Detection and molecular analysis of Pseudorabies virus strains isolated from dogs and a wild boar in Italy

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ABSTRACT

Aujeszky's disease (AD) is one of the most economically important diseases of farmed pigs. Wild boars can act as reservoirs and might represent a potential threat for domestic animals, including dogs. The aim of this study was to report the results of an AD survey based on the Pseudorabies virus (PRV) genome detection in samples of dogs clinically suspected of AD and of wild boars collected during four consecutive hunting seasons in the period 2010–2014. Genomic characterization was based on the partial gC sequence of the Italian strains and the comparison with those from domestic pigs and European PRV strains circulating in wild boars. The Italian PRV strains were mainly distributed into three different clusters and revealed two interesting findings. First, there was a clear distinction between the viral strains that were isolated from dogs used for hunting and subsequently traced back to wild boars and the strains that were isolated from working dogs and subsequently found to be closely related to domestic pigs. Second, the Italian epidemiological situation was found to be different from those of European countries in that the Italian situation was characterized by the presence of both the typical Italian clades 1 and 2 and supported by new patterns of aa deletions/insertions. Italian clade 1 included strains from hunting dogs and two Italian wild boars, and Italian clade 2 grouped with recent strains from dogs that were unable to hunt and domestic pigs that were related to one old reference strain (S66) and not included elsewhere. Molecular and phylogenetic analyses of PRV strains are therefore necessary to improve the understand- ing of the distribution of the PRV clusters and their evolution.

1. Introduction

Pseudorabies virus(PRV) is the causal agent of Aujeszky's disease (AD), an animal disease primarily affecting pigs but also known to occur occasionally in cattle, sheep, goats, horses, dogs and cats. Suids are the natural reservoir of the virus, and the disease is self-limiting in the other species. AD is a contagious infection and is mainly transmitted by direct and indirect contact between pigs.

The causative agent is an enveloped DNA virus, which belongs to the Alphaherpesvirinae subfamily of the Herpesviridae family. AD is a notifiable disease that causes substantial economic losses to the swine industry and has major economic impact due to trade implications and income losses for farmers.

Italian pig production is concentrated (over 80%) in the four Northern Italian regions of Lombardia, Emilia- Romagna, Piemonte and Veneto. The Lombardia region is the major producer, with 50% of the national pig production (Maiorano, 2009). Compared with the pig production of other countries, Italian pig production differs in the high live weight of pigs at slaughter; they are slaughtered at 10–12 months of age when they reach a live weight of 150–170 kg and provide carcasses of 125–140 kg maintaining proper adiposity.

The European general policy is to eradicate AD in order to support free intra-EU trade. However, eradication may take several years depending on the epidemiological situation in countries where the disease is endemic. In Italy, an AD national monitoring program was implemen- ted in 1997. However, since 2011, with the issuing of Decree 30/12/2010 and the latest update in 2014, the National Authority has put in place more strict measures to ensure a substantial reduction in virus circulation on pig farms. Thereafter, extraordinary regional control plans were implemented, especially in the Northern Italian regions with the highest concentrations of pig industry (Lombardia Region, D.d.s. 9/05/2014 - n. 3822; Emilia Romagna Region, Delibera Giunta Regionale n. 1588–13/ 10/2014; Veneto Region, Delibera Giunta Regionale n. 2061–11/10/2012), to achieve AD eradication status and to be included in annex two of the EU Decision 2008/185/EC. Important tools in AD eradication plans included the use of DIVA gE-deleted vaccines, movement restrictions, more intensive serological testing and the application of stamping out or slaughter policies to remove infected animals. Only the Bolzano province was classified in this annex that includes member states or regions that applied disease controls programs and have already eradicated AD or are in an advanced stage of eradication.

Although PRV has been eliminated in domestic pigs in many European countries, AD is being continuously reported in wild boar populations and in related hunting dogs (Albina et al., 2000; Gortazar et al., 2002; Lari et al., 2006; Leuenberger et al., 2007; Lutz et al., 2003; Muller et al., 2010; Pannwitz et al., 2012; Roic et al., 2012; Steinrigl et al., 2012; Verin et al., 2014; Vengust et al., 2005; Verpoest et al., 2014). Consequently, the possible impact of wild boars on the application and success of AD eradication programs and the risk they pose to the PRV- free status should be taken into account (Boadella et al., 2012). The role of wild boars as potential reservoirs of PRV has become increasingly important; thus, a deeper investigation on the distribution of PRV strains in wild boars and their genomic characterization at a regional level became necessary. AD surveillance plans were carried out by testing for the presence of anti-PRV antibodies in sera samples collected within the wildlife national monitoring program in different regions of Italy (Lari et al., 2006, Montagnaro et al., 2010, Verin et al., 2014) and by attempting PRV genome detection in wild boar samples voluntary submitted by hunters of some provinces of North Italy. In a previous study conducted on swine and dog strains isolated before 2010, we showed a clear distinction between the strains isolated from hunting dogs exposed to wild boars and those originated from domestic pigs (Sozzi et al., 2014). In the following years, we continued to monitor the epidemiology of AD in Italy to better understand the distribution of the virus clusters and their evolution. In this study, we report the results of an AD survey based on PRV genome detection in samples from dogs clinically suspected of AD and from wild boars collected during four consecutive hunting seasons, 2010– 2014. Moreover, the genome characterization of two strains isolated from dogs in 1993 and 1994 was included. Dogs in which AD was clinically suspected were examined by using histopathological and virological methods, resulting in the isolation of 13 PRV strains from dead dogs. One strain isolated from a wild boar during the same period and region was also genetically characterized. The phylogenetic analysis was based on a partial sequence of the gC gene, and the results were compared with the sequences available in GenBank.

2. Materials and methods

2.1. Animals

A total of 11 dogs of different breeds, which were conferred to IZSLER laboratories in 2010–2014, and two dogs collected in 1993 and 1994 were included in the study. Of the dogs, eight were hunting dogs, four were dogs living in or close to pig farms (farm dogs), and although the last dog's exposure was unknown, the dog was unable to hunt. The origins and years of the PRV strain identifications are reported in Table 1. All of the hunting dogs had been used for hunting in the days immediately before the onset of clinical signs. Epidemiological investigations were conducted by the field veterinary services, and in all cases, the dogs' owners declared that animals had direct contact with wild boars or had been fed PRV-infected meat and/or offal. The clinical signs in the dogs included neurological signs, such as tremor, trismus, spasms of the muscles of the larynx and pharynx, dyspnea, vomiting and pruritus. Death occurred within 24–48 h.

Wild boar tissue samples of lungs and tonsils were voluntarily collected by hunters in several provinces of North Italy during the period 2011–2014. The sampling sites were divided according two different ecologic areas, the Alps (AP) and the Apennines (AN). A total of 176 samples originated from AP and 155 from AN with the following per annum distribution: 2011- 86 AP, 3 AN; 2012- 89 AP, 3 AN; 2013- 1 AP, 99 AN; 2014- 48 AN.

2.2. Laboratory investigations

Complete necropsies followed by virological examina- tions of selected organs were performed.

The presence of PRV DNA in the field samples was systematically determined by real-time PCR tests based on the specific detection of the gE gene as described by Yoon et al., 2005.

Table 1
Data of the Italian PRV strains used in the study.

Year	N8	Sample n.	Province of origin	Species (attitude)–Breed	Age (years)	gC clade	Accession number gC
1993	1	3718	Piacenza	Farm Dog- n.a.	n.a.	Clade 3	KP780805
1994	2	736	Piacenza	Farm Dog- n.a.	n.a.	Clade 3	KP780806
2010	3	294871	Piacenza	Hunting Dog–n.a.	n.a.	Italian Clade 1	KP862621
2011	4	286509	Brescia	Hunting Dog- Maremma-Hound	4	Italian Clade 1	KP862611
	5	286672	Pavia	Hunting Dog - Mongrel	4	Italian Clade 1	KP862612
	6	290422	Brescia	Hunting Dog- Hound	3	Italian Clade 1	KP862613
	7	309516-2	Brescia	Wild Boar	n.a.	Italian Clade 1	KP893284
	8	310919/1	Brescia	Hunting Dog- Jura hound dog	>5	Italian Clade 1	KP862614
	9	310919/2	Brescia	Hunting Dog- Maremma -Hound	>5	Italian Clade 1	KP862615
2012	10	4966	Cremona	Farm Dog–n.a.	n.a.	Italian Clade 2	KP862616
	11	22640	Milano	Farm Dog–n.a.	<1	Italian Clade 2	KP862617
	12	160938	Napoli	Dog–n.a.	n.a.	Italian Clade 2	KP862618
2014	13	299424	Piacenza	Hunting Dog	2	Italian Clade 1	KP862619
	14	360167	Forlı`	Hunting Dog	n.a.	Italian Clade 1	KP862620

n.a.: not available.

Virus isolation was performed on PCR-positive samples as previously described (Sozzi et al., 2014).

Histological and immunohistochemical investigations were completed only on fresh dog samples taken from well-preserved carcasses. A pool of three MAbs (1F2, 2E12 and 3D5) was used. Of these, MAb 1F2 recognized the gC protein, whereas the other two recognized the gE protein (Grieco et al., 1997).

2.3. Phylogenetic and molecular analyses

Partially sequencing of the gC genes was performed. Genomic DNA extraction from the cells

culture super- natants was performed using an RNeasy kit (Qiagen, Hilden, Germany), and the PCR protocol described by Fonseca et al. (2010) was employed. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia; CA, USA). DNA sequencing was performed using a Big-Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same primers that were used for amplification. The sequencing reactions were run by capillary electrophoresis on an automatic sequencer (ABI 3130 Genetic Analyser; Applied Biosys- tems, Foster City, CA, USA). The sequences were edited using the SeqMan program (DNASTAR, Madison, USA). The sequence alignments were performed using the ClustalW W method (DNASTAR, Madison, USA). The best-fit model of the nucleotide substitution was determined using the jModelTest v.0.1.1 (Posada D, 2008). All of the models were compared using two criteria; i.e., the Akaike's information criterion (AIC) and the Bayesian information criterion (BIC). The preferred model was the HKY85 + I + G model. The phylogenetic tree was constructed with the maximum likelihood (ML) method within the IQ-tree software with bootstrap analyses involving 1000 replicates (Minh et al., 2013). The topologies were verified with the neighbor- joining method and the Kimura two-parameter model using MEGA 5.0 (Tamura et al., 2011). The Italian isolates were compared with the sequences of the references and field PRV strains that originated from other countries and are available at GenBank. The deduced amino acid sequences were aligned using ClustalW, and the alignment was manually optimized. For the molecular analyses, the presence of aa deletions or insertions relative to the gC prototype sequence YP068347 was analyzed.

The predicted glycosylation sites on the gC proteins of the Italian viruses were identified with NetNGlyc 1.0. A threshold value of a 0.5 average potential score was set to predict the glycosylated sites.

The sequences described in this report have been deposited in the GenBank database under the accession numbers reported in Table 1.

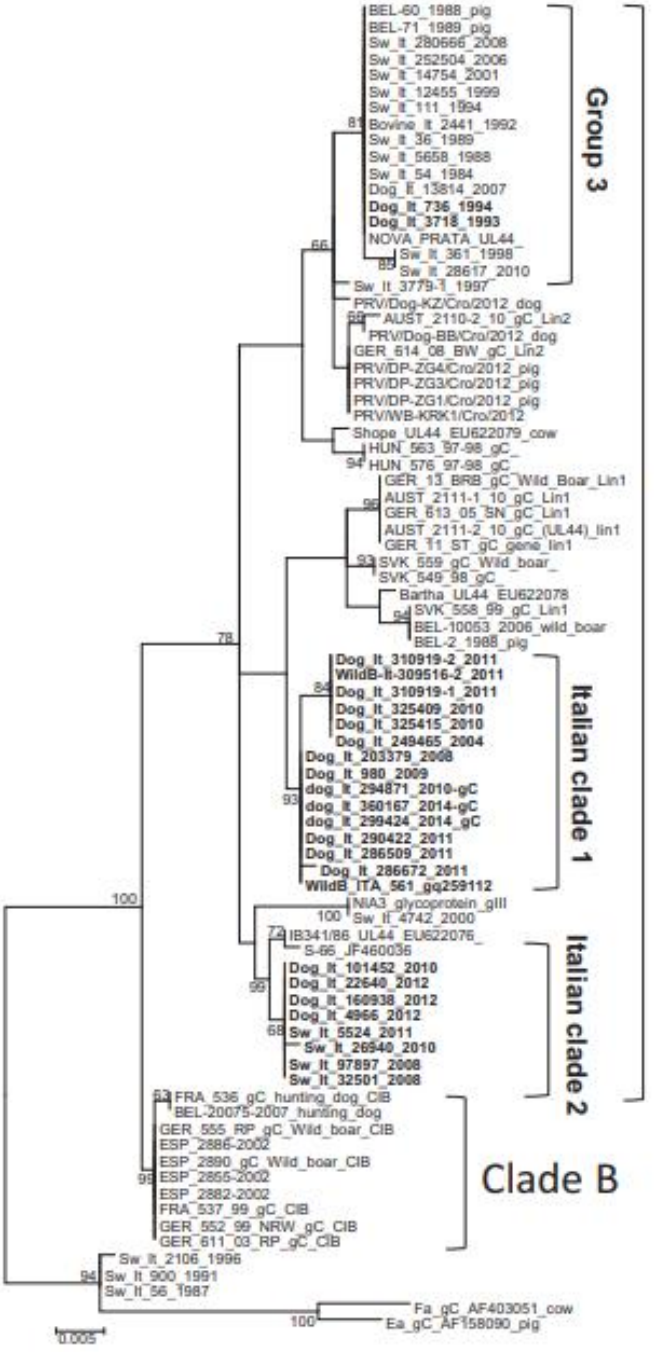
3. Results

3.1. Laboratory investigations

During the necropsies, the external examinations of the carcasses revealed that the majority of animals had periorbital and facial abrasions that likely resulted from intense pruritus. Relevant internal gross lesions were not observed with the exception of mild to diffuse acute pulmonary alveolar emphysema and edema. Thirteen PRV strains were obtained from dog samples through inocula- tion onto PK-15 cells at the first passage, and the virus isolation was confirmed by PRV real-time PCR. Ten wild boar samples tested PCR-positive with the following spatiotemporal distribution: 2011- 5 AP, 2012- 2 AP, 2013- 2 AN, 2014 -1 AN. PCR-positive wild boar samples were also inoculated onto PK-15 cells, but the virus isolation and PCR for sequencing was attempted only in one case (WB-It-309516-2-2011). Immunohistochemistry performed in some dog samples revealed viral antigens in the cytoplasm of both neurons and glial cells within inflamed brain tissue.

3.2. Phylogenetic and molecular analyses

The phylogenetic analysis revealed that the Italian strains were divided into the following three clades. Clade 1 included eight strains from hunting dogs and one strain from a wild boar, and they were closely related to another


BEL-60_1988_pig
BEL-71_1989_pig
Sw_It_280666_2008
Sw_It_252504_2006
Sw_It_14754_2001
Sw_It_12455_1999
Sw_It_111_1994
Bovine_It_2441_1992
Sw_It_36_1989
Sw_It_5658_1988
Sw_It_54_1984
Dog_It_13814_2007
Dog_It_736_1994
Dog_It_3718_1993
NOVA_PRATA_UL44_
Sw_It_361_1998
Sw_It_28617_2010
Sw_It_3779-1_1997
PRV/Dog-KZ/Cro/2012_dog
AUST_2110-2_10_gC_Lin2
PRV/Dog-BB/Cro/2012_dog
GER_614_08_BW_gC_Lin2
PRV/DP-ZG4/Cro/2012_pig
PRV/DP-ZG3/Cro/2012_pig
PRV/DP-ZG1/Cro/2012_pig
PRV/WB-KRK1/Cro/2012
Shope_UL44_EU622079_cow
HUN_563_97-98_gC_
HUN_576_97-98_gC_
GER_13_BRB_gC_Wild_Boar_Lin1
AUST_2111-1_10_gC_Lin1
GER_613_05_SN_gC_Lin1
AUST_2111-2_10_gC_(UL44)_lin1
GER_11_ST_gC_gene_lin1
SVK_559_gC_Wild_boar_
SVK_549_98_gC_
Bartha_UL44_EU622078
SVK_558_99_gC_Lin1
BEL-10053_2006_wild_boar
BEL-2_1988_pig
Dog_It_310919-2_2011
WildB-It-309516-2_2011
Dog_It_310919-1_2011
Dog_It_325409_2010
Dog_It_325415_2010
Dog_It_249465_2004
Dog_It_203379_2008
Dog_It_980_2009
dog_It_294871_2010-gC
dog_It_360167_2014-gC
dog_It_299424_2014_gC
Dog_It_290422_2011
Dog_It_286509_2011
Dog_It_286672_2011
WildB_ITA_561_gq259112
NIA3_glycoprotein_gIII
Sw_It_4742_2000
IB341/86_UL44_EU622076_
S-66_JF460036
Dog_It_101452_2010
Dog_It_22640_2012
Dog_It_160938_2012
Dog_It_4966_2012
Sw_It_5524_2011
Sw_It_26940_2010
Sw_It_97897_2008
Sw_It_32501_2008
FRA_536_gC_hunting_dog_CIB
BEL-20075-2007_hunting_dog
GER_555_RP_gC_Wild_boar_CIB
ESP_2886-2002
ESP_2890_gC_Wild_boar_CIB
ESP_2855-2002
ESP_2882-2002
FRA_537_99_gC_CIB
GER_552_99_NRW_gC_CIB
GER_611_03_RP_gC_CIB
Sw_It_2106_1996
Sw_It_900_1991
Sw_It_56_1987
Fa_gC_AF403051_cow
Ea_gC_AF158090_pig
0.005
Group 3
Clade A
Italian clade 1
Italian clade 2
Clade B

strain (ITA561) that was previously isolated from Italian wild boar in 1993 (Capua et al., 1997) and other hunting dogs (Sozzi et al., 2014). This clade formed a separate Italian group (Italian clade 1) that was clearly distinguishable within Muller's clade A, which included the PRV European feral strains. Clade 2 was formed by two strains that were from two farm dogs and one non-hunting dog of unknown exposure; these strains were closely related to Italian clade 2, field strains that were isolated in 2008–2011 from pigs and other farm dogs. This group exhibited a high percentage of similarity (99%) to the reference strain S66 that was isolated in Sweden and a Brazilian strain (IB341/86) that had not previously been included elsewhere. Clade 3 included the two strainsisolated in the nineties fromfarm dogs which were closely related to pig strains isolated in Europe and America within the last 20 years (Fig. 1).

The analysis of deduced amino acid (aa) sequences revealed that the Italian strains showed three different patterns of aa deletions or insertions that corresponded to the three phylogenetic clades. Italian clade 1 was charac- terized by only a single aa deletion at position 25. Italian clade 2 carried two deletions at positions 25 and 39. Clade 3 contained two aa deletions at positions 25 and 39 and, relative to the prototype sequence, and contained one insertion between positions 184 and 185 (184A).

The analysis was then focused on the hot spot region located in the gC protein between residues 180 and 185, which showed the largest aa variations and were associated with changes in the hydrophobicity profile (Fonseca et al., 2012). Two different aa profiles were identified in the Italian strains; the first profile (VVVE) was related to the wild boar strains and was present in Italian clades 1 and 2. Interestingly, Italian clade 2 included only strains isolated recently from domestic pigs and from dogs unable to hunt and hence not exposed to the wild boar population. The second profile (ALDDD) was present in clade 3 and was closely related to the pig strains. The analysis of the predicted glycosylation sites in the gC protein showed a highly conserved pattern in all of the PRV strains. It was characterized by three glycosylation sites at positions 40, 84 and 192 according to the prototype sequence YP068347 numbering (Fig. 2).

4. Discussion

Based on the gC analysis, the Italian PRV strains were primarily distributed into three different clusters, and this distribution revealed interesting findings. There was a clear distinction between both the wild boar strains (and those isolated from dogs that were used for hunting and subsequently traced back to wild boars) and the strains isolated from working farm dogs (and found to be closely related to strains in domestic pigs). The genetic results also indicated that the hunting dog and wild boar strains exhibited high homology to the PRV strains that were circulating in the 70 s and 80 s in pig farms. Indeed, these strains have nearly completely disappeared from com- mercial pig herds but are still circulating in feral pig populations and thus demonstrate that, in wild popula- tions, the replacement of older strains with more recent PRV swine strains has not yet taken place.

The Italian epidemiology was also found to be different from those of other European countries and was charac- terized by the presence of two typical Italian clades. Strains originated from wild boars and epidemiologically related to hunting dogs belonged to clade A (Muller et al., 2010), although these formed a clearly distinguishable group (Italian clade 1). No strains included in clade B were found. These two clades, A and B, seem to overlap geographically in Central Europe but not in Italy, as isolates of both clades were found in German, Belgium and France (Muller et al., 2010; Verpoest et al., 2014). Strains related to those found in domestic pigs were conversely distributed in two different clades. One of these grouped strains, isolated until 2010, were related to pig strains isolated in Europe and America within the last 20 year.

Studies previously conducted in Italy detected a high PRV seroprevalence mainly in the in wild boar populations of the Apennine Mountains (Lari et al., 2006; Montagnaro et al., 2010; Verin et al., 2014). In contrast to this seroprevalence, PCR-positive samples were detected in a very few cases originating only from the Alps. PCR examination was performed on pooled wild boar tissues and in some cases only lung tissue was available. Similar to our results, the detection of a low number of PCR positive

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Fig. 1. Phylogenetic tree based on the partial nucleotide sequences of the gC gene (690 bp) of the Italian pseudorabies virus (PRV) isolates (boldface) and the corresponding sequences of the selected PRV strains from GenBank. The tree was obtained using the maximum likelihood method and the HKY85 + I + G model with 1000 bootstrap replicates. The bootstrap percentage values are indicated at the nodes. Virus isolated in this work are listed below. For each strain accession number, specie, country, reference number and year of isolation are reported. JQ768151.Sw_Italy_5658_1988; JQ768152.Sw_Italy_36_1989; JQ768154.Sw_Italy_3718_1993;JQ768153.Bov_Italy_2441_1992; JQ768156.Dog_Italy_736_1994; JQ768155.Sw_Italy_111_1994; JQ768114.Sw_Italy_3779-1_1997; JQ768161.Sw_Italy_361_1998; JQ768162.Sw_Italy_12455_1999. JQ768150.Sw_Italy_14754_2001; JQ768126.Sw_Italy_252504_2006; JQ768125.Dog_Italy_13814_2007; JQ768138.Sw_Italy_280666_2008; JQ768111.Sw_Italy_97897_2008; Q768137.Sw_Italy_28617_2010; JQ768110. Sw_Italy_32501_2008; JQ768112. Sw_Italy_26940_2010; JQ768113.Sw_Italy_4742_2000; JQ768116.Sw_Italy_56_1987; JQ768117.Sw_Italy_900_1991; JQ768118.Sw_Italy_2106_1996; KP893283. Sw_Italy_5224_2011; JQ081291.AUST_2111-1_10/HD*; JQ081292.AUST_2111-2_10/HD; JQ081289.AUST_2110-2_10/HD; Q259093.1/GER11ST/ Germany/1996/WB8; GQ862778.GER614BW/Germany/2008/HD; GQ259095.GER13BRB/Germany/1995/WB; GQ259113.HUN563_97-98/WB; GQ259114.HUN576_97-98/WB; GQ259101.SVK549_98/WB; GQ259109.SVK558_99/WB; GQ259110.SVK559_99/WB; GQ259112.ITA561_93/WB; GQ259118.1/GER613SN/Germany/2005/WB; EU622078 Bartha; EU622079 Shope cow; EU622070 NOVA PRATA; GQ259099.FRA536/France/1999/HD; GQ259119. ESP 2885/Spain/2002/WB; GQ259123. ESP 2882/Spain/2002/WB; GQ259124. ESP 2886/Spain/2002/WB; GQ259125. ESP 2890/Spain/2002/WB; GQ259116. GER611NRW/Germany/2003/WB; GQ259104. GER 552NRW/Germany/1999/WB; GQ259107. GER555RP/Germany/2000/WB; GQ259100. FRA 537/France/1999/HD; EU622076. IB341/86/Brazil/1986; D49437. NIA-3/Northern Ireland/1971; JF460036. S66_1966/Sweden; AF158090 Ea; AF403051 Fa.; KF779458. BEL20075-2007/HD; KF779463. BEL60_1988/Belgium/pig; KF779468 BEL71_1989/Belgium/pig; KF779456. EL10053_2006/Belgium/WB; KF779460. BEL2_1988/Belgium/pig; KC865680. PRV/Dog-KZ/Croatia/2012 dog; KC865675. PRV/DP-ZG4/Croatia/2012 pig; KC865674. PRV/DP-ZG3/ Croatia/2012 pig; KC865672. PRV/DP-ZG1/Croatia/2012 pig; KC865678. PRV/WB-KRK1/Croatia/2012 WB;.
*WB: Wild boar; 8HD: hunting dog.

Fig. 2. Amino acid alignment of deduced gC protein sequences from three strains representatives of the Italian clades 1 and 2 and group 3. The position of deletions and insertions are reported respect to the prototype sequence YP068347. The hot spot region is indicated by a box. N-glycosylation sites are shown in bold face.

samples was previously reported (Steinrigl et al., 2012; Lutz et al., 2003) when non-CNS tissues were analyzed. Unfortunately, several samples were not well preserved and only one strain could be isolated and sequenced. PCR results observed in this study were biased by sampling, thus likely not representative of the AD prevalence, and were used only to perform a better genomic characteriza- tion of PRV strains circulating in our country.

All of the other isolates were obtained from clinically affected dogs that were divided into hunting dogs (n. 8) epidemiologically related to wild boars and dogs living in pig farms (n. 4) and then related to domestic pigs. Moreover, for one dog, no data were available, but it was unable to hunt and thus not exposed to wild boars. Sequence analysis further supported these two different epidemiologic linkages, showing a clear relationship between hunting dogs and wild boars and a distinct distribution for strains related to domestic pigs.

AD infections could be transmitted from domestic pigs to wild boars and vice versa as demonstrated by the presence of some domestic pig and wild boar isolates that cluster together (Muller et al., 2010; Verpoest et al., 2014). Moreover, the transmission between both populations was confirmed in experimental infections (Muller et al., 2001). Despite these considerations, the role of wild boars as AD reservoirs for domestic pigs has been rejected by Muller et al. (2011) because genomic analysis evidenced genomic differences between virus strains in the two populations. Our results are in line with this hypothesis, suggesting the presence in Italy of two distinct infection cycles related to wild boars and domestic pigs. Although the region used for sequence analysis is a small part of the complete SHV1 genome and deeper studies of genomic characterization should be taken into account, our results suggested that Italian wild boars and domestic pigs could provide two distinct ecological niches for the Italian AD strains.

Molecular analysis and the presence of amino acid insertions/deletions were consistent with the phylogenetic analysis and confirmed the presence of two Italian clades, which were characterized by two patterns not described in other European strains. Additionally, the amino acid sequence in the hot spot region is different in the two Italian clades. It has been hypothesized that the complete substitution (from VVVE to ALDDD), evidenced in the recent domestic pig strains and in our group 3, is related to changes in the hydrophilicity profile and then the region could be more antigenically exposed (Fonseca et al., 2012). Interestingly, Italian clade 2, which included recent domestic pig strains and epidemiologically related dog strains, did not show these substitutions and showed a profile similar to the wild boar strains. Sequencing of more extended parts of genome should be taken into account to better characterize these Italian strains. Moreover, it would be interesting to perform additional in vivo infection experiments in wild boars and pigs using strains belonging to the Italian clades to deeper investigate their pathogenesis in different hosts.

Although several regions of Northern Italy have implemented AD eradication programs in domestic pigs, the risk of transmission from wild boars to domestic pigs should not be completely ruled out and may be considered a constant threat. If preventive measures are disregarded, transmission between wild boars and free-range pigs or outdoor pigs might be possible. Therefore, the epidemiol- ogy in domestic pigs and wild boars should be continu- ously monitored and assessed.

Legal norms

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6. Commission Decision 2008/185/EC regarding additional guarantees in intra-Community trade of pigs related to Aujeszky's disease and criteria for listing a Member State or a region thereof as free from Aujeszky's disease or as having an approved disease control program. http://ec.europa.eu/food/animal/diseases/controlmea- sures/docs/guidance_2008-185-EC_en.pdf

Acknowledgements

This study was supported by grants from the Current research project 2011004 of the Italian Ministry of Health 'Studies on Italian Suid Herpesvirus (SHV-1) isolates: molecular characterisation and infection characteristics in swine and wild boars'. We would like to thank Anna Tirelli and Sonia Manenti for their technical collaboration.

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