Porcine Epidemic Diarrhea Virus and Discovery of a Recombinant Swine Enteric Coronavirus, Italy

M. Beatrice Boniotti,[1] Alice Papetti,[1] Antonio Lavazza, Giovanni Alborali, Enrica Sozzi, Chiara Chiapponi, Silvia Faccini, Paolo Bonilauri, Paolo Cordioli,[2] Douglas Marthaler

Porcine epidemic diarrhea virus (PEDV) has been detected sporadically in Italy since the 1990s. We report the phylogenetic relationship of swine enteric coronaviruses collected in Italy during 2007–2014 and identify a drastic shift in PEDV strain variability and a new swine enteric coronavirus generated by recombination of transmissible gastroenteritis virus and PEDV.

Porcine epidemic diarrhea virus (PEDV) and *Transmissible gastroenteritis virus* (TGEV) (family Coronaviridae, genus *Alphacoronavirus*) are enveloped viruses that contain a single-stranded, positive-sense RNA genome of ≈28 kb. Infection with these viruses causes watery diarrhea, dehydration, and a high mortality rate among suckling pigs. Coronaviruses (CoVs) are prone to genetic evolution through accumulation of point mutations and homologous recombination among members of the same genus (*1*). Porcine respiratory coronavirus (PRCV), a mutant of TGEV, appeared in pigs in the 1980s (*2*). The spread of PRCV, which conserved most of the antigenic sites and causes cross-protection against TGEV (*3*), led to the gradual disappearance of TGEV. Newly emerging CoVs pose a potential threat to human and animal health because multiple human CoV infections have been derived from animal hosts. Emerging swine coronaviruses are of great concern to swine health because of the potential increase in viral pathogenesis.

In 1978, PEDV was first identified in Europe; subsequent reports occurred in many countries in Asia, including China, Japan, Korea, and Thailand. In 2010–2012, genetically different PEDV strains emerged, causing severe outbreaks in China (*4*). PEDV spread to the United States, Canada, and Mexico in 2013–2014, and genetically related strains were detected in South Korea and Taiwan (*5–7*). The PEDV outbreak caused large global economic losses to the swine industry. In Europe, a severe PEDV epidemic occurred in Italy during 2005–2006 (*8*), and in 2014–2015, PEDV was detected in Germany, France, and Belgium. These strains have a high nucleotide identity to PEDV strains that contain distinct insertions and deletions (INDELs) in the S gene (S-INDELs) from the United States (*9–11*). We report the detection and genetic characterization of swine enteric CoVs circulating in Italy during 2007–2014. We also report a recombinant TGEV and PEDV strain (identified as the species *Swine enteric coronavirus* [SeCoV]) circulating from June 2009 through 2012. Finally, we describe the phylogenetic relationship of the 2014 PEDV S-INDELs to the recent PEDV strains circulating in Europe.

The Study

During 2007–2014, we collected 27 fecal and 24 intestinal samples from pigs with suspected PEDV or TGEV infections; the pigs came from swine farms in northern Italy (Po Valley), which contains the regions of Piemonte, Lombardia, Emilia Romagna, and Veneto (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/1/15-0544-Techapp1.pdf). The Po Valley contains 70% of Italy's swine. Clinical signs included watery diarrhea in sows and a death rate in piglets of 5%–10%, lower than is typical with PEDV or TGEV infections. Samples were submitted for testing by electron microscopy, PEDV ELISA, viral isolation, pan-CoV reverse transcription PCR (RT-PCR), and RT-PCR for PRCV and TGEV; selected positive pan-CoV samples were sequenced (*12–14*) (online Technical Appendix).

Results of electron microscopy showed that 25 (49%) of the 51 samples contained CoV-like particles, but all samples were negative for viral isolation. Although only 38 samples (74%) were positive by pan-CoV RT-PCR, 47 (92%) were positive by the PEDV ELISA (Table 1) (*12,13*). Of the 38 pan-CoV–positive samples, 18 were selected for partial RNA-dependent RNA polymerase (RdRp), spike (S1) (*14*), and membrane (M) sequencing (Table 1). All samples were negative for PRCV and TGEV by RT-PCR, ruling out co-infection with PEDV and TGEV or PRCV (*15*).

Author affiliations: Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, Brescia, Italy (M.B. Boniotti, A. Papetti, A. Lavazza, G. Alborali, E. Sozzi, C. Chiapponi, S. Faccini, P. Bonilauri, P. Cordioli); University of Minnesota Veterinary Diagnostic Laboratory, St. Paul, Minnesota, USA (D. Marthaler)

DOI: http://dx.doi.org/10.3201/eid2201.150544

[1]These first authors contributed equally to this article.

[2]Deceased.

Table 1. Distribution of test results of samples from pig farms in study of swine enteric coronaviruses in northern Italy, 2007–2014*

Sample no.	Farm no.	Year	Region	EM	PEDV ELISA	Pan-CoV RT-PCR	TGEV/ PRCV S1	Sequences		
								RdRp	S1	M
222654	1	2007	Emilia Romagna	–	+	+	NA	NA	NA	NA
1448	2	2007	Emilia Romagna	–	+	–	NA	NA	NA	NA
19908	3	2007	Emilia Romagna	–	+	+	–	Cluster I	Cluster I	Cluster I
70323	4	2007	Lombardia	+	+	+	NA	NA	†	NA
114372	5	2007	Lombardia	+	+	+	NA	NA	NA	NA
200079	6	2007	Lombardia	–	+	+	NA	NA	†	NA
320855/5	7	2007	Lombardia	+	+	+	–	Cluster I	Cluster I	Cluster I
320855/6	7	2007	Lombardia	+	+	+	NA	†	†	NA
3936/1	8	2008	Lombardia	–	+	+	–	Cluster I	Cluster I	Cluster I
3936/2	8	2008	Lombardia	–	+	+	NA	†	NA	NA
29177	9	2008	Veneto	+	+	+	–	Cluster I	Cluster I	Cluster I
43853	10	2008	Lombardia	+	+	–	NA	NA	NA	NA
7239‡	11	2009	Lombardia	–	+	+	–	Cluster I	Cluster I	Cluster I
20001	12	2009	Lombardia	–	+	+	–	Cluster I	Cluster I	Cluster I
20416	13	2009	Lombardia	–	+	+	NA	†	†	NA
22603	14	2009	Lombardia	–	+	+	–	Cluster I	Cluster I	Cluster I
26199/2	15	2009	Lombardia	–	+	–	NA	NA	NA	NA
87565	16	2009	Emilia Romagna	–	+	+	NA	NA	†	NA
111357/7	17	2009	Lombardia	NA	+	–	NA	NA	NA	NA
137442	18	2009	Lombardia	+	+	+	–	Cluster II	Cluster II	Cluster II
205396	19	2009	Lombardia	+	+	–	NA	NA	NA	NA
208995	20	2009	Lombardia	+	–	+	NA	†	NA	NA
213306‡	21	2009	Lombardia	+	+	+	–	Cluster II	Cluster II	Cluster II
244945	22	2009	Emilia Romagna	+	+	–	NA	NA	NA	NA
245242	22	2009	Emilia Romagna	+	+	+	NA	†	NA	NA
274771	23	2009	Veneto	+	–	–	NA	NA	NA	NA
307121	24	2009	Emilia Romagna	+	+	+	–	Cluster II	Cluster II	Cluster II
315994	25	2009	Lombardia	+	–	–	NA	NA	NA	NA
320695	26	2009	Lombardia	+	+	+	NA	NA	†	†
320825	26	2009	Lombardia	+	+	+	–	Cluster II	Cluster II	Cluster II
324345	27	2009	Lombardia	+	+	+	NA	†	†	†
324374	27	2009	Lombardia	+	+	+	NA	†	†	†
324397	27	2009	Lombardia	+	+	+	–	Cluster II	Cluster II	Cluster II
324507/1	28	2010	Lombardia	+	+	+	–	Cluster II	Cluster II	Cluster II
324507/2	28	2010	Lombardia	+	+	+	NA	NA	†	†
324507/3	28	2010	Lombardia	+	+	+	NA	NA	NA	†
324507/4	28	2010	Lombardia	+	+	+	NA	NA	†	†
5448/2	29	2011	Emilia Romagna	NA	+	–	NA	NA	NA	NA
28607	30	2012	Lombardia	–	+	+	NA	NA	†	†
29742	30	2012	Lombardia	+	+	+	–	Cluster II	Cluster II	Cluster II
30917	31	2012	Lombardia	+	+	–	NA	NA	NA	NA
35621/1	32	2012	Lombardia	+	+	–	NA	NA	NA	NA
35621/2	32	2012	Lombardia	–	+	+	NA	NA	NA	NA
41906	33	2012	Lombardia	–	+	+	NA	NA	NA	NA
44833	34	2012	Lombardia	NA	+	+	–	Cluster II	Cluster II	Cluster II
67322	8	2012	Lombardia	–	+	+	NA	NA	NA	†
273992	35	2012	Lombardia	–	+	+	–	Cluster II	Cluster II	Cluster II
32961	36	2013	Piemonte	–	+	–	NA	NA	NA	NA
32963	36	2013	Piemonte	+	+	–	NA	NA	NA	NA
178509	37	2014	Emilia Romagna	NA	NA	+	–	Cluster III	Cluster III	Cluster III
200885	38	2014	Emilia Romagna	+	+	+	–	Cluster III	Cluster III	Cluster III

*Cluster I represents strains circulating from 2007 through mid-2009; cluster II represents strains circulating from mid-2009 through 2012; and cluster III represents strains circulating since 2014. EM, electron microscopy; M, membrane; pan-CoV RT-PCR, pan-coronavirus reverse transcription PCR; PEDV, porcine epidemic diarrhea virus; PRCV, porcine respiratory coronavirus; RdRp, RNA-dependent RNA polymerase; S1, spike; TGEV, transmissible gastroenteritis virus; +, positive; –, negative; NA, not tested or sequenced.
†Sequence available but not included in this study.
‡Samples selected for whole genome sequencing.

On the basis of the partial sequences from RdRp and the S1 and M genes, the strains from Italy clustered into 3 temporally divided groups, suggesting 3 independent virus entries. Cluster I represents strains circulating from 2007 through mid-2009; cluster II represents strains circulating from mid-2009 through 2012; and cluster III represents strains circulating since 2014 (online Technical Appendix Figure 2, panels A–C). Cluster I was identified in Emilia Romagna (n = 1), Lombardia (n = 5), and Veneto (n = 1). Cluster II was identified in Emilia Romagna (n = 1) and Lombardia (n = 8). Cluster III was identified in Emilia Romagna at 2 swine farms. To help explain the temporal clustering, a

single S1 gene segment was sequenced from clusters I and II (PEDV/Italy/7239/2009 and SeCoV/Italy/213306/2009, respectively). Because of the recent outbreak of PEDV in Europe, the 2 positive samples from cluster III (PEDV/Italy/178509/2014 and PEDV/Italy/200885/2014) were sequenced (Figure 1, panel A).

One strain from each cluster was selected for whole genome sequencing (online Technical Appendix). Unfortunately, the whole genome was obtained from only clusters I and II (PEDV/Italy/7239/2009 and SeCoV/Italy/213306/2009, respectively; Figure 1, panel B). Recombination analysis was conducted on the 2 whole genomes and was not detected in PEDV/Italy/7239/2009. Recombination was detected in SeCoV/Italy/213306/2009 at position 20636 and 24867 of PEDV CV777 and at position 20366 and 24996 of TGEV H16 (Figure 2), suggesting the occurrence of a recombination event between a PEDV and a TGEV. The complete S gene of SeCoV/Italy/213306/2009 shared 92% and 90% nt identity with the prototype European strain PEDV CV777 and the original highly virulent North American strain Colorado 2013, respectively, and the remaining genome shared a 97% nt identity with the virulent strains TGEV H16 and TGEV Miller M6. Whole-genome analysis of PEDV/Italy/7239/2009 showed that it grouped with the global PEDV strains (*6*) and shared ≈97% nt identity with PEDV strains CV777, DR13 virulent, and North American S-INDEL strain OH851 (Table 2).

Conclusions

During 2007–2014, most (92%) samples collected from the Po Valley in Italy were positive for PEDV by ELISA; only 72% were positive by pan-CoV PCR. However, because we were investigating the presence of PEDV or TGEV in samples with clinical signs of diarrhea, the high occurrence of PEDV may not reflect the actual prevalence of PEDV in Italy. The increased percentage

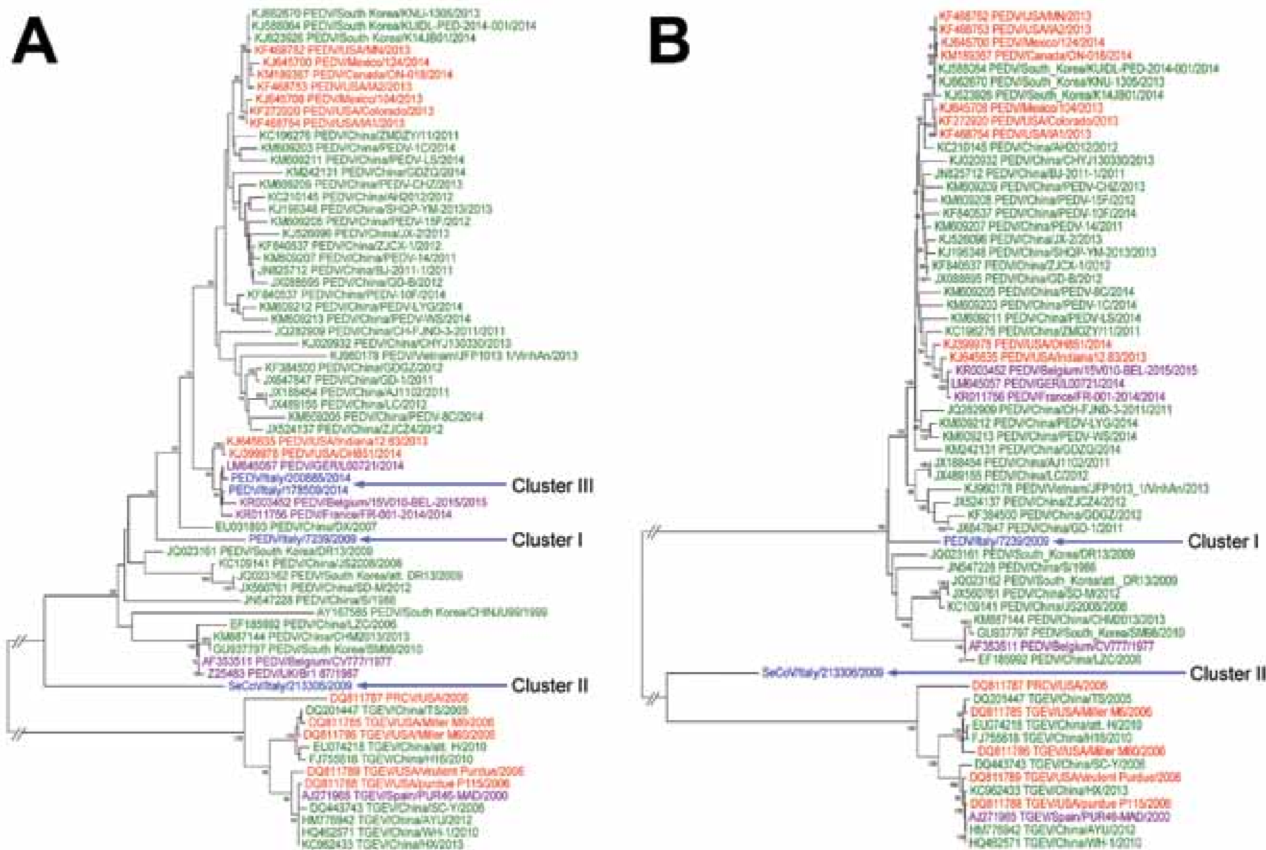


Figure 1. Phylogenetic analyses of swine enteric coronaviruses in Italy. A) Analysis performed on the basis of the nucleotide sequence of the complete spike (S1) gene of 4 representative strains from the 3 clusters and B) whole genome of 2 positive strains from clusters I and II. Cluster I represents strains circulating from 2007 through mid-2009; cluster II represents strains circulating from mid-2009 through 2012; and cluster III represents strains circulating since 2014. Bootstrap values >70% (1,000 replicates) are indicated. Reference sequences are identified by GenBank accession no. and strain name. The strains from this study are represented in blue; strains from China are green; strains from North America are red; and strains from Europe are purple. PEDV, porcine epidemic diarrhea virus; PRCV, porcine respiratory coronavirus; TGEV, transmissible gastroenteritis virus; SeCoV, swine enteric coronavirus.

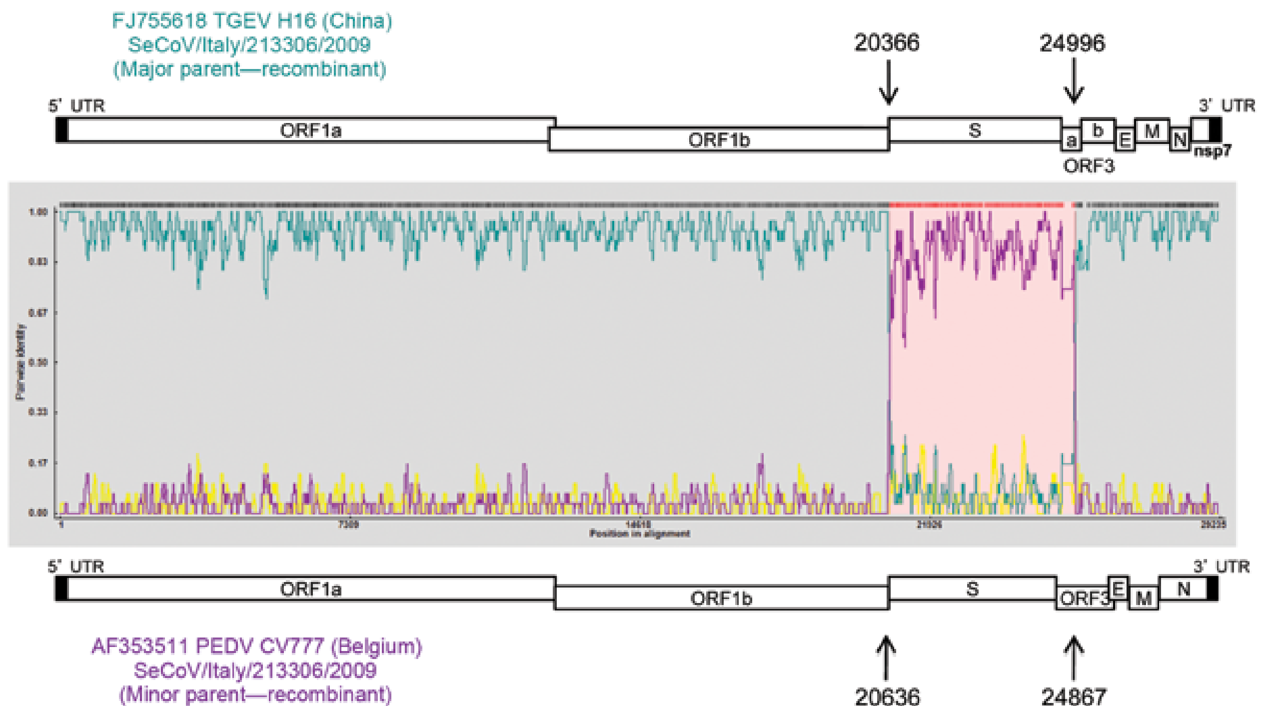


Figure 2. Potential recombination points in the SeCoV strains in study of swine enteric coronaviruses in Italy. The potential parent strains H16 (TGEV) and CV777 (PEDV) are shown in teal and purple, respectively. Arrows indicate recombinant breakpoints. UTR, untranslated region; ORF, openreading frame; S, spike; E, envelope; M, membrane; N, nucleocapsid; nsp, nonstructural protein; PEDV, porcine epidemic diarrhea virus; TGEV, transmissible gastroenteritis virus; SeCoV, swine enteric coronavirus.

of PEDV found in samples tested by ELISA, compared with the proportion found by PCR, may be explained by the number of ambiguous bases in the pan-CoV primers; the ambiguous bases severely reduce the efficiency of the reaction. The swine enteric CoV strains from Italy in our study, including the recombinant strain, were reported in pigs with mild clinical signs, indicating that PEDV and SeCoV have been circulating in Italy and likely throughout Europe for multiple years but were underestimated as a mild form of diarrhea.

To understand the evolution of PEDV in Italy, the partial RdRp, S, and M genes were sequenced from 18 samples and grouped in 3 different temporal clusters. Cluster I (2007–mid 2009) resembles the oldest PEDV strains; cluster II resembles a new TGEV and PEDV recombinant variant; and cluster III, identified from 2 pig farms in northern Italy in 2014, resembles the PEDV S-INDEL strains identified in Germany, France, Belgium, and the United States. The >99.3% nt identity of the S1 gene within cluster III and in previously identified strains could suggest the spread of the S-INDEL strain into Europe. However, directionality of spread cannot be determined because of a lack of global and temporal PEDV sequences.

Although our findings could indicate 3 introductions of PEDV in Italy, the results more likely suggest the high ability of natural recombination among CoVs and the

Table 2. Nucleotide identities of strains PEDV/Italy/7239/2009 and SeCoV/Italy/213306/2009, representative of clusters I and II, respectively, in study of swine enteric coronaviruses in Italy*

Strain identification	ORF1		Spike		ORF3		Envelope		Membrane		Nucleocapsid	
	I	II	I	II	I	II	I	II	I	II	I	II
PEDV/Belgium/CV777/1977	97.3	57.8	96.3	92.7	98.1	43.6	97.6	43.0	97.6	55.9	96.6	42.5
PEDV/South Korea/DR13 vir/2009	98.1	58.0	97.3	93.1	99.3	43.7	99.6	43.0	97.7	55.6	97.6	43.3
PEDV/USA/Colorado/2013	98.0	57.9	94.6	90.7	98.6	44.0	99.2	43.0	97.8	55.3	96.8	43.4
PEDV/USA/OH851/2014	98.1	57.9	96.9	91.9	98.7	43.8	99.2	43.0	97.7	55.5	96.7	43.4
PEDV/L00721/GER/2014	98.0	57.9	97.0	92.0	98.6	43.7	99.2	43.0	97.8	55.3	96.8	43.4
TGEV/USA/Miller M6/2006	57.9	96.8	52.0	52.5	40.0	89.1	42.6	96.4	56.1	97.1	43.1	96.3
PRCV/USA/ISU-1/2006	58.0	96.5	47.7	48.2	53.0	76.6	43.8	96.0	56.2	96.3	42.8	95.7

*Cluster I represents strains circulating from 2007 through mid-2009; cluster II represents strains circulating from mid-2009 through 2012. Ger, Germany; ORF, open reading frame; PRCV, porcine respiratory coronavirus; PEDV, porcine epidemic diarrhea virus; SeCoV, swine enteric coronavirus; TGEV, transmissible gastroenteritis virus.

continued emergence of novel CoVs with distinct pathogenic properties. Further investigation is needed to determine the ancestor of the SeCoV strain or to verify whether the recombinant virus was introduced in Italy. Recombinant SeCoV was probably generated in a country in which both PEDV and TGEV are endemic, but because the presence of these viruses in Europe is unclear and SeCoV has not been previously described, it is difficult to determine the parental strains and geographic spread of SeCoV. Future studies are required to describe the pathogenesis of SeCoV and its prevalence in other countries.

This work was funded by the Italian Ministry of Health (project PRC2010010- E87G11000130001).

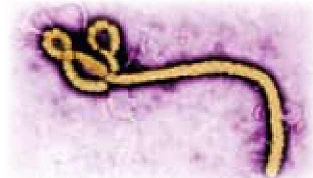
Dr. Boniotti is a scientist at the Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna; her primary research interests include molecular diagnosis and epidemiology of viral and bacterial infectious diseases. Dr. Papetti is a research scientist at the Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna; her major research interests are molecular diagnostic development and phylogenetic analysis of infectious diseases.

References

1. Enjuanes L, Brian D, Cavanagh D, Holmes K, Lai MMC, Laude H, et al. Coronaviridae. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, et al., editors. Virus taxonomy. Classification and nomenclature of viruses. New York: Academic Press; 2000. p. 835–9.
2. Pensaert M, Callebaut P, Vergote J. Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis. Vet Q. 1986;8:257–61. http://dx.doi.org/10.1080/01652176.1986.9694050
3. Wesley RD, Woods RD. Immunization of pregnant gilts with PRCV induces lactogenic immunity for protection of nursing piglets from challenge with TGEV. Vet Microbiol. 1993;38:31–40. http://dx.doi.org/10.1016/0378-1135(93)90073-G
4. Huang YW, Dickerman AW, Pyñeyro P, Li L, Fang L, Kiehne R, et al. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. mBiol. 2013; 4:00737-13. http://dx.doi.org/10.1128/mBio.00737-13
5. Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, et al. Emergence of porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest. 2013;25:649–54. http://dx.doi.org/10.1177/1040638713501675
6. Vlasova AN, Marthaler D, Wang Q, Culhane MR, Rossow KD, Rovira A, et al. Distinct characteristics and complex evolution of PEDV strains, North America, May 2013–February 2014. Emerg Infect Dis. 2014;20:1620–8. http://dx.doi.org/10.3201/eid2010.140491
7. Sun M, Ma J, Wang Y, Wang M, Song W, Zhang W, et al. Genomic and epidemiological characteristics provide new insights into the phylogeographical and spatiotemporal spread of porcine epidemic diarrhea virus in Asia. J Clin Microbiol. 2015;53:1484–92. http://dx.doi.org/10.1128/JCM.02898-14
8. Martelli P, Lavazza A, Nigrelli AD, Merialdi G, Alborali LG, Pensaert MB. Epidemic of diarrhoea caused by porcine epidemic diarrhoea virus in Italy. Vet Rec. 2008;162:307–10. http://dx.doi.org/10.1136/vr.162.10.307
9. Hanke D, Jenckel M, Petrov A, Ritzmann M, Stadler J, Akimkin V, et al. Comparison of porcine epidemic diarrhea viruses from Germany and the United States, 2014. Emerg Infect Dis. 2015;21:493–6. http://dx.doi.org/10.3201/eid2103.141165
10. Grasland B, Bigault L, Bernard C, Quenault H, Toulouse O, Fablet C, et al. Complete genome sequence of a porcine epidemic diarrhea S gene indel strain isolated in France in December 2014. Genome Announc. 2015;3:e00535-15. http://dx.doi.org/10.1128/genomeA.00535-15
11. Theuns S, Conceição-Neto N, Christiaens I, Zeller M, Desmarets LMB, Roukaerts IDM, et al. Complete genome sequence of a porcine epidemic diarrhea virus from a novel outbreak in Belgium, January 2015. Genome Announc. 2015; 3:e00506-15. http://dx.doi.org/10.1128/genomeA.00506-15
12. Sozzi E, Luppi A, Lelli D, Moreno Martin A, Canelli E, Brocchi E, et al. Comparison of enzyme-linked immunosorbent assay and RT-PCR or the detection of porcine epidemic diarrhoea virus. Res Vet Sci. 2010;88:166–8. http://dx.doi.org/10.1016/j.rvsc.2009.05.009
13. Lelli D, Papetti A, Sabelli C, Rosti E, Moreno A, Boniotti MB. Detection of coronaviruses in various bat species in Italy. Viruses. 2013;5:2679–89. http://dx.doi.org/10.3390/v5112679
14. Kim SY, Song DS, Park BK. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by duplex RT-PCR. J Vet Diagn Invest. 2001;13:516–20. http://dx.doi.org/10.1177/104063870101300611
15. Kim L, Chang KO, Sestak K, Parwani A, Saif LJ. Development of a reverse transcription–nested polymerase chain reaction assay for differential diagnosis of transmissible gastroenteritis virus and porcine respiratory coronavirus from feces and nasal swabs of infected pigs. J Vet Diagn Invest. 2000;12:385–8. http://dx.doi.org/10.1177/104063870001200418

Address for correspondence: M. Beatrice Boniotti, Research and Development Laboratory, Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna, via Bianchi 9, 25124 Brescia (BS), Italy; email: mariabeatrice.boniotti@izsler.it

Emerging Infectious Diseases Journal Podcasts



Dr. Mike Miller reads an abridged version of the article, **Biomarker Correlates of Survival in Pediatric Patients with Ebola Virus Disease.**

http://www2c.cdc.gov/podcasts/player.asp?f=8633631

Article DOI: http://dx.doi.org/10.3201/eid2201.150544

Porcine Epidemic Diarrhea Virus and Discovery of a Recombinant Swine Enteric Coronavirus, Italy

Technical Appendix

Additional Methods and Details

Clinical Samples

Fecal and intestinal samples were collected from pigs with clinical signs of diarrhea. The specimens were homogenized in phosphate-buffered saline (10% wt/vol), centrifuged at 2,000 g for 10 min, and the supernatant was poured into a new tube and stored at −80°C.

Virus Detection

Negative staining electron microscopy was performed as previously described (*1*). A double antibody sandwich ELISA was performed by using monoclonal antibodies (*2*). Virus isolation of Porcine epidemic diarrhea virus (PEDV) was attempted on Vero cells in minimum essential medium Eagle (MEM) (Sigma-Aldrich, Saint Louis, Missouri, USA), in the presence of trypsin as previously described (*3*).

RNA Extraction and Reverse Transcription-PCR (RT-PCR)

RNA was extracted from the supernatant by using the TRIzol method (Invitrogen, Carlsbad, CA, USA), and RT-PCR was performed by using the QIAGEN OneStep RT-PCR kit (QIAGEN, Hilden, Germany), performed with the following cycling conditions: 50°C for 30 min and 95°C for 15 min for the RT reaction, followed by 40 cycles of amplification at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. Samples were tested with the pan-CoV primers for the RNA-dependent RNA polymerase (RdRp) gene (*4*). If the samples were positive by the pan-CoV RT-PCR, a larger fragment of the RdRp (349 nt), the

spike (553 nt), and M (439 nt) genes were amplified by RT-PCR (Technical Appendix Table 1). The M gene primers were designed from the conserved regions of 30 Transmissible gastroenteritis virus (TGEV), Porcine respiratory coronavirus (PRCV), and PEDV sequences present in the GenBank database while the RdRp primers were designed from 41 *alpha*, *beta*, *gamma*, and *delta* CoVs sequences from GenBank (Technical Appendix Table 1). Amplification of partial S1 (553 nt) was performed by primers described by Kim et al. 2001 (*5*), and the S1 segment (2785 nt) was designed by using the conserved regions of 18 available PEDV sequences (Technical Appendix Table 1). The amplicons were sequenced with the initial amplification primers by using ABI PRISM 3130 Automated Capillary DNA Sequencer (Applied Biosystems, Waltham, Massachusetts, USA). The 18 sequenced samples were then tested by PCR for TGEV and PRCV by using the previously described RT-PCR kit and thermal cycling conditions (*6*). The phylogenetic trees for the partial RdRp, S1, and M genes, the complete S1 gene and full-length genomes were constructed by using the neighbor-joining method p-distance model and bootstrap test of 1,000 replicates in MEGA 5 (http://www.megasoftware.net/).

Next Generation Sequencing Assembly

Whole genome sequencing was performed on 1 strain from each genetic cluster because of limited available volume of sample. Seven milliliters of a 20% fecal homogenate were ultracentrifuged on 5 ml of 30% sucrose cushion at 35,000 rpm for 1 h at 4°C. The pellet was resuspended in 1x TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) and treated with DNase (RNase-free DNase set, QIAGEN, Hilden, Germany). Purified viral RNA was extracted by using the protocol described above. Viral cDNA synthesis and whole genome amplification of nucleic acids were performed as described by Victoria and colleagues (*7*). The sequencing libraries were prepared by using the NEXTERA-XT DNA library Preparation kit (Illumina Inc. San Diego, CA, USA), and purified according to the manufacturer protocol and sequenced on an Illumina MiSeq by using the Miseq Reagent Kit v2, 250-cycle paired-end run (Illumina Inc. San Diego, CA, USA). Reads were assembled by using PEDV or TGEV reference genomes. When mean coverage was <5 or polymorphisms were present in more than 20% of the reads, Sanger sequencing was performed by primers (Technical Appendix Table 2).

Nucleotide Sequence Accession Numbers

Partial and complete genome sequences obtained in this study were deposited in GenBank under accession nos. KT027383-KT027398 (RdRp), KT027399-KT027414 (S1), KT027415-KT027430 (M), KR061458 (PEDV/Italy/7239/2009) and KR061459 (SeCoV/Italy/213306/2009).

Recombination Analysis

Recombination breakpoints were determined by using the Recombination Detection Program (RDP) 4.43 (http://web.cbio.uct.ac.za/~darren/rdp.html), with BOOTSCAN, GENECONV, MAXCHI, CHIMAERA, SISCAN, 3SEQ and LARD. Statistical significance was determined by using p-values <0.05.

References

1. Martelli P, Lavazza A, Nigrelli AD, Merialdi G, Alborali LG, Pensaert MB. Epidemic of diarrhoea caused by porcine epidemic diarrhoea virus in Italy. Vet Rec. 2008;162:307–10. PubMed http://dx.doi.org/10.1136/vr.162.10.307

2. Sozzi E, Luppi A, Lelli D, Moreno Martin A, Canelli E, Brocchi E, et al. Comparison of enzyme-linked immunosorbent assay and RT-PCR or the detection of porcine epidemic diarrhoea virus. Res Vet Sci. 2010;88:166–8. PubMed http://dx.doi.org/10.1016/j.rvsc.2009.05.009

3. Hofmann M, Wyler R. Propagation of the virus of porcine epidemic diarrhea in cell culture. J Clin Microbiol. 1988;26:2335–9. PubMed

4. Lelli D, Papetti A, Sabelli C, Rosti E, Moreno A, Boniotti MB. Detection of coronaviruses in various bat species in Italy. Viruses. 2013;5:2679–89. PubMed http://dx.doi.org/10.3390/v5112679

5. Kim SY, Song DS, Park BK. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by duplex RT-PCR. J Vet Diagn Invest. 2001;13:516–20. PubMed http://dx.doi.org/10.1177/104063870101300611

6. Kim L, Chang KO, Sestak K, Parwani A, Saif LJ. Development of a reverse transcription–nested polymerase chain reaction assay for differential diagnosis of transmissible gastroenteritis virus and porcine respiratory coronavirus from feces and nasal swabs of infected pigs. J Vet Diagn Invest. 2000;12:385–8. PubMed http://dx.doi.org/10.1177/104063870001200418

7. Victoria JG, Kapoor A, Dupuis K, Schnurr DP, Delwart EL. Rapid identification of known and new RNA virus from animal tissues. PLoS Pathog. 2008;4:e1000163. PubMed http://dx.doi.org/10.1371/journal.ppat.1000163

Technical Appendix Table 1. Primers used for sequencing for the S1, M and RdRp genes*

Primers	Oligonucleotide sequence (5′-3′)	Position†
PEDV_S_1F	GGTAAGTTGCTAGTGCGTAA	20570–50589 nt
PEDV_S_1R	TCCCATGTTATGCCGACAA	21116–21134 nt
PEDV_S_2F	TAATGATGTTACAACAGGTC	21039–21058 nt
PEDV_S_2R	AGCAGTATGAAGTACAATTG	21629–21648 nt
PEDV_S_3F	GGCTCTGAGGTTTAATATTA	21579–21598 nt
PEDV_S_3R	GGTAAATTGTCTAGTGTCAA	22259–22278 nt
PEDV_S_4F	TGAGTTGATTACTGGCAC	22503–22520 nt
PEDV_S_4R	GCCATTAGTAACCACTTTAT	23336–23355 nt
M_1F	AYCTTRSAAACTGGAAYTTC	25722–25741 nt
M_1R	ACATAGWAAGCCCAWCCAGT	26258–26277 nt
RdRp gene CoV_8F	GGNTGGGAYTAYCCNAARTGYGA	14426–14448 nt
RdRp gene CoV_10R	TGYTGNGARCARAAYTCRTG	15008–15027 nt

*M, membrane; RdRp, RNA-dependent RNA polymerase; S1, spike.
†Nucleotide position refers to PEDV CV777(AF353511).

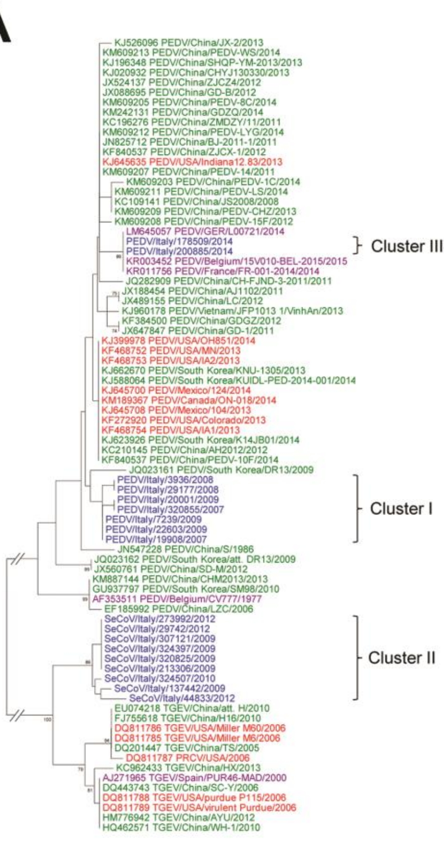
Technical Appendix Table 2. Primers used to close the genome

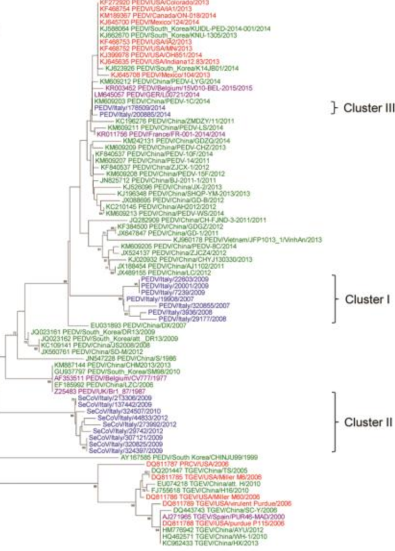
Primers	Oligonucleotide sequence (5′-3′)	Position*
213306_1_for	AGTAAAGTGAGTGTAGCGT	1 –19 nt
213306_1_rev	ACAAGGGCATCTAAGAGTA	1014–1032 nt
213306_2_for	TATGGGAAATGGTGACTGT	957–975 nt
213306_2_rev	GATGTCGTAAGATGGAACT	2055–2073 nt
213306_3_for	GATTGGTAATGGTGTGAAAGT	2517–2537 nt
213306_3_rev	CATTACCAGGAGCAATAGATT	4466–4486 nt
213306_4_for	TCATTGTCACACATACTACAG	5279–5299 nt
213306_4_rev	ACTACAATCACAAACTCAGC	6391–6410 nt
213306_5_for	ATAGTAGTCAAGAGGTTCTC	6809–6828 nt
213306_5_rev	CATAGCAGCAGATTCAAAGT	8675–8692 nt
213306_6_for	TACATGGGCTAAAACTAACAGT	9624–9645 nt
213306_6_rev	GAGGGCTAACATCATTTTTCTTA	11046–11068 nt
213306_7_for	TAGTGGAAAGGCTCTTATG	11634–11652 nt
213306_7_rev	TGACAATGTAGTAGGCATC	12480–12498 nt
213306_8_for	CGTTGCGTGTATTGGTAAA	12416–12434 nt
213306_8_rev	CGTCATTGGTATTGTCATAG	13237–13256 nt
213306_9_for	AGACAGTAAAACCAGGTCA	13507–13525 nt
213306_9_rev	ATGGTGTGATAGACATAATGAA	15201–15222 nt
213306_10_for	AGGCTATCACACTCTATTTTG	17078–07098 nt
213306_10_rev	CACAACTTTGGACACAACAT	19348–19367 nt
213306_11_for	CTCTATCACATAACTCAGTC	20167–20186 nt
213306_11_rev	TAGGTAGATAACCACCCAA	20499–20517 nt
213306_12_for	GTGACCACACCTATGAC	25375–25391 nt
213306_12_rev	CCAAGCGTAGTAGGTTTG	26812–26829 nt
213306_13_for	GTAGAGGTGATGTGACAA	27171–27188 nt
213306_13_rev	ATTGTTGGCTCGTCATAG	28017–28034 nt
7239_1_for	ATCTAYGGATAGTTAGCTCT	1–20 nt
7239_1_rev	TCACCAACAGTCCAATGA	1027–1044 nt
7239_2_for	GTGACGGGTTTTACAGAC	1977–1994 nt
7239_2_rev	ACAAAAGAAGTTTCAATGACGC	2790–2811 nt
7239_3_for	TGTCCAGGAAGATGTTCA	4672–4689 nt
7239_3_rev	TCCAATTTGTTGTCCATAAGTA	5233–5254 nt
7239_4_for	GTAATGGTAACGGTGTTGT	5913–5931 nt
7239_4_rev	GGAAAGGCATCACATTAC	6591–6608 nt
7239_5_for	GAAGTTGGTAATGTTGTCAAAC	7031–7052 nt
7239_5_rev	TATCACTGCTAACCTGAGTA	7921–7940 nt
7239_6_for	CTATGACCTACTGTCGT	8442–8458 nt
7239_6_rev	TAGGCGTATGGACATTGT	9543–9560 nt
7239_7_for	AGATTTCTTCCGTTCAGTCTA	10995–11015 nt
7239_7_rev	CACAAGCGCCTACCTTA	12996–13012 nt
7239_8_for	CTAAGCGTAACATCCTGC	14150–14167 nt
7239_8_rev	TGGAGTGATGGACAAAATGAAT	15474–15495 nt
7239_9_for	CAAGGAGGAGAGCGTTA	15774–15790 nt
7239_9_rev	GAAACCCATAAACGAGATAAC	17305–17325 nt

Primers	Oligonucleotide sequence (5′-3′)	Position*
7239_10_for	AGGGATCACTTAGCCTTAA	17891–17909 nt
7239_10_rev	CAAGGTTCTAAACACATACG	19765–19784 nt
7239_11_for	CTAATGTGCTGGGTGTTTC	23230–23248 nt
7239_11_rev	ATAAACTCTATACAAACGCCCTA	25579–25601 nt
7239_12_for	CAGTGTAGTTGAGATTGTTGAAC	26738–26760 nt
7239_12_rev	TAGGCTCGTCAAGCGGAT	27918–27935 nt

*Nucleotide position refers to complete genome of the samples 7239 and 213306.

Technical Appendix Figure 1. Geographic distribution of the ELISA and pan-CoV RT-PCR results (red and black, respectively) from this study.





Technical Appendix Figure 2. Phylogenetic trees from the study of porcine epidemic diarrhea virus and a recombinant swine enteric coronavirus. A) Partial RNA-dependent RNA polymerase gene; B) spike (S1) gene; and C) membrane (M) gene. Bootstrap values >70% (1,000 replicates) are indicated. Reference sequences are identified by GenBank accession no. and strain name. The 18 strains from Italy identified from this study are represented in blue; strains from China are green; strains from America are red; and

strains from Europe are purple. PEDV, porcine epidemic diarrhea virus; PRCV, porcine respiratory coronavirus; TGEV, transmissible gastroenteritis virus; SeCoV, swine enteric coronavirus.