of genotype 9, Papenburg lineage


C
B
A
2009
2010
2011
2012
2013
2014
2015
human IAV
European swine IAV
Italian human IAV
Italian swine IAV


A
B
C
2008
2009
2010
2011
2012
2013
2014
2015
human IAV
European swine IAV
Italian human IAV
Italian swine IAV

FIGURE 5 Time-scaled Bayesian MCC tree inferred for the NP segment of A(H1N1)pdm09 IAV lineage. Posterior probabilities >0.8 are included. Human and swine viruses are represented by branch colours. The strains cited in the text are marked with asterisks (*, **, ***). Clade A: 2011 human Italian IAVs, clade B: 2011–2015 Italian and European viruses. Clade C: 2009–2010 H1N1pdm09 human viruses, H1N2 swine strains of genotype 9, Papenburg lineage

The sequences of swIAV NP and HA gene segments of H1N1pdm09 lineage were aligned using ClustalW as described above. The predicted amino acid sequence was obtained for each gene. In addition, amino acid positions 53 and 289 were analysed for NP (Liang et al., 2014; Manz et al., 2013) and amino acid positions 159, 172, 183, 200, 202, 204 for HA (Lange et al., 2013).

2.3 | Phylogenetic analysis and molecular clock

The evolutionary model that best fitted the data (GTR + G for the HA dataset gene segments and HKY + I for the NP region) was selected using an information criterion implemented in JmodelTest (Posada, 2008) (freely available at http://darwin.uvigo.es/software/jmodeltest.html).

The evolutionary rates were estimated using a Bayesian MCMC method implemented in BEAST 1.8.0 (Drummond, Suchard, Xie, & Rambaut, 2012) under strict and relaxed clock conditions, and an uncorrelated log normal rate distribution model. Four demographic models of population growth were compared as coalescent priors: constant size, exponential growth, logistic growth and a piecewise-constant Bayesian skyline plot (BSP) (Drummond, Rambaut, Shapiro, & Pybus, 2005).

A Bayes factor (BF, using marginal likelihoods) implemented in Beast selected the best-fitting models (Suchard, Weiss, & Sinsheimer, 2001). In accordance with Kass et al. (Kass & Raftery, 1995), only values of 2lnBF ≥6 were considered significant. Two independent MCMC chains were run for 150 million generations (with sampling every 15,000th generation) for the HA portion, and 90 million generations were sampled every 90,000 steps for NP sequence gene segments and were combined using the LogCombiner 1.80 included in the BEAST package. Convergence was assessed on the basis of the effective sampling size (ESS) after a 10% burn-in using Tracer software version 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Only ESSs of ≥200 were accepted.

Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals.

The obtained trees were summarized in a maximum clade credibility tree using the Tree Annotator program included in the BEAST package, and the tree with the maximum product of posterior probabilities (maximum clade credibility: MCC) after a 10% burn-in was displayed using Figtree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

3 | RESULTS

We examined 179 IAV strains collected from 2010 to 2015 in Northern Italy. Seventy-five were isolated from humans (32 H1N1pdm09 and 44 H3N2) and 104 from pigs (31 H1N1, 38 H1N2, 26 H3N2 and nine H1N1pdm09).

A preliminary phylogeny of the whole genomes of Italian isolates, with the arrangement of the genes, allowed us to further assign each virus to its lineage, genotype and origin (Figure 1 for H1 and N2 gene trees, Supporting Information S1 for H3, N1 and PB2 gene trees, not shown for PB1, PA, NP, M and NS genes).

We detected two different genotypes, namely H1N1pdm09 and H3N2 (genotypes 5 and 13), in humans, and 12 genotypes in swine with 22 reassortant strains (21.2%) (Figure 2). The highest variability was found among H1N2 strains with the presence of six genotypes (Figure 3) made by the circulation of three lineages of HA-H1 (EA avianH1—Clade 1C.2, H1N1pdm09—Clade 1A.3.3.2, H1hu-A/swine/Italy/4675/2003—Clade 1B.1.2.2) and two lineages of NA-N2 (swH3N2-A/swine/Gent-1/1984 H3N2 and huH3N2 A/Hong/Kong/280/1997 H3N2) (Figures 1 and 2) .

Preliminary genetic characterization of the subtypes showed that human and pig H3N2 IAVs segregated separately; none of the human H3N2 isolates included in this study was of swine origin, and only one 2011 swine H3N2 isolate was a double reassortant with a constellation of HA, NA, PB1, NP and M of EA swIAV origin and PB2, PA and NS genes of H1N1pdm09 origin (genotype 12).

The only genetic exchange between human and pigs found in this study concerned genes from the H1N1pdm09 lineage; therefore, we focused the analysis on the phylogeny of full-length HA and NP genes—representative of external and internal segments, respectively—of H1N1pdm09 origin from H1N1 and H1N2 IAVs isolated in Italy from 2010 to 2015.

Dated trees were built by comparing a strict clock versus a relaxed lognormal model.

The BF analysis showed that the relaxed clock fitted the data significantly better than the strict clock (2lnBF = 328.8 for the HA dataset gene segments, 2lnBF = 21.6 for the NP region). Moreover, under the relaxed clock the BF analysis showed that the Bayesian skyline model was better than the other models for all two datasets of sequences (2lnBF always >91.2 for BSP).

The presence of H1N1pdm09 genes among swine H1N1 and H1N2 strains was evident from 2010 onwards. The phylogenetic analysis of H1N1pdm09 HA (Figure 4) and NP (Figure 5) showed the distribution of the Italian sequences in three main clades, A, B and C. Clade A included human Italian IAVs isolated in 2011, while clade B included Italian and European viruses circulating in the period 2011–2015. Clade C comprised human H1N1pdm09 viruses of the pandemic season (2009/2010), several H1N2 swine strains of genotype 9 belonging to the so-called Papenburg lineage established in Germany since 2010 (Lange et al., 2013), one Italian swine H1N2 strain isolated in 2009 (Moreno et al. 2011), and two swine H1N2 strains isolated in 2014 in Italy. This analysis showed the circulation in the pig populations of similar reassortant strains with a common H1N1pdm09 ancestor which had been introduced to pig populations in 2009–2010 (Figures 1, 4 and 5 clade C).

TABLE 1 Amino acid substitutions in positions 53 and 289 of the NP putative protein of the swIAV strains of the study. Each strain is labelled with the assigned genotype. Mutated sites are marked in grey

Strain	Subtype	Genotype	53	289
A/California/04/2009	H1N1	5	D	H
A/swine/Italy/105389/2014	H1N1	5	E	H
A/swine/Italy/161692/2015	H1N1	5	D	H
A/swine/Italy/179057/2015	H1N1	5	E	H
A/swine/Italy/23721/2015	H1N1	5	D	H
A/swine/Italy/282866/2013	H1N1	5	E	H
A/swine/Italy/321118-6/2014	H1N1	5	D	Y
A/swine/Italy/150383-1/2014	H1N2	7	E	H
A/swine/Italy/327497-7/2014	H1N2	7	E	H
A/swine/Italy/56341/2015	H1N2	7	E	H
A/swine/Italy/124953/2014	H1N2	9	D	Y
A/swine/Italy/246087/2014	H1N2	9	D	Y

We did not find any evidence of swine-to-human transmission but we found three separate spillover events from human H1N1pdm09 viruses to swine populations in 2013 and 2015, represented by H1N1 strains of genotype 5 (* Figures 4 and 5). Interestingly, three different genotype 5 strains (** Figures 4 and 5) identified in 2014 and 2015 appeared to be introduced from humans to pig populations some years before their isolation (2010–2011 and 2012, respectively).

These data were confirmed by the NP analysis (Figure 5) and by PB2, PB1, PA, M and NS phylogenies (data not shown). Moreover, the genotype 9 strains of cluster C, isolated in Italy in 2014, showed amino acid mutation 289Y in the NP putative protein (Table 1), as a sign of viral adaptation to swine (Liang et al., 2014), and shared with the Papenburg strains four of five of the unique substitutions (G172E, I183V, S200P, S202N, D204S) in the antigenic site of HA (Lange et al., 2013). It was also possible to detect three H1N2 viruses of genotype 7 (*** Figure 5) in 2014 with endemic Italian external genes and H1N1pdm09 internal genes, all harbouring the pig adaptive hallmark, D53E mutation, in NP putative protein (Table 1). The introduction of H1N1pdm09 internal genes from two different human clusters, A and B, to this viral population could be dated back to approximately 2010–2011 (Figure 5).

4 | DISCUSSION

We examined the genetic composition of IAVs isolated from 2010 to 2015 during the passive surveillance of respiratory illness in humans and pigs in Northern Italy, an area with a high density of human and swine populations. The genetic study of human and swine IAVs isolated in Italy from passive surveillance monitoring did not find signs of transmission from swine to humans. A single zoonotic event was detected in an immunocompromised patient infected by an Italian swine H3N2 IAV circulating at the same time, which did not spread to other people (Piralla et al., 2015). On the other hand, events of viral dissemination from humans to swine occurred.

The introduction of the H1N1pdm09 in the swine population contributed to expand genetic diversity among swIAVs increasing the chance of reassortment events. The circulation of different lineages of HA-H1 and NA-N2 in the Italian pig population made the H1N2 subtype the most variable (six genotypes). The HA-H1 detected in H1huN2hu subtypes was uniquely A/swine/Italy/4675/2003-like (Clade 1B.1.2.2), confirming the region-specific circulation of this haemagglutinin (Anderson et al., 2016; Moreno et al., 2013; Watson et al., 2015).

The analysis of the genome constellation of IAVs circulating in the pig population showed evidence of several viral introductions from humans to pigs, confirming the data collected in the European framework (Watson et al., 2015).

The NP analysis showed a situation similar to that observed in persistent swIAV lineages isolated in China (Liang et al., 2014). While for human IAVs of the H1N1pdm09 lineage the NP always had D53 and 289H amino acid mutations, we showed the pig adaptive characters D53E or 289Y in 4/6 of swIAVs of the H1N1pdm09 lineage (genotype 5). In particular, D53E or 289Y were found in all H1N2 reassortant strains (5/5) of genotypes 7 and 9 (Table 1). This analysis was performed using cultured viruses, and further sequencing studies from original clinical samples will be performed to confirm the data.

Despite signs of viral adaptation to pigs, the H1N1pdm09 IAV and its reassortant-derived strains do not yet seem to have spread to the swine population more than the well-adapted enzootic H1avN1, H1huN2hu and H3N2 strains in Italy. On the other hand, the detection of H1N1pdm09 strains in pigs, with signs of introduction some years before their isolation, could be a consequence of a subclinical, rarely detectable circulation of pandemic strains in the Italian swine population. This hypothesis should be confirmed through the monitoring by active surveillance of swine farms. The commercial exchange of live animals produces viral introductions from other European countries to Italy. This kind of animal handling could explain the detection in Italian pig farms of strains occurring in Northern Europe (i.e. Papenburg lineage).

IAVs evolve differently in human hosts than in swine. In humans, selective pressure of immunity (acquired either by natural infection or vaccination) drives the virus evolution through antigenic drift. In swine, the antigenic evolution of IAVs occurs through long and

divergent evolutionary paths. The immune pressure is less important in pigs because of their short life span (Furuse et al. 2010, de Jong et al. 2007): only adult sows could contribute to viral endemicity and slow antigenic drift of swIAVs.

The average level of yearly influenza vaccination in Italian people in the last years ranged between 12% and 19% (Ministero della Salute. 2016); the coverage rates of influenza vaccination in farm and veterinary workers—who are recommended targets for vaccination—are low. In Italy, inactivated bivalent (H1N1, H3N2) and trivalent (H1N1, H1N2, H3N2) vaccines are available for pigs immunization, vaccination is not a common practice in Italian swine herds and often only sows in breeding herds are vaccinated.

The frequent reassortment between endemic swIAV strains and H1N1pdm09 contributes to generating new antigenic and potentially pandemic variants. In fact, the immune status of the swine population could generate different gene combinations and different swIAVs with new antigenic characteristics, potentially hazardous for a non-immune population as humans are.

The analysis of the whole genome represents an important tool for following and monitoring these evolving situations that could not be highlighted by antigenic or molecular HA/NA subtyping only. Viral surveillance should be implemented with active surveillance plans, at least at the farm level in pigs, but also in swine-exposed workers. In fact, we showed that multiple reassortment events occur in swine, and mixed infections may be underestimated in this species.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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