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PORCINE EPIDEMIC DIARRHOEA VIRUS WITH A RECOMBINANT S GENE DETECTED IN HUNGARY, 2016

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Porcine epidemic diarrhoea virus (PEDV) can cause a severe enteric disease affecting pigs of all ages. In January 2016, diarrhoea with occasional vomiting was observed in a small pig farm in Hungary. All animals became affected, while mortality (of up to 30%) was only seen in piglets. Samples from different age groups and the carcass of a piglet were examined by various methods including pathology, bacteriology and molecular biology. PEDV was confirmed by PCR and its whole genome sequence was determined. The sequence PEDV HUN/5031/2016 showed high identity with recently reported European viruses. Differences were found mostly in the S gene, where recombination was detected with a newly identified and already recombinant swine enteric coronavirus (Se-CoV) from Italy. The present report describes the first porcine epidemic diarrhoea outbreak in Hungary after many years and gives an insight into the genetics of the Hungarian PEDV.

Key words: Porcine epidemic diarrhoea virus, spike glycoprotein, recombination, Hungary

Porcine epidemic diarrhoea virus (PEDV) belongs to the family *Coronaviridae*. Coronaviruses possess a positive-sense, single-stranded RNA genome of 26.4 to 31.7 kb, which makes them the largest enveloped RNA viruses (Woo et al., 2010). The genome of PEDV is approximately 28 kb long and consists of open reading frame (ORF) 1ab, which encodes non-structural proteins. Only one

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third of the genome encodes structural proteins (Kocherhans et al., 2001), namely spike (S), envelope (E), membrane (M) and nucleocapsid (N). In addition, there is another ORF named ORF3 between the S and E genes, encoding an ion channel, which possibly regulates virus production (Wang et al., 2012). Among these the main research interest is focused on the S gene and its glycoprotein product, which in the presence of proteolytic trypsin is cleaved into two functionally distinct subunits, S1 and S2, responsible for receptor binding and fusion mechanisms, respectively, which makes the spike protein the primary target for neutralising antibodies (Wicht et al., 2014).

PEDV was first described as the causative agent of porcine epidemic diarrhoea (PED) in the 1970s in England and Belgium (Wood, 1977; Pensaert and De Bouck, 1978). Thereafter the virus spread throughout Europe; however, the disease became infrequent in the last two decades, and epidemic outbreaks were reported only sporadically (Martelli et al., 2008). In contrast, PED still causes significant economic losses in Asian countries, especially since 2010, when new, highly pathogenic variants of PEDV were found (Li et al., 2012). Genetic analyses assume that in the spring of 2013 PEDVs of Chinese origin emerged in the United States of America (USA), where prior to that PED had been considered an exotic disease (Huang et al., 2013). After its introduction PED spread rapidly within the USA and through other parts of the American continent (EFSA AHAW Panel, 2014). However, not only these highly pathogenic viruses have emerged in the USA, new variants were also found, which were introduced into North America presumably at the same time as the previously identified ones. These variants do not cause severe clinical signs and have certain insertions and deletions in their S gene, which coined the designation S INDEL strains (Vlasova et al., 2014). The Asian and American outbreaks again attracted attention to PED in Europe; however, it is still notifiable only in a few countries (EFSA, 2016). In France, an outbreak caused by an S INDEL strain was reported in 2015 by Graland and Bigault. Similar cases affecting mostly fattening pigs with mild diarrhoea were reported from Belgium (Theuns et al., 2015), Germany (Stadler et al., 2015), Austria (Steinrigl et al., 2015), Portugal (Mesquita et al., 2015), Slovenia (Toplak et al., 2016) and Italy (Bertasio et al., 2016), while in Ukraine a highly pathogenic virus caused an outbreak with nearly 100% mortality rate among piglets under 10 days of age (Dastjerdi et al., 2015). In Hungary, the first PED outbreak was reported in 1977 by Benyeda et al., and the last PEDV-positive pigs were found in 2009, although there is no report describing that case (EFSA AHAW Panel, 2014). Here, we report the first detection of PEDV in Hungary after many years and provide information about the details of the outbreak and the genetics of the newly identified Hungarian virus.

Materials and methods

At the end of January 2016, greenish to brownish, watery diarrhoea of varying intensity occurred in a 60-sow farrow-to-finish pig farm located in western Hungary. Morbidity of breeding animals reached 100% with no subsequent mortality, but severe hypogalactia became very frequent among lactating sows. All newborn piglets had severe diarrhoea and occasional vomiting, and the mortality of suckling piglets had reached 30% by the end of the outbreak. Several different antibiotics were used without particular effect, still the clinical signs faded away after about three weeks. The carcass of a piglet and 12 rectal swabs from different age groups (growing pigs, boars, pregnant and lactating sows) were submitted to the Department and Clinic for Production Animals, University of Veterinary Medicine Budapest for pathological and standard microbiological diagnostic examinations.

Rectal swabs and intestinal samples were sent further to the Department of Microbiology and Infectious Diseases for testing for coronaviruses by polymerase chain reaction (PCR). All samples were prepared for PCR using Viral Nucleic Acid Extraction Kit III (Geneaid Biotech Ltd., New Taipei, Taiwan) and RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the manufacturer's instructions. PCR of the S gene was performed as described previously (Kim et al., 2001). PCR-positive products were submitted to BaseClear B.V. (Leiden, The Netherlands) for sequencing in both directions by Sanger sequencing methodology. Sequences were edited and analysed by using BioEdit version 7.2.5 (Ibis Biosciences, Carlsbad, California, USA). Alignments were built and a phylogenetic tree was computed using the software MEGA version 6.0 (Tamura et al., 2013). Possible recombination events were detected by the use of RDP4 (Martin et al., 2015).

The full-length genome was determined using the intestinal samples by the Laboratory for Molecular Biology of the National Food Chain Safety Office, Veterinary Diagnostic Directorate. RNA extraction was carried out with the help of the MagAttract Virus Mini M48 Kit (Qiagen, Hilden, Germany) on a King Fisher 96 Flex instrument (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the manufacturer's instructions. PCR was performed according to Song et al. (2015). All PCR products were submitted to BaseClear B.V. (Leiden, The Netherlands), but five sequences did not give clear results. These PCRs were repeated and products were submitted to Biomi Ltd. (Gödöllő, Hungary) for sequencing in the same way as mentioned above. Sequences were edited, analysed and the complete genome assembled with the programs included in the software DNASTAR version 13 (DNASTAR, Inc., Madison, Wisconsin, USA). The complete genome sequence was submitted to GenBank under accession number KX289955.

Results

Necropsy of the piglet revealed signs of diarrhoea and marked dehydration, together with acute gastroenteritis with small intestinal villous atrophy and crypt hyperplasia. Aerobic bacteriological culture of the small intestinal contents yielded non-haemolytic *E. coli* in almost pure culture. Rapid diagnostic test of the intestinal contents for F4, F5, F18 *E. coli* fimbriae, rotaviruses, *Clostridium difficile* and *Cryptosporidium* sp. (Bio K 353 Rainbow Piglet Scours, Bio-X Diagnostics S.A., Rochefort, France) yielded negative results. Standard aerobic culture of the submitted rectal swabs did not yield pathogenic bacteria, while anaerobic culture for *Brachyspira* species and pre-enrichment culture for *Salmonella* species were also negative.

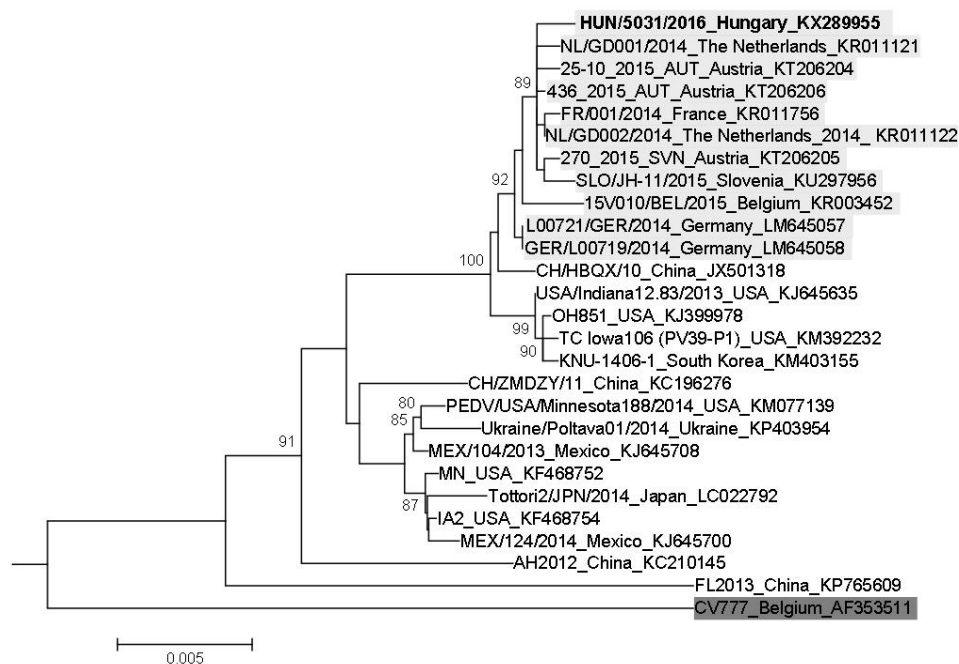


Fig. 1. Neighbour-joining phylogenetic tree computed with MEGA6 using the Maximum Composite Likelihood method based on the alignment of the S gene of PEDV from 27 sequences downloaded from GenBank and from the virus newly reported in this study. Numbers along the branches indicate the percentage of 1000 bootstrapping replicates (bootstrap support < 80% is not shown). For each sequence, the name is followed by the country and the GenBank accession number. European sequences are marked with light grey and the originally discovered PEDV with dark grey shading. The Hungarian PEDV is indicated in bold

PCR of the S gene was positive in five rectal swabs and in the intestinal sample. The rectal swabs did not contain enough material for further testing, therefore the intestinal sample was chosen for full-length genome analysis. The

sequenced PEDV HUN/5031/2016 shared 99.6% nucleotide (nt) identity with the recently detected European virus FR/001/2014 (Fig. 1). Difference count was found mostly in the S gene (Table 1), located mainly in an approximately 400 nt long section, which showed the highest identity of 96% and 95% with swine enteric coronavirus (SeCoV) Italy/213306/2009 and SeCoV/GER/L00930/2012, respectively, and only 89% to 91% identity with the above-mentioned European strains. The difference count at amino acid level is shown in Table 2.

Alignments of the S genes of these SeCoVs along with European, American and Asian sequences were analysed by several methods included in RDP4. A significant ($P < 0.05$) recombination event was detected in HUN/5031/2016 between positions 248 and 640 with the Belgian PEDV 15V010/BEL/2015 as the major parent and the SeCoV Italy/213306/2009 as the minor parent (Fig. 2).

Discussion

In this report, a PED outbreak in Hungary affecting pigs of all ages is described. The possible source of infection remains unknown, as the potential introduction routes of PEDV were not investigated thoroughly, because the owner of the farm rejected further co-operation and terminated operation after the clinical signs ceased. This was a solitary outbreak, as no similar cases have been reported to us or to other laboratories in Hungary since then. In contrast to recently reported European cases, the Hungarian outbreak occurred in a small farrow-to-finish operation, so suckling piglets and breeding animals were also involved. The viruses were quite similar at the genetic level: PEDV sequence HUN/5031/2016 shared at least 99% nt identity with all recently reported European viruses in Fig. 1, and the differences were found mainly in the S gene (Table 1). In that, a possible recombination was detected between PEDV sequence 15V010/BEL/2015 found in diarrheic fattening pigs in Belgium in 2015 (Theuns et al., 2015), and SeCoV Italy/213306/2009 reported from Italy in a study of swine enteric coronaviruses, including PEDV and transmissible gastroenteritis virus (TGEV), which also belongs to the genus *Alphacoronavirus* and causes quite similar clinical signs (Boniotti et al., 2016). This study also reported a recombinant TGEV and PEDV sequence identified as SeCoV, which became the possible minor parent regarding the new Hungarian virus sequence reported in this paper. Recently, another SeCoV resembling the Italian virus was found in diarrhoeic faecal samples collected in 2012 in Germany (Akimkin et al., 2016). In this study, it is suggested that these viruses can be targets of recombination events, which is a possibility in our case. In the same year, a third SeCoV was found in Central Eastern Europe (Belsham et al., 2016), but its sequence information was not yet available at the time of analysing the Hungarian virus, therefore it did not affect the results described in this study. The position of the possible recombination in HUN/5031/

Table 1

Sequence nucleotide difference count correlated to HUN/5031/2016 (GenBank Acc. No. KX289955). ORF: open reading frame, S: spike, E: envelope, M: membrane, N: nucleocapsid

Name	GenBank Acc. No.	Complete genome	All coding regions	ORF1	S	ORF3	E	M	N	Reference
FR/001/2014	KR011756	101	85	27	54	0	1	2	1	Grasland and Bigault, 2015
L00721/GER/2014	LM645057	112	92	31	56	0	1	1	3	Stadler et al., 2015
GER/L00719/2014	LM645058	113	93	32	56	0	1	1	3	Stadler et al., 2015
SLO/JH-11/2015	KU297956	136	113	50	58	0	1	1	3	Toplak et al., 2016
I5V010/BEL/2015	KR003452	157	136	61	66	1	2	2	4	Theuns et al., 2015
OH851	KJ399978	250	228	142	66	1	1	2	16	Wang et al., 2014
FL2013	KP765609	665	626	285	247	28	4	10	52	Zhang et al., 2015
CV777	AF353511	895	859	552	203	22	8	13	61	Koehlerhans et al., 2001
Italy/213306/2009	KR061459	11131	10733	8639	313	432	142	405	802	Boniotti et al., 2016

Table 2

Sequence amino acid difference count correlated to HUN/5031/2016 (GenBank Acc. No. KX289955). ORF: open reading frame, S: spike, E: envelope, M: membrane, N: nucleocapsid

Name	GenBank Acc. No.	All coding regions	ORF1	S	ORF3	E	M	N	Reference	
FR/001/2014	KR011756	29	12	16	0	0	0	0	1	Grasland and Bigault, 2015
L00721/GER/2014	LM645057	32	12	18	0	0	0	0	2	Stadler et al., 2015
GER/L00719/2014	LM645058	33	13	18	0	0	0	0	2	Stadler et al., 2015
SLO/JH-11/2015	KU297956	46	24	20	0	0	0	0	2	Toplak et al., 2016
I5V010/BEL/2015	KR003452	57	31	22	1	0	0	0	3	Theuns et al., 2015
OH851	KJ399978	66	37	22	1	0	0	0	6	Wang et al., 2014
FL2013	KP765609	185	60	97	9	1	3	15	17	Zhang et al., 2015
CV777	AF353511	218	123	65	9	1	3	17	17	Koehlerhans et al., 2001
Italy/213306/2009	KR061459	4201	3383	102	189	61	140	326	326	Boniotti et al., 2016

201 suggests that it can change the amino acid sequence of the S1 subunit, although further studies are needed to determine the potential consequences in receptor binding and antibody production reflecting these differences.

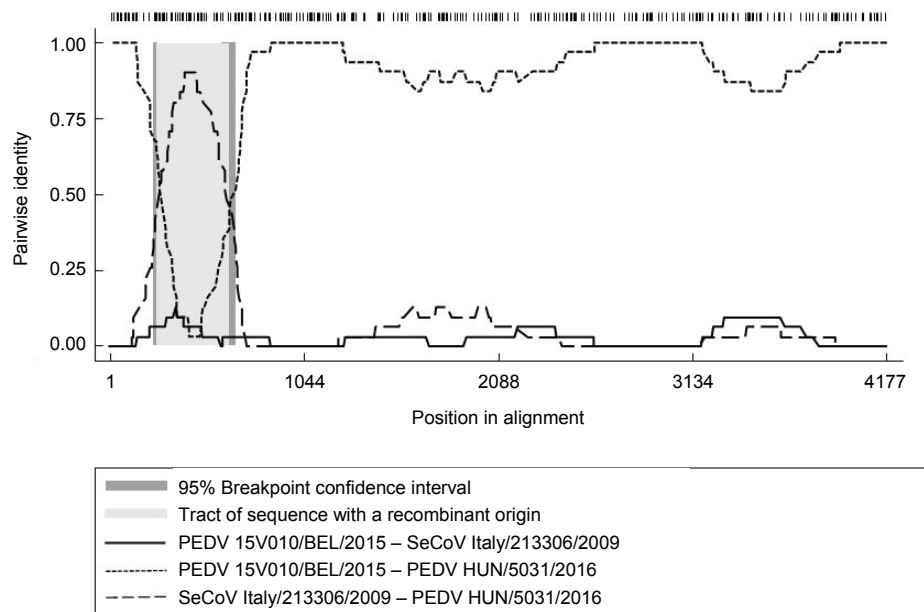


Fig. 2. Pairwise identity plot achieved by screening multiple sequence alignments of the S gene of PEDV with Recombination Detection Program (RDP). The pairwise identities of the potential recombinant HUN/5031/2016, the major parent PEDV 15V010/BEL/2015 and the minor parent SeCoV Italy/213306/2009 determine the potential recombinant region with 95% confidence interval. The bold dashes on top indicate the positions of informative sites, which are not identical or different in all three sequences

In conclusion, we detected a novel Hungarian PEDV with a possible recombination in its S gene. More swine enteric coronaviruses from Europe, including PEDV, TGEV and SeCoV should be identified and full-length genomes submitted to GenBank to determine the phylogenetic relations and the potential origin of the recombination events. As coronaviruses can be potential subjects to such events, confirmed also by this study, diagnostic difficulties can be expected. Further studies are needed to help overcome these diagnostic problems, as well as improve our knowledge regarding the epidemiological situation of PEDV with a focus on prevention through co-operation between field veterinarians and different laboratories.

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