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1 **Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus**

2

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14 **Abstract**

15 Swine influenza monitoring programs have been in place in Italy since the 1990's and from 2009 testing for
16 the pandemic H1N1/2009 virus (H1N1pdm) virus was also performed on all the swine samples positive for
17 type A influenza. This paper reports the isolation and genomic characterization of a novel H1N2 swine
18 influenza reassortant strain from pigs in Italy that was derived from the H1N1 pdm virus.

19 In May 2010, mild respiratory symptoms were observed in around 10% of the pigs raised on a fattening farm
20 in Italy. Lung homogenate taken from one pig showing respiratory distress was tested for Influenza type A
21 and H1N1pdm by two real time RT-PCR assays. Virus isolation was achieved by inoculation of lung
22 homogenate into specific pathogen free chicken embryonated eggs (SPF CEE) and applied onto Caco-2
23 cells and then the complete genome sequencing and phylogenetic analysis was performed from the CEE
24 isolate.

25 The lung homogenate proved to be positive for both influenza type A (gene M) and H1N1pdm real time RT-
26 PCRs. Virus isolation (A/Sw/It/116114/2010) was obtained from both SPF CEE and Caco-2 cells.

27 Phylogenetic analysis showed that all of the genes of A/Sw/It/116114/2010, with the exception of
28 neuraminidase (NA), belonged to the H1N1pdm cluster. The NA was closely related to two H1N2 double
29 reassortant swine influenza viruses (SIVs), previously isolated in Sweden and Italy. NA sequences for these
30 three strains were clustering with H3N2 SIVs. The emergence of a novel reassortant H1N2 strain derived
31 from H1N1pdm in swine in Italy raises further concerns about whether these viruses will become established
32 in pigs. The new reassortant not only represents a pandemic (zoonotic) threat but also has unknown
33 livestock implications for the European swine industry.

34

35 Keywords: swine influenza, reassortant H1N2 strain, pandemic H1N1/2009 virus, pig, Italy

36 1- Introduction

37 Influenza type A viruses are currently circulating in the European pig population with high prevalence of
38 H1N1, H1N2 and H3N2 subtypes. In April 2009 a new virus, identified as pandemic H1N1/2009 strain of
39 influenza A (H1N1pdm), was reported to have caused a human influenza outbreak in Mexico (Centers for
40 Disease Control and prevention, 2009, pp. 400-2). By June 2009 the H1N1pdm had rapidly spread among
41 humans, leading the WHO to the declaration of the first influenza pandemic of the 21st century (WHO, phase
42 of pandemic alert, 2009). The virus is a quadruple reassortant and contains mostly genes from the triple
43 reassortant H1N1 viruses found in swine in North America as well as two genes, Neuraminidase (NA) and
44 Matrix (M) from Influenza viruses found in swine in Europe and Asia (Schnitzer et al., 2009). In Italy, swine
45 influenza monitoring programs, based on genome detection, virus isolation and sequencing of all respiratory
46 forms, have been in place since the nineties, especially in North Italy where more than 75% of the swine
47 industry is located. Responding to the emergence of the new pandemic virus and consequently to the recent
48 reports on human to animal transmission (Hofshagen et al., 2009; OIE, 2009), testing for the H1N1pdm was
49 also performed on all the swine samples which resulted positive to influenza type A. In the last two years,
50 systematic virological surveillance of influenza viruses in pigs has led to the isolation of 67 swine influenza
51 viruses (SIVs) belonging to the following subtypes: 24 H1N1, 25 H1N2, 18 H3N2 SIVs (Moreno et al., 2010,
52 pp.73), 2 H1N1pdm viruses (Moreno et al., 2010, pp. 52-56) and one new reassortant strain derived from
53 H1N1pdm. This paper reports the first isolation of a new H1N2 reassortant strain on a Italian pig farm. The
54 molecular characterization and phylogenetic analysis of the new reassortant strain revealed that all the
55 genes except the neuraminidase (NA) belonged to the H1N1pdm viruses and the NA gene was closely
56 related to two H1N2 SIV strains, previously isolated in Sweden (A/Sw/Sweden/1021/2009) and Italy
57 (A/sw/Italy/58769/2010).

58 2- Materials and methods

59 2.1 - Clinical samples

60 In May 2010, a clinical outbreak was reported on a pig fattening farm with 4000 heads located in the
61 province of Mantua (North Italy). The clinical signs observed were coughing, dyspnoea, fever, anorexia and
62 depression with 10% morbidity, 0,5% mortality. This farm used a continuous flow system of management
63 with monthly introduction of animals and fortnightly departures to the slaughterhouse. SIV vaccination
64 program had not been in place at the farm. One dead pig was submitted to the laboratory for respiratory
65 disease diagnostic tests.

66 2.2 - Influenza type A antigen detection

67 Lung homogenate was screened for the presence of influenza A viruses by M gene real time RT-PCR (rt RT-
68 PCR) (Spackman et al., 2002). Positive samples were further tested for the H1N1pdm by rt RT-PCR,
69 according to the CDC procedure (Centers for Disease Control and prevention, 2009).

70 2.3 - Detecting swine respiratory pathogens

71 The lung homogenate was cultured using different media for the most common swine respiratory bacterial
72 pathogens. The presence of Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine
73 circoviruses type 2 (PCV2) and *Mycoplasma hyopneumoniae* was determined by using either RT-PCR,
74 multiplex PCR and PCR assays respectively, as previously described (Calsamiglia et al., 1999; Ouardani et
75 al., 1999; Suarez et al., 1994).

76 2.4 - Virus isolation and subtype determination

77 The novel SIV isolate was obtained from a 10% lung homogenate applied onto Caco-2 cells and further
78 inoculated through the allantoic sac route of 9-11 day-old SPF chicken embryonated eggs (CEE). Culture
79 supernatant (CS) after observed cytopathic effect and allantoic fluid (AF) were tested with haemagglutination
80 assay (HA) using chicken erythrocytes performed as described (Swine influenza, OIE Manual, 2010) and for
81 influenza type A and H1N1pdm by real time RT-PCR assays. The subtype of the isolate was determined
82 from culture supernatant, allantoic fluid as well as from lung tissues by three multiplex RT-PCR assays using
83 primers to amplify H1, H3, N1 and N2 SIVs (Chiapponi et al., 2003) and primers described by CDC to amplify
84 fragment 4 of HA gene of H1N1pdm (WHO sequencing primers and protocol, 2009).

85 2.5 - Genome sequencing and phylogenetic analysis

86 For genomic sequencing viral RNA was extracted from allantoic fluid of A/sw/It/116114/2010 using QIAamp
87 ViralRNA Mini Kit (Qiagen, Hilden, Germany). SuperScript III One-Step RT-PCR System with Platinum Taq
88 High Fidelity (Invitrogen Carlsbad, CA, USA) was used to perform RT-PCR of 46 amplicons representing the
89 entire viral genome using the Influenza A H1N1pdm genome Primer Set (Applied Biosystems, Foster City,
90 CA, USA). Sequencing reactions were performed using BigDye Terminator Cycle Sequencing kit v3.1 and
91 resolved by an ABI 3130 DNA automatic sequencer. DNA sequences were combined and edited using the
92 Lasergene sequencing analysis software package (DNASTAR, Madison, WI). Multiple sequence alignments
93 were made using ClustalW and maximum parsimony phylogenetic trees were created using MEGA4
94 (Tamura et al., 2007). Each tree is a consensus of 500 bootstrap replicates.

95 The identification as a novel reassortant strain was confirmed by full length sequencing of HA and NA genes
96 performed directly on lung homogenates.

97 2.6 - Nucleotide sequence accession numbers

98 GenBank accession numbers of the complete genome of A/sw/Italy/116114/2010 were assigned CY067659
99 to CY067666. GenBank accession numbers of A/sw/Italy/58769/2010 were assigned HM771274 to
100 HM771279, HQ168025 and HQ168026.

101 3- Results

102 3.1 - Virus isolation and subtype determination

103 Lung homogenates from one pig carcass showing purple areas of consolidation in the apical lobes of the
104 lungs, interlobular oedema, and fibrinous pleuritis resulted positive for influenza type A and H1N1pdm by real
105 time RT-PCR assays. The clinical specimen was further inoculated in SPF CEE and onto Caco-2 cells for
106 virus isolation.

107 The presence of influenza A and the H1N1pdm was detected on AF and Caco-2 CS by real time RT-PCRs.

108 A multiplex RT-PCR specific for subtype determination was further used to subtype both AF and CS that
109 unexpectedly resulted to be H1pdmN2. The presence of the H1pdmN2 virus in the lung tissues was also
110 confirmed by RT-PCR and was the only H-N combination detected in the sample. The isolate was named
111 A/Sw/It/116114/2010. Respiratory pathogens such as bacteria, *M. hyopneumoniae*, PRRSV or PCV2, which
112 are involved in the porcine respiratory complex, were not observed. Only a *Streptococcus suis* was isolated
113 from the lungs and therefore could be the cause of the fibrinous pleuritis.

114 3.2 - Genomic sequencing and phylogenetic analysis

115 HA and NA sequences were obtained from the clinical specimen and compared with the Influenza
116 sequences available in Genbank, confirming the results of subtype determination obtained by the RT-PCR
117 and real time RT-PCR assays. Full genome sequencing was also obtained from the virus isolate, confirming
118 the results for HA and NA obtained from the original sample.

119 Phylogenetic analysis showed that all the genes of A/Sw/It/116114/2010, with the exception of
120 neuraminidase (NA), belonged to the H1N1pdm clusters. In particular H1 showed more than 99% homology
121 to H1pdm sequences. The phylogenetic tree of the HA gene shows that A/Sw/It/116114/2010 grouped
122 together with H1N1pdm sequences in cluster 7, as most of the H1N1pdm human isolates in Italy (Potdar et
123 al., 2010; Valli et al., 2010) (figure n.1). By contrast, NA was closely related to H1N2 SIV double reassortant
124 viruses, isolated in Sweden (A/Sw/Sweden/1021/2009) (Bálint et al., 2009) and recently also in Italy
125 (A/Sw/It/58769/2010) with 93,8% and 95,5% homology respectively. In the phylogenetic tree, the NA gene of
126 these two H1N2 SIVs grouped together with A/Sw/It/116114/2010 and were located in the cluster of H3N2
127 SIVs (figure n.2). This Italian strain was isolated in 2010 from piglets with respiratory lesions of SI, raised on
128 a breeder farm located in North Italy.

129 Full genome sequencing of A/Sw/It/116114/2010 confirms a very high similarity (from 99-100%) through the
130 seven genes to H1N1pdm circulating in human and animal species. Comparison with the H1N1pdm
131 sequences available in GenBank shows in A/Sw/It/116114/2010 three unique amino-acid (aa) changes
132 respectively in HA (N260K) (H3 numbering) and NP (A85V and G404S) proteins, not yet associated with
133 any well characterized phenotype marker of influenza viruses. Two other aa changes of interest were also
134 found. The first one in PB2 protein (K340N), frequently co-occurred with the potentially virulent HA-D222G
135 mutation (WHO, 2009), although the latter is absent in A/Sw/It/116114/2010. The second aa change is the
136 E67K substitution in NS2 protein, previously correlated with a possible modification of the protein function in
137 viral nucleocapsid export from the nucleus (Wu et al., 2009). Comparison between NA proteins of
138 A/Sw/It/116114/2010 and A/Sw/It/58769/2010 revealed changes in different phylogenetically important
139 regions (PIRs) (Fanning et al., 2000): PIR-A-Y40C and C42R, PIR-F N339H, PIR-G D346N , PIR-K N401D.
140 Combinations of HA and NA subtypes need to be functional to recognize the same type of sialic acid
141 modifications to allow smooth interplay of the two proteins, which is important for the viral cycle (Wagner et
142 al., 2002).

143 Complete genome sequences of the novel H1N2 strain were compared to those of A/California/4/2009 H1N1
144 pdm and A/Sw/It/58769/2010 H1N2 SIV and the amino acid differences were reported in table n.1.

145 4 - Discussion

146 The identification of the new H1N2 reassortant virus demonstrates once again the important role played by
147 pigs as mixing vessels for animal and human influenza, providing a place for reassortment and host
148 adaptation to take place. These results show that the introduction of H1N1pdm in the swine population has
149 provided opportunities for reassortment with the risk that the H1N1pdm virus could alter transmissibility and
150 increase virulence. The success of interspecies transmission of influenza virus depends on the viral gene
151 constellation. Successful transmission between species can follow genetic reassortment with a progeny virus
152 containing a specific gene constellation with the ability to replicate in the new host. The new reassortant virus
153 could have the capability to replicate efficiently in humans, considering its specific gene constellation. This is
154 characterized by 7 genes belonging to H1N1pdm, well-adapted to humans and the NA gene closely related
155 to H1N2 SIVs.

156 The isolation of H1N1pdm from pigs in different countries also raises further concerns about the possible
157 establishment of H1N1pdm in the swine population. This could lead to a scenario where a co-circulation of
158 influenza A viruses within swine that could act as mixing vessels for the reassortment between influenza
159 viruses of both mammal and avian origin, with unknown implications for public health. All these facts could

160 have a great impact in the Northern part of Italy where over 70% of swine and 65% of poultry are raised and
161 several epidemics of avian influenza, H5 and H7 subtypes, occurred in the last ten years (Alexander et al.,
162 2007).

163 The immune selection is not considered important in pigs due to the continuous availability of susceptible
164 pigs. Previous studies reported that human H1N1 and H3N2 viruses could be frequently transmitted to pigs
165 but fail to persist (Van Reeth, 2007). This could be explained by the fact that virus strains with different
166 antigenic characteristics could be disadvantaged compared to the well-adapted established viruses which
167 are circulating among the susceptible population. However, some cases, such as the emergence of the
168 H1N2 subtype in UK with a HA closely related to human H1N1 viruses (Brown et al., 1998) and subsequently
169 spread in the European pig population, suggest that the genes of human viruses may persist after
170 reassortment with one or more influenza viruses in pigs and following adaptation to pigs may often be
171 associated with clinical disease. Moreover, the recent emergence and persistence of reassortant H1N2
172 strains in Italy since 2003, with a NA closely related to recent H3N2 human viruses (Moreno et al., 2010,
173 pp.73), could be another example supporting these hypotheses.

174 In the case we are reporting, after the isolation of the reassortant strain the three following groups of animals
175 sent to the slaughterhouse were tested for influenza type A. Nasal swabs, collected from 60 pigs, were
176 tested by M gene real time RT-PCR and resulted negative.

177 The unusual circulation of H1N1pdm in swine herds worldwide raises concern about the generation and
178 transmission to humans of virus reassortants with key molecular determinants of pathogenicity lacking in the
179 current H1N1pdm (Neumann et al., 2009).

180 Recently, swine influenza surveillance in Hong Kong has given raise to the isolation of a reassortant virus
181 containing seven genes derived from SIVs circulating in China, and the H1N1pdm NA gene (Vijaykrishna et
182 al., 2010).

183 These independent isolation events underline the importance of surveillance and complete genetic
184 characterization of influenza viruses isolated in the swine population worldwide.

185 Conflicts of interest statement

186 All authors have declared that no conflicts of interest exist.

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269

270 Table n.1 - Amino acid substitutions in HA and internal proteins of H1N1pdm viruses A/California/04/2009 -
 271 A/Sw/It/290272/09 and H1N2 reassortant A/Sw/It/116114/10.

Gene segment	Residue number	A/California/04/2009 (H1N1pdm)	A/Sw/It/290272/09 (H1N1pdm)	A/Sw/It/116114/10 (H1N2 reassortant virus)
PB2	340	K	K	N
	405	S	T	S
PB1	386	K	R	K
	535	I	V	I
	728	V	V	I
	736	K	G	K
PA	14	I	V	V
	224	P	S	S
	256	K	Q	K
HA	100	P	S	S
	214	T	A	A
	220	S	T	T
	277	N	N	K
	391	E	K	E
	544	V	V	I
	85	A	A	V
NP	100	V	I	I
	101	D	N	D
	375	D	D	N
	404	G	G	S
NS1	123	I	V	V
NS2	67	E	E	K

272

273

274 Figure n.1 - Phylogenetic relationship of the HA gene of the swine reassortant isolate
275 (A/sw/Italy/116114/2010 H1N2) with database retrieved pandemic H1N1/2009 viruses. The unrooted tree
276 was created by maximum parsimony method and bootstrapped with 500 replicates. Only bootstrap values
277 higher than 50% are shown.

278

279 Figure n.2 - Phylogenetic relationship of the NA gene of the swine reassortant H1N2 Italian isolate
280 (A/sw/Italy/116114/2010 H1N2) with other Italian SIV viruses and database retrieved H1N2 and H3N2 swine
281 and H3N2 human viruses. The unrooted tree was generated as described in the legend of figure n.1.

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Figure n.1 - Phylogenetic relationship of the HA gene of the swine reassortant isolate (A/sw/Italy/116114/2010 H1N2) with database retrieved p H1N1 viruses. The unrooted tree was created by maximum parsimony method and bootstrapped with 500 replicates. Only bootstrap values higher than 50% are shown.

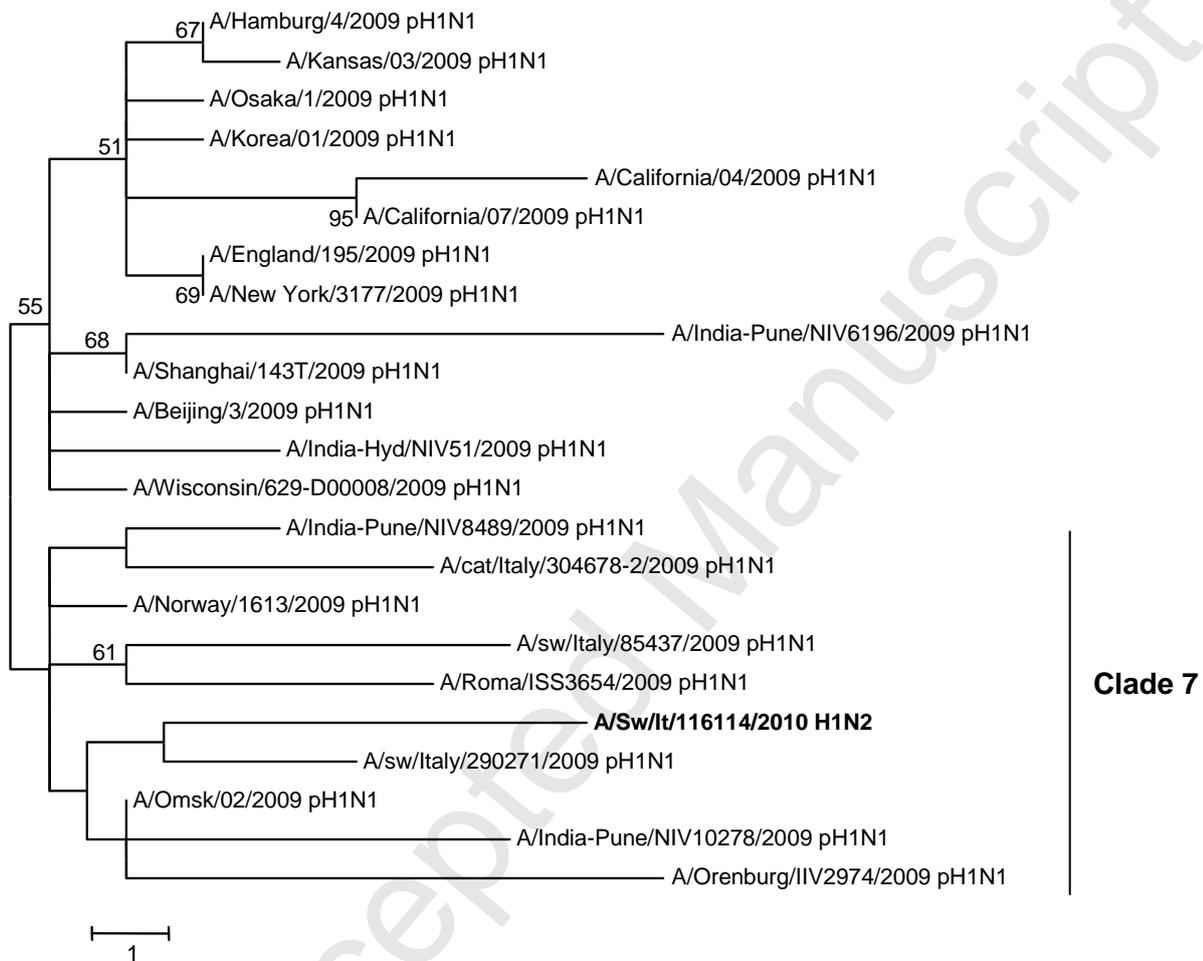


Figure n.2 - Phylogenetic relationship of the NA gene of the swine reassortant H1N2 Italian isolate (A/sw/Italy/116114/2010 H1N2) with other Italian SIV viruses and database retrieved H1N2 and H3N2 swine and H3N2 human viruses. The unrooted tree was generated as described in the legend of figure n.1.

