DR. PEDRO JOSÉ GÓMEZ DE NOVA (Orcid ID : 0000-0001-9725-6747)

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A retrospective study of porcine epidemic diarrhoea virus (PEDV) reveals the presence of swine enteric coronavirus (SeCoV) since 1993 and the recent introduction of a recombinant PEDV-SeCoV in Spain

Running title. SeCoV and PEDV in Spain

Pedro J.G. de Nova[1], Martí Cortey[2], Ivan Díaz[3], Héctor Puente[1], Pedro Rubio[1], Marga Martín[2,3], Ana Carvajal[1]

[1]Department of Animal Health, Facultad de Veterinaria, Universidad de León, 24071 León, Spain

[2]Departament de Sanitat i Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain

[3]IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain

Correspondence

Pedro José Gómez de Nova, Department of Animal Health, Facultad de Veterinaria, Universidad de León, 24071 León, Spain.

Email: pgomn@unileon.es

Summary (up to 300 words)

A retrospective evaluation of PEDV positive samples recovered in Spain before and after the re-emergence of this coronavirus in several European countries was carried out. We described for the first time recombinant SeCoV circulating in Spain between 1993 and 2014 and its misidentification as PEDV when diagnostic assays based on the S-protein or S-gene of the PEDV were used. The complete S-gene sequence of 7 Spanish SeCoV and 30 PEDV Spanish isolates was phylogenetically analysed including the S-gene sequences of the three SeCoV and a representative selection of the PEDV strains with complete genome sequences available in the GenBank. The tree showed a common ancestor for the S-gene of the PEDV and SeCoV, but no evolution from any known PEDV clade was shown for the SeCoV strains. Moreover, complete genome sequences were obtained from 23 PEDV strains recovered in Spanish swine farms since 2014. The phylogenetic tree showed the INDEL type genogroup of these Spanish strains, supporting the lower pathogenicity of this genogroup since no significant economic losses were reported in the affected Spanish swine farms. Four subgroups were detected among PEDV strains in Spain, closely related to the recent European strains. Moreover, eight of the most recent Spanish PEDV isolates formed a subclade together with three European strains from 2015, showing a new evolution branch with a recombinant virus.

KEYWORDS: SeCoV, swine enteric coronavirus, PEDV, porcine epidemic diarrhoea, epidemic diarrhoea

1 INTRODUCTION

Coronaviruses (CoVs) belong to the *Nidovirales* order, the *Coronaviridae* family and the *Orthocoronavirinae* subfamily. Four genera are recognised based on phylogenetic clustering: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The CoVs are enveloped viruses and their genome is composed of a non-segmented positive sense RNA with a size of approximately 30 kb (Fehr and Perlman, 2015). From the 5'-end to the 3'-end, their genomic structure comprises six open reading frames (ORFs) named ORF1a, ORF1b, spike (S), envelope (E), membrane (M) and nucleocapsid (N). The ORF1a and ORF1b encode non-structural polyproteins, whereas the remaining genes encoded structural proteins. In addition, at least one accessory gene is present in each virus. Among the six CoVs which infect pigs, porcine epidemic diarrhoea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine respiratory

coronavirus (PRCV) and the recently described swine acute diarrhoea syndrome-coronavirus (SADS-CoV) belong to the *Alphacoronavirus* genera (Wang et al., 2019) while porcine hemaglutinating encephalomyelitis virus is a *Betacoronavirus* and porcine deltacoronavirus is included in the *Deltacoronavirus* genera (Saif et al., 2019).

PEDV causes watery diarrhoea, vomiting, anorexia and depression in pigs. In less than two weeks old piglets, the virus can produce up to 100% of mortality due to the severe dehydration, producing important economic losses (Pospischil et al., 2002; Carvajal et al., 2015; Saif et al., 2019). The virus was first associated to a diarrhoea outbreak in the UK in 1971 but it was not isolated until 1977 (Chasey and Cartwright, 1978; Pensaert and De Bouck, 1978; Song and Park, 2012). Subsequently, it spread throughout most European and Asian pig producing countries in the 1980s and 1990s. Since then, sporadic outbreaks have been reported in Europe (Nagy et al., 1996; Pritchard et al., 1999), with the only exception of an epidemic outbreak affecting more than 60 farms in northern Italy in 2005-2006 (Martelli et al., 2008); while PEDV has remained as a significant cause of diarrhoea outbreaks in swine herds in Asia (Takahashi et al., 1983; Kweon et al., 1993). In the Americas, PEDV was first detected in 2013 in the USA (Stevenson et al., 2013) and spread rapidly through the country, as well as to other countries in North, Centre and South America (Pasick et al., 2014; Jarvis et al., 2016; Barrera et al., 2017; Lara-Romero et al., 2018). Infection resulted in high mortality in piglets (up to 90-95%) and caused huge economic losses to the pig industry (Bevins et al., 2018).

After PEDV emergence in the USA, outbreaks have been reported in several European countries such as Germany (Stadler et al., 2015), Ukraine (Dastjerdi et al., 2015), the Netherlands (Dortmans et al., 2018), France (Grasland et al., 2015), Austria (Steinrigl et al., 2015), Portugal (Mesquita et al., 2015), Belgium (Theuns et al., 2015) and Slovenia (Toplak et al., 2016). Two main PEDV genogroups, named S INDEL or INDEL and non-S INDEL or non-INDEL strains, have been described, based on the presence or absence of certain insertions and deletions in the S1 subunit of the S-gene (Vlasova et al., 2014; Gallien et al., 2018). Both types of strains have been described in Asia and America, while in Europe there are no evidences of the presence of the non-INDEL variant, with the only exception of an Ukrainian isolate (Dastjerdi et al., 2015).

On the other hand, a novel enteric coronavirus named swine enteric coronavirus (SeCoV) was described in Italy in 2009 (Boniotti et al., 2016), subsequently in 2012 in Germany (Akimkin et al., 2016) and in 2016 in Central Eastern Europe (Belsham et al., 2016; Mandelik et al., 2018). A recombinant origin, with the backbone sequence of TGEV and the S-gene from PEDV, has been

proposed for this new SeCoV (Boniotti et al., 2016). It causes the same clinical signs of PEDV and TGEV, but lower mortality in piglets, about 5% to 10% (Belsham et al., 2016; Boniotti et al., 2016). Because of the recombinant nature of SeCoV, a diagnosis based on the detection of a particular protein or its sequence for both PEDV and TGEV, may lead to incorrect identification of the viral agent and the presence of SeCoV may be unnoticed. In addition, a small segment spanning 400 bp in the 5' end of the SeCoV S-gene has been proposed as a parent in the description of a recent recombinant PEDV group in Hungary and Slovenia (Valkó et al., 2017, 2019).

In Spain, PEDV was first described in 1985 and was a relevant cause of diarrhoea during the 90s (Carvajal et al., 1995a; Carvajal et al., 1995b) reappearing in 2014-2015 (Benito Zuñiga et al., 2016; EFSA, 2016). The aim of this study was to characterize a collection of PEDV positive samples recovered in Spain between 1993 and 1999, as well as after the re-emergence of PEDV between 2014 and 2019 in order to establish the evolutionary relationships between the strains. A second aim would be to identify and characterize the presence of SeCoV in Spain. This study is the first in depth evaluation of PEDV and SeCoV in Spain.

2 MATERIALS AND METHODS

2.1 Samples and CoV identification

A selection of 37 PEDV positive faecal samples were obtained from the collection maintained at -80ºC at the Department of Animal Health, University of León (Spain) (35 samples) and at the Department of Animal Health and Anatomy of the Autonomous University of Barcelona (Spain) (2 samples). Samples were collected from diarrhoea outbreaks in swine farms in Spain since 1993 (Table 1). PEDV detection was carried out using a direct ELISA based on two monoclonal antibodies against the S-protein of PEDV (samples from 1993 to 1999) (Carvajal et al., 1995a), or using a one-step RT-PCR assay targeting a 651 bp fragment of the S-gene, with the primers P1 and P2 previously described (Kim et al., 2001) and the Verso 1 Step RT-PCR ReddyMix kit (Thermo Scientific) (samples from 2014 to 2019).

Samples were thawed and diluted 1:5 (v/v) in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4), homogenized by vortex mixing and centrifuged for 10 min at 6000 g. The RNA was extracted from 140 µl of the supernatant using

QIAMP Viral RNA Mini Kit (QIAGEN) following the instructions of the manufacturer. All samples were subjected to an additional one-step RT-PCR to discriminate between PEDV and SeCoV by amplifying a 612 bp fragment of the TGEV N-gene as previously described (Kim et al., 2000). The reaction conditions for both PEDV and SeCoV RT-PCRs were: 50ºC for 15 min, 95ºC for 2 min, 35 cycles at 95ºC for 30 s, 50ºC for 30 s and 72ºC for 30 s, followed by a final extension step at 72ºC for 10 min.

The RT-PCR products were visualized under UV light after they were resolved using electrophoresis in a 2% agarose gel, containing RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc.) in 1xTAE buffer (40 mM Tris/acetate buffer and 1 mM EDTA, pH 8.0).

2.2 Whole genome sequencing

The whole sequence of 23 PEDV strains were obtained using two approaches: nine genomes were obtained by direct amplification of eight overlapping fragments using the SuperScript® One-Step RT-PCR for Long Templates kit (Invitrogen) according to the manufacturer's recommendations and primers previously designed (Huang et al., 2013). After the amplification, the RT-PCR products were purified using the Ilustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and were equimolar mixed, fragmented and sequenced in an external service (AC-Gen), by next-generation sequencing (NGS) using the Ion Torrent technology. The remaining 14 PEDV and the SeCoV complete genomes were obtained from the total RNA extraction, without any amplification step, applying a RNA virus-specific tailor-made NGS protocol (Cortey et al., 2018; Vidal et al., 2018). NGS runs were performed by the Genomics Bioinformatics Service (SGB) of the UAB using an Illumina Miseq Platform. Although full-length genomic sequencing was attempted in all the strains, it was not achieved in seven PEDV and all but one SeCoV isolates due to the poor preservation of the samples.

2.3 S-gene sequencing

The complete sequences of the spike gene (S-gene) were obtained by sequencing in both directions by Sanger methodology for both PEDV and SeCoV positive samples which could not be completely sequenced. Four overlapping fragments from the S-gene were amplified using the primers described in Table 2 and the Verso 1-Step RT-PCR ReddyMix kit (Thermo Scientific).

The thermal cycler and electrophoretic conditions were carried out as in the PEDV or SeCoV identification and the RT-PCR products were purified using the Ilustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare).

2.4. Recombination analysis

Considering the existence of several recombinant strains among swine coronaviruses, the presence of recombinant signals among the S-genes obtained was evaluated with the RDPv4.99 program (Martin et al., 2015), running six methods implemented (RDP, GeneConv, BootScan, MaxChi, Chimaera and SiScan). The six methods used the following general settings: window size = 20, highest acceptable P value = 0.001 and Bonferroni correction.

2.5 Phylogenetic analysis

In order to compare the S-gene or the complete genome sequences from this study with those previously described in other countries, the PEDV and SeCoV complete genome sequences available in the GenBank database were aligned together with sequences obtained in this study using the Clustal Omega command included in the unified bioinformatics toolkit Unipro UGENE (Okonechnikov et al., 2012). After the alignment, MEGA-X software (Kumar et al., 2018) was used to check and correct the alignment and to construct the phylogenetic tree. The neighbour joining method (Saitou and Nei, 1987) using the maximum composite likelihood method (Tamura et al., 2004) to compute the evolutionary distances in the units of the number of base substitutions per site, and the pairwise deletion option using 1000 bootstrap replicates (Felsenstein, 1985) were used to infer the evolutionary history.

3 RESULTS

A collection of 37 PEDV positive faecal samples based on the detection of the S-gene sequence or protein kept at the Department of Animal Health of the University of León and at the Department of Animal Health and Anatomy of the Autonomous University of Barcelona and recovered from diarrhoea outbreaks in Spanish swine farms since 1993 was analysed. Surprisingly, all samples recovered between 1993 and 1999, as well as a sample from a diarrhoea

outbreak in 2014 were identified as SeCoV by means of the simultaneous amplification of 651 bp of the PEDV S-gene (Kim et al., 2001) and 612 bp of the TGEV N-gene (Kim et al., 2000) (Figure 1). The possibility of a mixed TGEV-PEDV infection was ruled out as no amplification of the remaining genes of the PEDV was obtained with the primers described before for the complete genome sequence of the PEDV (Huang et al., 2013) and the observation that after NGS no TGEV or PEDV complete sequences were detected. Sequence analyses of the amplified TGEV N-gene fragments showed up to 97.7% identity to previously described TGEV isolates and 99% to SeCoV isolates. The distribution of positive PEDV and SeCoV strains included in the study are shown in Table 1.

In order to clarify the phylogenetic origin of the S-gene of the recombinant SeCoV with respect to those of the PEDV strains, a phylogenetic tree was constructed using the S-gene sequences from this study and those of the complete genome of the three SeCoV from Italy 2009 (Boniotti et al., 2016), Germany 2012 (Akimkin et al., 2016) and Central Eastern Europe 2016 (Belsham et al., 2016) and PEDV available in GenBank (Figure 2). The phylogenetic tree showed the strong association of the previously described SeCoV strains with the seven Spanish SeCoV strains from 1993 to 1999 and 2014. This clade was clearly segregated from the PEDV strains and does not group within any INDEL or non-INDEL clade. In addition, the tree showed two branches within the SeCoV clade, one containing Spanish strains from 1993 to 1999 and the other containing the Spanish strains of 1998 and 2014 together with the strains from Italy of 2009, Germany of 2012 and Central Eastern Europe of 2016.

The 23 PEDV and the SeCoV complete genomes obtained were phylogenetically compared with the complete genome sequences from GenBank (Figure 3); as already observed in the S-gene tree, only PEDV of the INDEL genogroup were circulating in Spain since 2014, while the Spanish SeCoV isolate clustered with the other SeCoV in a clearly separated branch together with PRCV and TGEV isolates.

With regard to the Spanish strains, four subgroups named SP1, SP2, SP3 and SP4 were found within the INDEL 2 clade in both trees, but no geographic relationship of the subgroups was observed. The subgroup SP1 was formed by Spanish strains of 2014 and 2015 joined to some European strains from the same years. The subgroups SP2 and SP3 contained Spanish strains from 2014 to 2019 and from 2014 to 2016, respectively, while the most recent subgroup, SP4, included Spanish strains from 2017to 2019 together with two Hungarian and two Slovenian strains of 2015, 2016 and 2018. This clade corresponded to the PEDV recombinant group described in those

countries (Valkó et al., 2017, 2019), and also by the RDP program. All six methods assayed consistently detected a recombinant segment located at the 5' end of the S-gene spanning 392 bp between positions 248 and 640 (Figure S1 available as supporting information), with PEDV and SeCoV being the major and minor parents, respectively. As can be seen in the phylogenetic tree constructed with the recombinant segment of 392 bp (Figure 4), eight PEDV Spanish strains isolated between 2017 and 2019 clustered together with SeCoV and PEDV recombinant isolates described in Hungary and Slovenia.

4 DISCUSSION

The results suggest a sporadic presentation of diarrhoea outbreaks due to this recombinant virus in Spain during the 1990s – 16 years prior to the oldest SeCoV strain identified nowadays (SeCoV Italy/213306/2009 KR061459) –, and also in 2014 when PEDV re-emerged in Spain and other European countries. The clinical signs associated with this recombinant virus have been described as less severe than those of PEDV, with a significantly lower mortality (Belsham et al., 2016; Boniotti et al., 2016) and would allow the infection to pass unnoticed in a certain number of farms. Moreover, the use of PEDV diagnostic tests targeted at the S-protein, including ELISA (van Nieuwstadt and Zetstra, 1991; Carvajal et al., 1995a) or PCR based assays (Kim et al., 2001), might have misidentified SeCoV as PEDV in some of the investigated outbreaks. According to our results, SeCoV circulated in Spain for years. The proposed combination of backbone genome sequence of the TGEV together with PEDV S gene in the SeCoV genome (Boniotti et al., 2016) involves a coinfection with these two enteric coronaviruses at one point in history. The emergence and widespread in Europe since 1984 of the porcine respiratory coronavirus (PRCV) which is a naturally occurring mutant of the TGEV with a great deletion in its genome and produces a mild respiratory disease in pigs (Pensaert et al., 1986), provide pig population cross-immunity against the enteric virus TGEV due to their similarity. Therefore, since then TGEV outbreaks have been uncommon in Europe (Saif et al., 2019). The detection of SeCoV in diarrhoea outbreaks in Spain in 1993 suggests that the recombinant event between TGEV and PEDV would have occurred long time ago, even before the spread of PRCV, making it advisable to perform retrospective studies for the detection of SeCoV in faecal samples collected before 1984.

A total of 11 PEDV isolates were recovered in 2014, followed by six in 2015, three in 2016, three in 2017, four in 2018 and three in 2019, suggesting the re-emergence of PEDV in Spain, as it

has been described in several European countries (Dastjerdi et al., 2015; Grasland et al., 2015; Stadler, et al., 2015; Steinrigl et al., 2015; Dortmans et al., 2018), soon after its first detection and emergence in the USA (Stevenson et al., 2013). In order to understand the origin and evolution of PEDV isolates in Spain, the complete genome sequences were phylogenetically compared with the PEDV complete genome sequences available in the GenBank. The sequences from cell-adapted isolates were excluded to avoid the presence of non-natural variants with mutations derived from the cell-culture of the virus as previously described (Park et al., 2007; Chen et al., 2015; Lee et al., 2017). Therefore, a representative selection of the 457 complete genome sequences from Europe, Asia and America were included. After the sequence analysis, only the presence of the PEDV INDEL genogroup was observed among the Spanish isolates. This genogroup of the virus has been described to cause a less severe disease than the non-INDEL genogroup (Wang et al., 2014; Vlasova et al., 2014; Lin et al., 2015; Chen et al., 2016). Also, a better horizontal transmission by direct contact as well as indirectly through the air for short distances of the non-INDEL strains has been proposed (Gallien et al., 2018). Thus, re-emergence of PEDV in Spain since 2014 has not been associated with significant economic impact on the swine industry, in contrast to the Asian and USA outbreaks (Song and Park, 2012; Alvarez et al., 2015; Jung and Saif, 2015), supporting the lower mortality as well as probably the lower transmission rate associated to these INDEL strains. However, differences in the virulence among PEDV strains belonging to the INDEL type have been reported in Germany (Stadler et al., 2015) and also observed in Spanish swine farms (Benito Zuñiga et al., 2016).

When the complete genome sequences were analysed, the PEDV isolates were grouped based on their S-gene genogroup (INDEL and non-INDEL), with the peculiarity of the INDEL genogroup which was segregated in two distant clades although visual differences in the alignment between their sequences and in the deletion zone that characterises this genogroup were not found. Regarding the INDEL strains, a common ancestor was found for the oldest European strains (CV777, Br1/87 and Germany 1978) and some PEDV strains detected in Asia before 2015 (INDEL 1 clade). An Italian strain from 2009 (Lombardia 2009-01), belonging to the INDEL genogroup, likely represents a transition between the INDEL 1 clade and the remaining strains. No geographic relationship was observed in the distribution of the strains in the trees, especially among the non-INDEL Asian and American isolates as well as for the INDEL 1 strains. However, the Spanish INDEL strains were clearly clustered within a European cluster in the INDEL 2 clade. As previously described, this clade showed a common ancestor with the non-INDEL strains,

different of the ancestor of the INDEL 1 clade (Guo et al., 2018; Zhang et al., 2018). The clear separation of the recent subgroup SP4 from the rest of the Spanish strains, showing a common ancestor with one Hungarian and two Slovenian strains, suggests the presence of two PEDV lines circulating in Europe since 2015, which are independently evolving within the INDEL 2 clade. According to our results, the PEDV recombinant subgroup SP4 is replacing other PEDV subgroups in recent years and since 2017, nearly all the PEDV strains belonged to this SP4 subgroup. The recombinant event in the 5' end of S-gene with SeCoV reported in these isolates was described previously (Valkó et al., 2017, 2019), and might provide some advantages, as the S protein is a key target for PEDV neutralizing antibodies (Li et al., 2017; Okda et al., 2017). Besides, the comparison of the sequences of this recombinant region indicated that the SP4 subgroup clusters together with the more modern SeCoV strains (after 2009), but not with the older Spanish ones (1993 to 1999), reinforcing the notion that the recombination event that generated the PEDV recombinant group occurred recently (the oldest PEDV recombinant strain was reported in Slovenia in 2015).

With regard to the tree topology based on the S-gene, there were no significant differences regarding the complete genome tree with the exception of the position of several isolated strains, supporting the use of the complete S-gene sequences to infer the phylogenetic relationships of PEDV as previously proposed (Zhang et al., 2014; Sung et al., 2015). However, although the alignment revealed the presence of the insertions and deletions of the PEDV INDEL genogroup in the SeCoV isolates, these strains form a segregated clade clearly separated from all the PEDV strains. This fact suggests the presence of a common ancestor which evolved in two ways; the first one originating the PEDV strains and the second the SeCoV. Finally, two main evolution branches of SeCoV were demonstrated suggesting that this virus has evolved in at least two clades since 1993. While the first only includes Spanish strains from 1993 to 1999, the second comprises European SeCoV from Italy, Germany, Central Eastern Europe and Spain between 2009 and 2016.

In summary, this study describes for the first time recombinant SeCoV circulating in Spain between 1993 and 2014. Misidentification of these isolates as PEDV occurred when diagnostic assays based on S-protein or S-gen were used and makes highly recommendable to carry out this diagnosis using a different target. The identification of the SeCoV requires the simultaneous use of one target for the S-gene of the PEDV and another target for the rest of the backbone of the TGEV. Alternatively, whole genome sequencing can be also used. Moreover, PEDV isolates circulating in Spain since 2014 were characterized by complete or S-gene sequencing. Only the

most recent clade 2 of PEDV INDEL genogroup was identified and the isolates were allocated into four subgroups within this clade, without any geographic relationship. The SP4 subgroup, which include eight Spanish isolates from 2017 to 2019 together with isolates recovered from Hungary and Slovenia in 2015, 2016 and 2018, was clearly segregated, suggesting a new independent evolution of PEDV in Europe from 2015 due to a recombination event in the S-gene between PEDV and SeCoV. The phylogenetic tree based on the sequence of S-gene showed an independent evolution of SeCoV and PEDV with a common ancestor but no evolution of SeCoV from any PEDV clade. A retrospective study of the presence of SeCoV in Europe before and after the emergence of PRCV in 1984 would allow the clarification of the origin of this recombinant virus.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest with respect to the research, authorship and publication of this article.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The regional guidelines for the care and use of animals were followed.

DATA AVAILABILITY STATEMENT

Data are available in the GenBank database and by direct contact with the correspondence author.

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TABLES

TABLE 1. Spanish porcine epidemic diarrhoea virus (PEDV) and swine enteric coronavirus (SeCoV) isolates used for the phylogenetic analyses of the complete genome or the S-gene with the global described strains from GenBank.

CoVs	Acc. No.	Isolate	Sample Origin	Collection time	Sequence
PEDV	MN692771	1453	Zaragoza	2014-04-24	Complete
PEDV	MN692772	1455	Zaragoza	2014-04-29	Complete
PEDV	MN692773	1456	Zaragoza	2014-04-29	Complete
PEDV	MN692774	1481	Pamplona	2014-06-03	Complete
PEDV	MN692775	1508	Zaragoza	2014-07-11	Complete
PEDV	MN692776	1521	Segovia	2014-09-30	Complete
PEDV	MN692777	1522	Segovia	2014-09-30	Complete
PEDV	MN692778	1524	Segovia	2014-10-07	Complete
PEDV	MN692779	1526	Zaragoza	2014-10-09	Complete
PEDV	MN692780	1569	Badajoz	2014-12-19	Complete
PEDV	MN692781	1776	Badajoz	2016-02-12	Complete
PEDV	MN692782	1873	Segovia	2016-09-03	Complete
PEDV	MN692783	1914	Pontevedra	2016-12-05	Complete
PEDV	MN692784	1931-1	Valladolid	2017-01-19	Complete
PEDV	MN692785	1931-2	Valladolid	2017-01-19	Complete
PEDV	MN692786	1931-3	Burgos	2017-01-19	Complete
PEDV	MN692787	2098	Malaga	2018-01-11	Complete
PEDV	MN692788	2118	Orense	2018-02-02	Complete
PEDV	MN692789	2149	Castellon	2018-03-02	Complete
PEDV	MN692790	2181	Castellon	2018-05-03	Complete
PEDV	MN692791	2330	Orense	2019-01-30	Complete
PEDV	MN692792	H3*	Barcelona	2019-03-07	Complete
PEDV	MN692793	H18*	Barcelona	2019-05-11	Complete

PEDV	MN692763	1556	Valencia	2014-11-28	S-gene
PEDV	MN692764	1573	Toledo	2015-01-13	S-gene
PEDV	MN692765	1576	Zamora	2015-01-20	S-gene
PEDV	MN692766	1587-1	Orense	2015-02-03	S-gene
PEDV	MN692767	1587-7	Lugo	2015-02-03	S-gene
PEDV	MN692768	1611	Murcia	2015-02-25	S-gene
PEDV	MN692769	1613	Murcia	2015-02-26	S-gene
SeCoV	MN692757	BU	Burgos	1993	S-gene
SeCoV	MN692758	EGV	Segovia	1993	S-gene
SeCoV	MN692759	SG1	Segovia	1994	S-gene
SeCoV	MN692760	VA	Valladolid	1994	S-gene
SeCoV	MN692761	MU2	Murcia	1998	S-gene
SeCoV	MN692762	AYL	Segovia	1999	S-gene
SeCoV	MN692770	1480	Murcia	2014-05-30	Complete

* All the strains were obtained from the collection of the Department of Animal Health, University of León with the exception of the two marked strains which were collected from the Department of Animal Health and Anatomy of the Autonomous University of Barcelona.

TABLE 2. Primers used in the amplification of the S-gene.

Name	**Sequence 5'-3'**	**Product size (pb)***	**Source**
S-F1	TGCTAGTGCGTAATAATGAC		(Huang et al., 2013)
ED-S1R	CGTCAGTGCCATGACCAGTG	1349	This study
ED-S2F	GGGAAATTGTCATCACCAAG		This study
PEDV-S1R	CTGGGTGAGTAATTGTTTACAACG	1289	(Chen et al., 2014)
ED-S3F	AGTACTAGGGAGTTGCCTGG		This study
ED-S3R	AACCATAACGCTGAGATTGC	1216	This study
ED-S4F	TTGAACACTGTGGCTCATGC		This study
S-R1	CATCTTTGACAACTGTGT	1128	(Huang et al., 2013)

* Product size is referred to the reference sequence of the CV777 strain (NC003436).

FIGURE LEGENDS

FIGURE 1.

Identification of the swine enteric coronavirus (SeCoV) by the visualization under UV light of the RedSafe staining 2% agarose gel electrophoresis of the amplification of 651 bp of the porcine epidemic diarrhoea virus (PEDV) S-gene (**A**) and 612 bp of the transmissible gastroenteritis virus (TGEV) N-gene (**B**). Isolates of BU-1993 (**1**), EGV-1993 (**2**), SG1-1994 (**3**), VA-1994 (**4**), MU2-1998 (**5**), AYL-1999 (**6**), 1480-2014 (**7**), 1613-2015 as a positive control for PEDV (**8**), positive control for TGEV (**9**) and non-template control (**10**) are shown.

FIGURE 2.

Phylogenetic analysis of the porcine epidemic diarrhoea virus (PEDV) and the swine enteric coronavirus (SeCoV) based on the complete S-gene sequences. The tree was performed using MEGA-X software with the neighbour joining method and 1000 bootstrap replicates based on the nucleotide sequence. The evolutionary distances were measured by the number of substitutions per site. GenBank accession numbers, country and date of isolation of each strain are shown. The genogroups, clades, groups and subgroups referred in the text are embraced on the right of the tree. Spanish strains are marked in bold and with black circles for PEDV and squares for SeCoV.

FIGURE 3.

Phylogenetic analysis of the porcine epidemic diarrhoea virus (PEDV) and the swine enteric coronavirus (SeCoV) based on the complete genome sequences. The tree was performed using MEGA-X software with the neighbour joining method and 1000 bootstrap replicates. The evolutionary distances were measured by the number of substitutions per site. GenBank accession numbers, country and date of isolation of each strain are shown. The genogroups, clades, groups and subgroups referred in the text are embraced on the right of the tree. Spanish strains are marked in bold and with black circles.

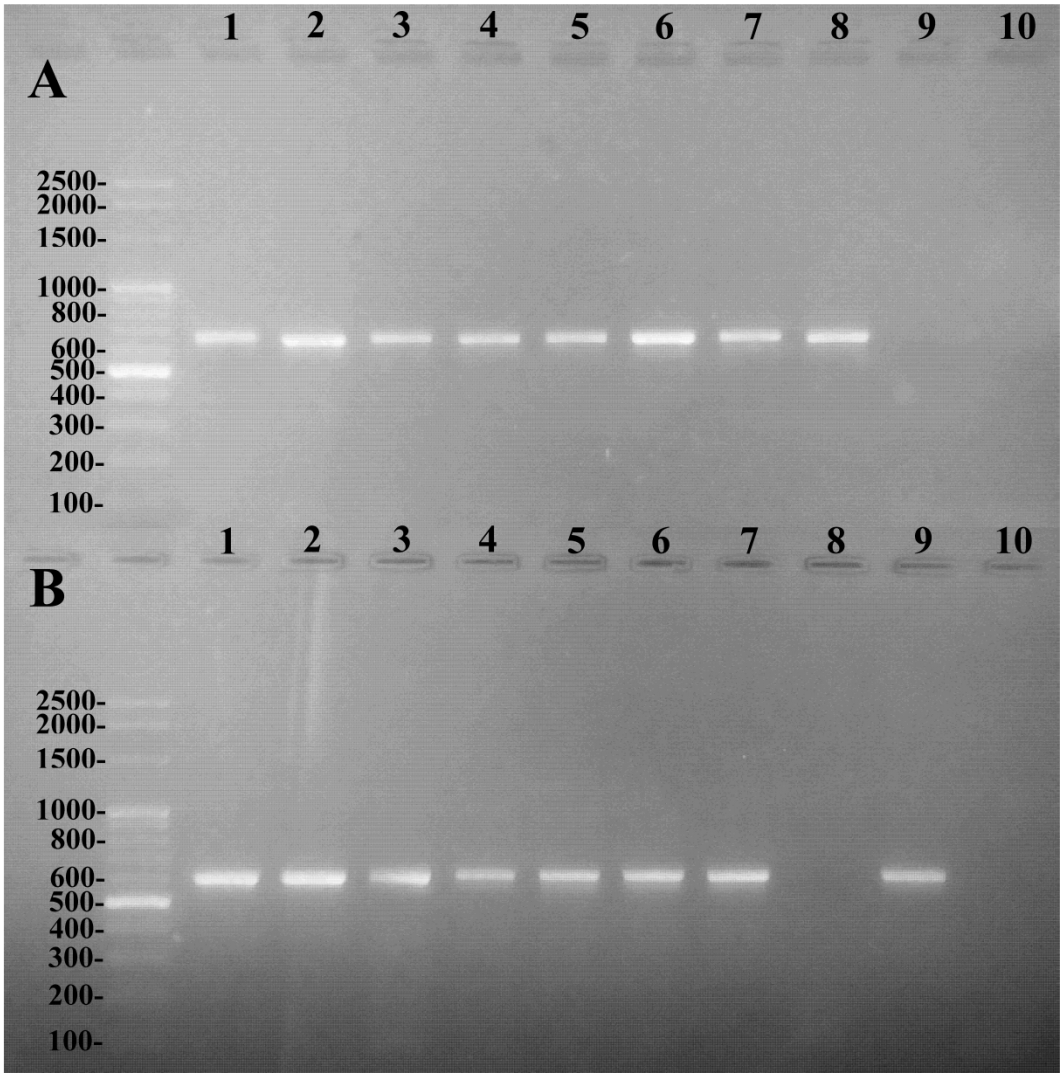
FIGURE 4.

Phylogenetic analysis of the porcine epidemic diarrhoea virus (PEDV) and the swine enteric coronavirus (SeCoV) based on the 392 bp from the positions 248 to 640 of the S-gene sequences. The tree was performed using MEGA-X software with the neighbour joining method and 1000 bootstrap replicates. The evolutionary distances were measured by the number of substitutions per

site. GenBank accession numbers, country and date of isolation of each strain are shown. The genogroups, clades, groups and subgroups referred in the text are embraced on the right of the tree. Spanish strains are marked in bold and with black circles for PEDV and squares for SeCoV.

SUPPORTING INFORMATION

Figure S1. SimPlot graph representing the recombination analysis of the S-gene in recombinant porcine epidemic diarrhoea virus (PEDV) Spanish isolates (subgroup SP4). The continuous and dashed lines represents the major (PEDV) and minor (swine enteric coronavirus SeCoV) parents, respectively, while the recombinant region is marked in grey.



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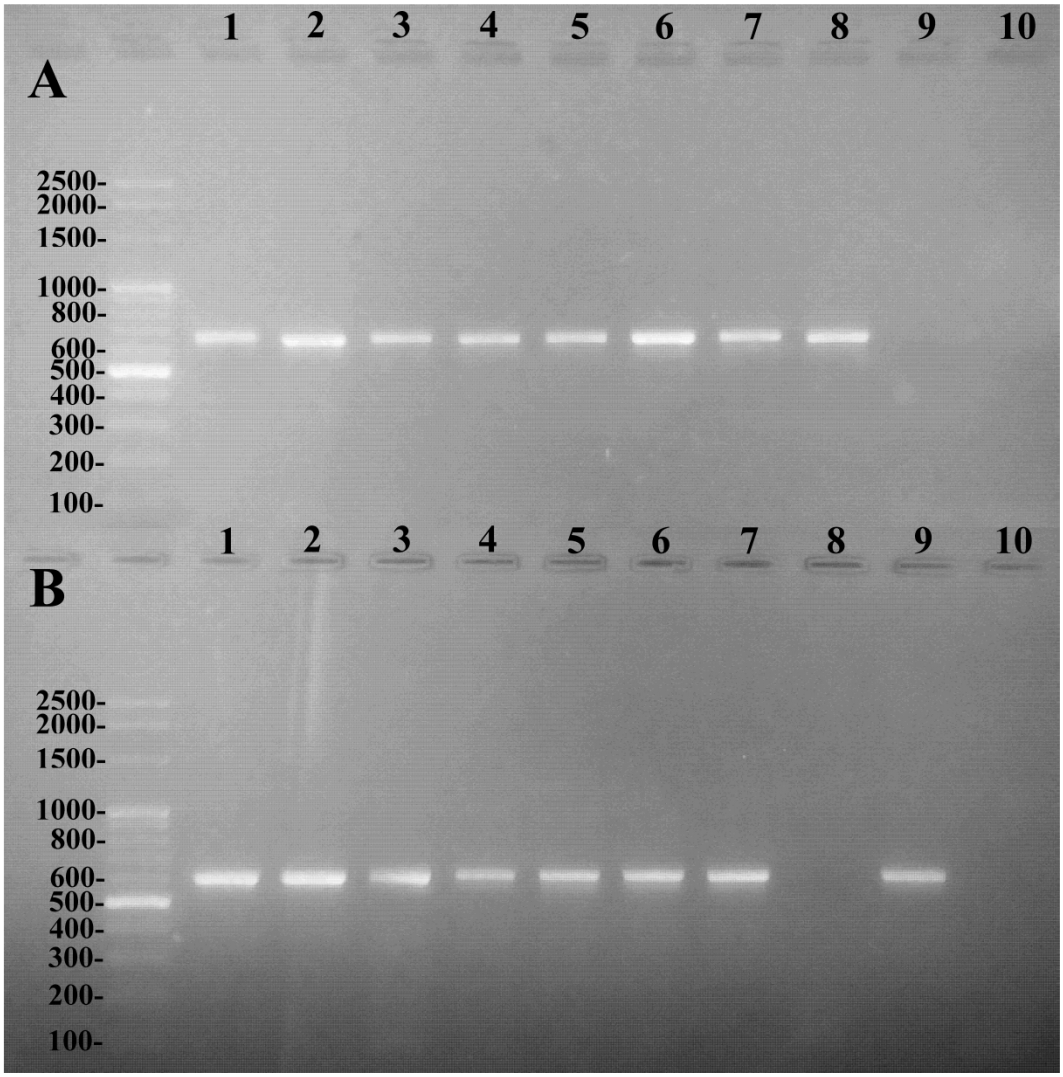

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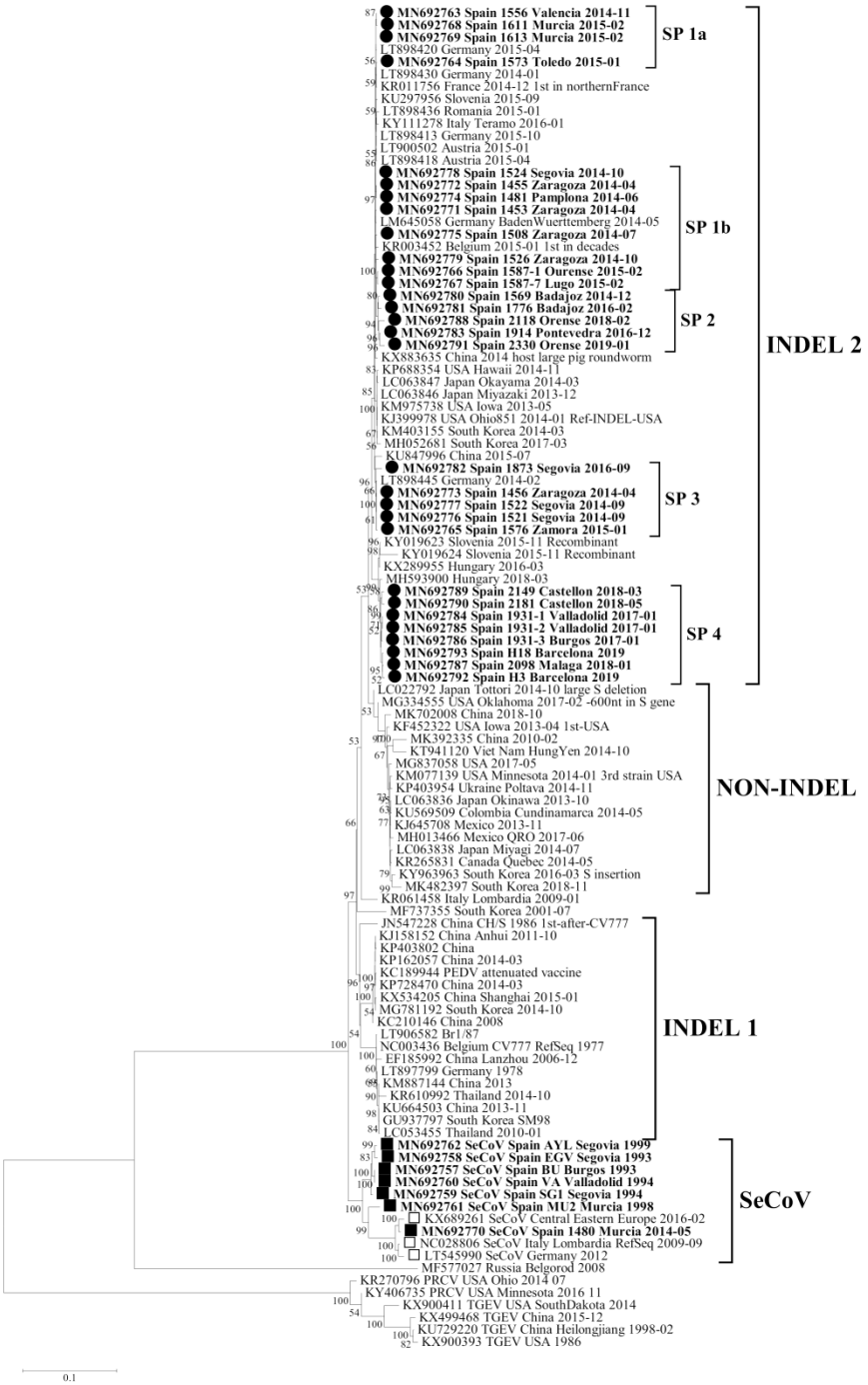

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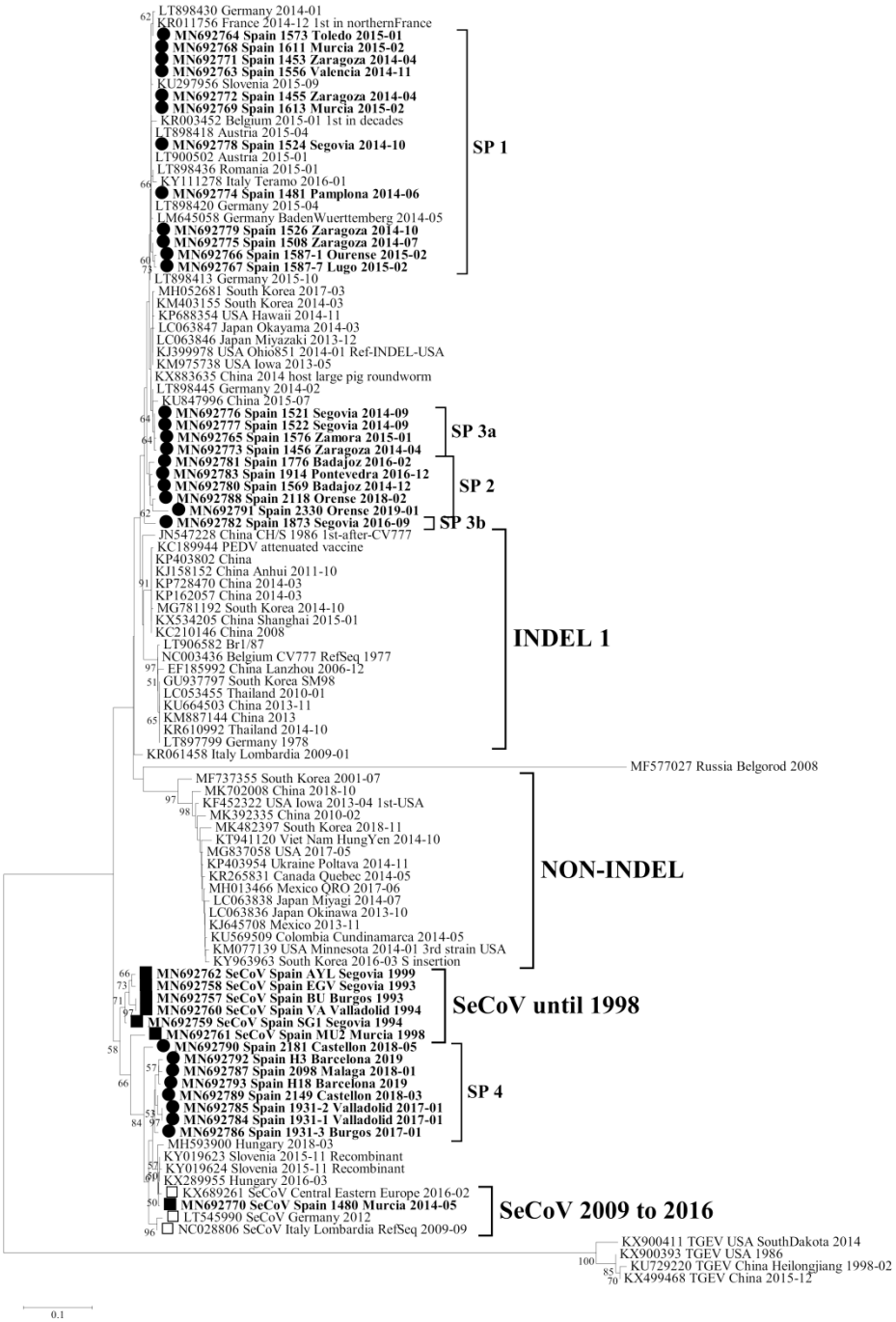

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