Molecular characterization of a porcine sapovirus strain isolated from a piglet with diarrhoea: a case report

L. Dufkova, P. Kulich, J. Prodelalova

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: Porcine sapoviruses, members of the family *Caliciviridae*, have been considered as an aetiological agent of gastroenteritis in pigs. In this study, we analysed 251 faecal samples obtained from 3 to 90 day-old diarrhoeic pigs in the Czech Republic between January 2005 and June 2010 and tested them by negative staining electron microscopy for the presence of sapoviruses. Only one sample showed the presence of viral particles with characteristic sapovirus morphology. The presence of sapovirus (SaV) was confirmed by an RT-PCR assay with primers specific for the sapoviral RNA polymerase and capsid genes. Phylogenetic analysis based on a partial sequence of the RNA polymerase gene placed the new Czech isolate into the GVII genogroup of porcine sapoviruses; however, analysis of a portion of the capsid gene sequence classified the isolate as GIII of the genus *Sapovirus*. These contradictory findings indicate that recombinant porcine sapovirus was identified. According to our knowledge this is the first description of porcine sapovirus in domestic pigs in the Czech Republic.

Keywords: calicivirus; RT-PCR; phylogenetic analysis; genogroup; enteritis

Sapoviruses (SaVs), members of the genus *Sapovirus*, are small, non-enveloped, positive sense, single-stranded RNA viruses that belong to the family *Caliciviridae* (Oliver et al., 2006). Porcine SaV was first identified in a faecal sample of a diarrhoeic piglet (*Sus scrofa* f. *domestica*) by electron microscopy in the United States in 1980 (Saif et al., 1980; Guo et al., 1999) and later the pathogenic potential of porcine SaV was demonstrated in experimental infections of gnotobiotic pigs (Flynn et al., 1988; Guo et al., 2001). Recent studies have considered SaVs as causative agents of gastroenteritis in swine (Kim et al., 2006; Jeong et al., 2007; Zhang et al., 2008). Since certain porcine SaV strains are closely related to human SaVs genetically (Wang et al., 2007), their zoonotic potential cannot be ruled out (Bank-Wolf et al., 2010).

Porcine SaVs have been detected in several European countries (Reuter et al., 2007, 2010; Martella et al., 2008; Mauroy et al., 2008; Collins et al., 2009), Asia (Kim et al., 2006; Yin et al., 2006; Yang et al., 2009) and the Americas (Martinez et al., 2006; Wang et al., 2006; Barry et al., 2008; L'Homme et al., 2009), indicating their worldwide distribution. Nevertheless, there is still a lack of information regarding SaV epidemiology because porcine SaVs are not included in the routine diagnosis of porcine pathogens (Collins et al., 2009).

SaVs are usually detected using electron microscopy and RT-PCR methods. For direct virus detection in faecal samples, electron microscopy based on the typical morphology of viral particles is conventionally used, but this method is relatively insensitive (Hazelton and Gelderblom, 2003). Sapoviruses have a classic cup-shaped morphology and can be clearly detected using electron microscopy in diarrheal faeces when the faecal sample is collected early in the course of an infection (Green et al., 2000; Atmar and Estes, 2001). For the detection of sapoviral RNA in faecal samples, RT-PCR based on the detection of RNA dependent RNA polymerase (RdRp) or capsid genes has been used

Supported by the Czech Science Foundation (Grant No. 524/09/P195) and the Ministry of Education, Youth and Sports of the Czech Republic (AdmireVet; Grant No. CZ.1.05/2.1.00/01.0006, ED0006/01/01).

in epidemiological studies (Jiang et al., 1999; Guo et al., 2001; Kim et al., 2006). The results of RT-PCR analysis of faecal samples from diarrhoeic piglets suggest that sapoviruses are associated with diarrhoea in piglets either alone or in combination with other enteric pathogens (Jeong et al., 2007; Reuter et al., 2007; Martella et al., 2008; Zhang et al., 2008). Using RT-PCR methods, porcine sapoviruses have been detected in both symptomatic and asymptomatic animals (Reuter et al., 2010).

Porcine SaVs are divided into four genogroups (GIII, GVI, GVII, GVIII) based on complete capsid gene sequences (Martella et al., 2008; Wang et al., 2005; Yin et al., 2006); nevertheless, the majority of porcine sapoviruses including the prototype strain Cowden, belong to GIII (Guo et al., 1999). Recently, two potential new genogroups, GIX and GX, were described by Reuter et al. (2010). Similar to other RNA viruses, recombination events complicate the classification of SaVs and both intra- and inter-genogroup recombinant SaV strains have been detected (Hansman et al, 2005; Wang et al. 2005).

Since the epidemiological situation of SaV in the Czech Republic is completely unknown, the aim of this study was to examine the occurrence of porcine SaVs in diarrhoeal faeces obtained from pigs on Czech farms.

Case description

Between January 2005 and June 2010, a total of 251 samples collected on 139 farms in the Czech Republic from piglets aged between three and 90 days showing symptoms of diarrhoea were examined by electron microscopy. The samples were screened in the laboratory for the presence of aetiological agents of viral gastroenteritis. The supernatants of 10% faecal suspensions in phosphate buffered saline (PBS, pH 7.2) were negatively stained with 2% ammonium molybdenate and observed with a Philips EM 208 transmission electron microscope.

RNA was extracted from faecal suspensions using TRI Reagent LS (Molecular Research Center, Cincinnati, USA) supplemented with Polyacryl Carrier (Molecular Research Center, Cincinnati, USA), according to the manufacturer's instructions. The RNA was stored at –80 °C until used. To ensure the suitability of isolated RNA prior to reverse transcription and PCR amplifications, internal quality control of extracted RNA (RECK; Nanogen Advanced Diagnostics) was performed. Reverse transcription was carried out in a 50 µl volume using SuperScript III Reverse Transcriptase (Invitrogen), random hexamers (Invitrogen) and the specific primers p290 and capsid F in accordance with the manufacturer's instructions. Two microlitres of RT product were used in PCRs performed using the HotStarTaq Master Mix Kit (Qiagen) and the P290/289 primer pair (Jiang et al., 1999) targeting the conserved sequences of the RdRp region and the F and R capsid primer pair specific for the capsid gene (Kim et al., 2006). RdRp PCR consisted of a hot start performed for 15 min, followed by 40 cycles of 30 s at 94 °C, 90 s at 49 °C and 60 s at 72 °C, and a final extension at 72 °C for 10 min. The capsid-specific PCR consisted of 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 7 min. Expected 331 bp and 757 bp amplicons were analyzed by ethidium bromide (0.5 µg/ml)-stained 1.5% agarose gel electrophoresis in TBE buffer and were visualized under UV light.

PCR products were purified using the Wizard SV Gel and PCR Clean up system (Promega), and sequenced (Elisabeth Pharmacon, Brno, Czech Republic) using the p290/289 primer pair and capsid F and R primers. At least two amplification products from two independent RT-PCR reactions were sequenced. Sequence editing, assembling and analysis were performed using BioEdit version 5.0.9 (Hall, 1999). Nucleotide sequences of porcine strains of the genus *Sapovirus* and selected caliciviruses available in the GenBank were compared with obtained sequences of the Czech sapovirus strain using BLAST utility (http://www.ncbi.nlm.nih.gov/BLAST). The classification and GenBank accession numbers of reference strains for phylogenetic analyses are listed in Table 1. Phylogenetic analyses were carried out by the unweighted pair group method with arithmetic mean (UPGMA) with bootstrap (1000 replicates) using the MEGA software package v4.0 (Tamura et al., 2007). The viral strains used for phylogenetic analyses are listed in Table 1.

Out of 251 samples analysed by electron microscopy, two samples, P1884/2 and H252, were found to be positive for calicivirus-like particles. However, only sample P1884/2 contained particles with characteristic sapovirus morphology with cup-shaped depressions (Figure 1). Both calicivirus-like particle-positive samples were tested by RT-PCR for calicivirus RNA. Sample H252 yielded a negative result in

Table 1. Summary of the sapovirus strains and reference strains for *Lagovirus*, *Norovirus*, and *Vesivirus* genera used in phylogenetic analysis

Strain	Genus/genogroup-genotype	Abbreviation	GenBank accession No.
Hu/Sapporo/82/JP	SaV/GI-1	Sapporo	HM002617
Hu/Parkville/94/US	SaV/GI-2	Parkville	U73124
Hu/Stockholm/97/SE	SaV/GI-3	Stockholm	AF194182
Hu/Bristol/98/UK	SaV/GII-1	Bristol	AJ249939
Hu/Mex340/90/MX	SaV/GII-2	Mex340	AF435812
Hu/Cruise ship/00/US	SaV/GII-3	Cruise ship	AY289804
Hu/Mc10/00/TH	SaV/GII-4	Mc10	AY237420
Hu/C12/00/JP	SaV/GII-5	C12	AY603425
Po/SaV/Cowden/80/US	SaV/GIII	Cowden	NC000940, AF182760
Po/SaV/Ch-Sw-Sa1/08/CHN	SaV/GIII	Ch-Sw-Sa1	EU59922
Po/SaV/8D/05/IRE	SaV/GIII	8D/05	FJ975602
SWECI/VA10/NL	SaV/GIII	VA10	AY615807
Po/SaV/OH-MM280/03/US	SaV/GIII	MM280	AY823308
Po/SaV/JB-SC55/04/Korea	SaV/GIII	JB-SC55	DQ389628
PEC/swine-Id3/2005/HUN	SaV/GIII	Id3	DQ383274
Po/SaV/JB-GC155/05/KO	SaV/GIII	JB-GC155	DQ389610
Hu/Hou7-1181/90/US	SaV/GIV	Hou7	AF435814
Hu/Argentina39/ARG	SaV/GV	Arg39	AY289803
Po/SaV/OH-JJ681/00/US	SaV/GVI	JJ681	AY974192
Po/SaV/MI-QW19/02/US	SaV/GVII	QW19	AY826424
SWECII/VA14	SaV/GVII	VA14	AY615810
Po/SaV/OH-LL26/02/US	SaV/GVIII	LL26	AY974195
Po/SaV/K7/JP	SaV/GVIII	K7	AB221130
Po/SaV/9/07/IRE	SaV/GVIII	9/07	FJ975601
Po/SaV/43/06-18p3/06/ITA	SaV/GVIII	43/06-18p3	EU221477
SWECIII/VA59/NL	SaVG?	VA59	AY615813
Sapovirus Po/B2194/Brazil	SaVG?	B2194	DQ359032
Po/SaV/K19/JP	SaVG?	K19	AB223004
SWECIII/VA112/NL	SaVG?	VA112	AY615814
Po/SaV/K11/JP	SaVG?	K11	AB223000
Po/SaV/K24/JP	SaVG?	K24	AB223005
SWECIII/VA24a/NL	SaVG?	VA24a	AY615812
Mink/SaV/151A/CAN	SaVG?	mink 151A	AY144337
Ra/RHDV/GH/1988/GE	*Lagovirus*	RHDV	M67473
Fe/FCV/F9/1958/US	*Vesivirus*	FCV	M86379
Hu/Norwalk/68/US	*Norovirus*	Norwalk	M87661

a PCR with RdRp- and capsid-specific primers. On the other hand, PCR analysis of the P1884/2 sample resulted in amplification with both primer pairs, in agreement with electron microscopy.

Sequencing and phylogenetic analysis of a partial capsid sequence obtained from sample P1884/2 showed that the Czech porcine enteric calicivirus strain P1884/2 was tightly clustered (bootstrap value

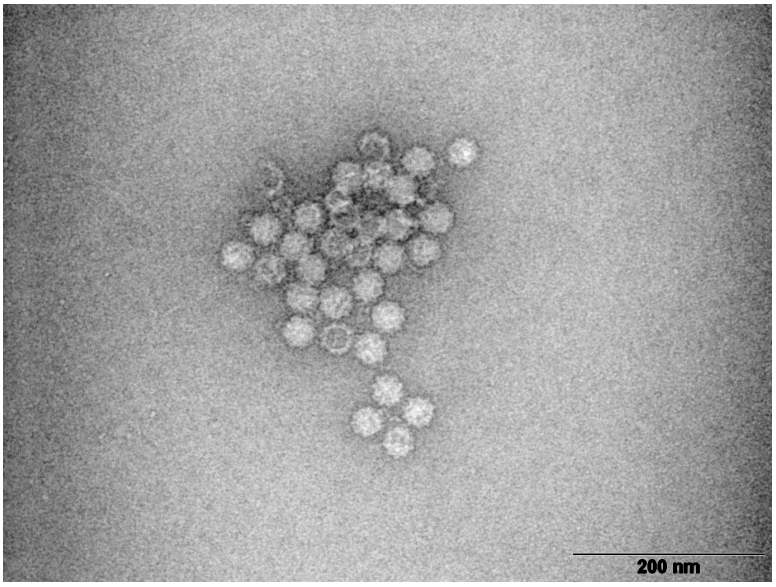

Figure 1. Electron micrograph showing *Sapovirus* particles from a swine faecal sample negatively stained with 2% ammonium molybdenate

100%) with the genogroup GIII strains JB-GC155/Korea, Cowden/US and MM280/US (Figure 2). Among GIII reference strains and the Czech isolate, the paired comparison showed the highest similarity between P1884/2 and JB-GC155/Korea (93%). Classification of sapoviruses is usually based on the capsid sequence and phylogenetic analysis of strain P1884/2 using the capsid sequence has confirmed its classification into the genus *Sapovirus* (Shuffenecker et al., 2001). The UPGMA phylogenetic analysis of the RdRp sequence surprisingly showed only 50–53 % nt identity with the GIII reference strains, but formed a tight cluster with strain K7/JP instead, which is a prototype of the GVII ge-

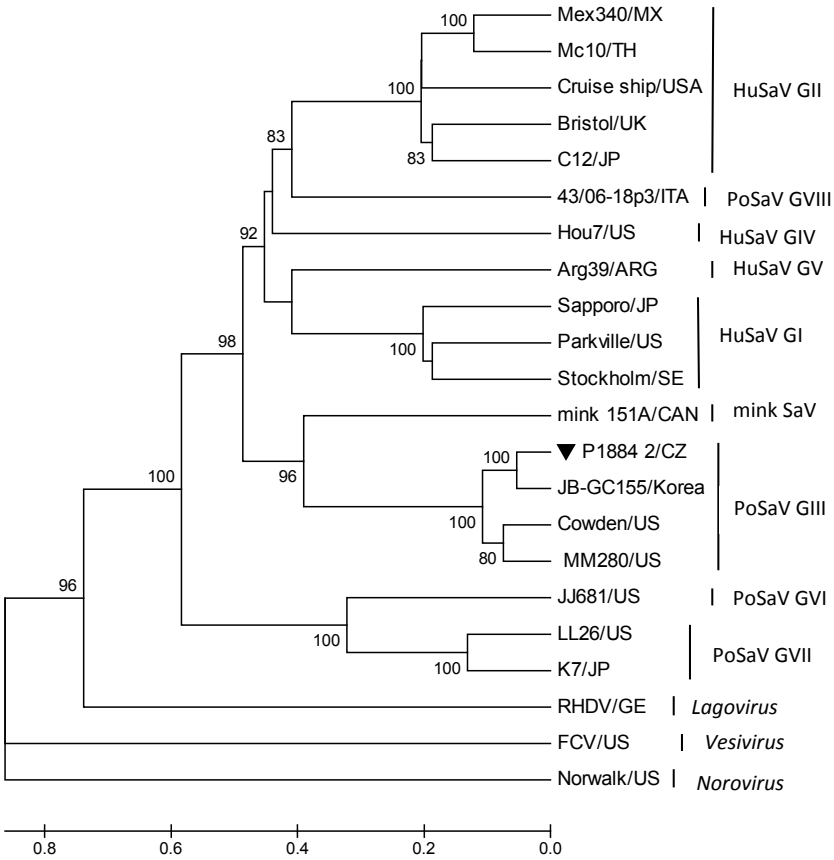

Figure 2. UPGMA phylogenetic tree based on a partial sequence of the capsid gene (674 nt; located at nucleotide positions 5771–6444 in the Cowden virus genome) of human and animal caliciviruses. The P1884/2 porcine sapovirus strain identified in this study is marked with a black triangle (▼). The scale represents genetic distances as units of expected nt substitutions per site

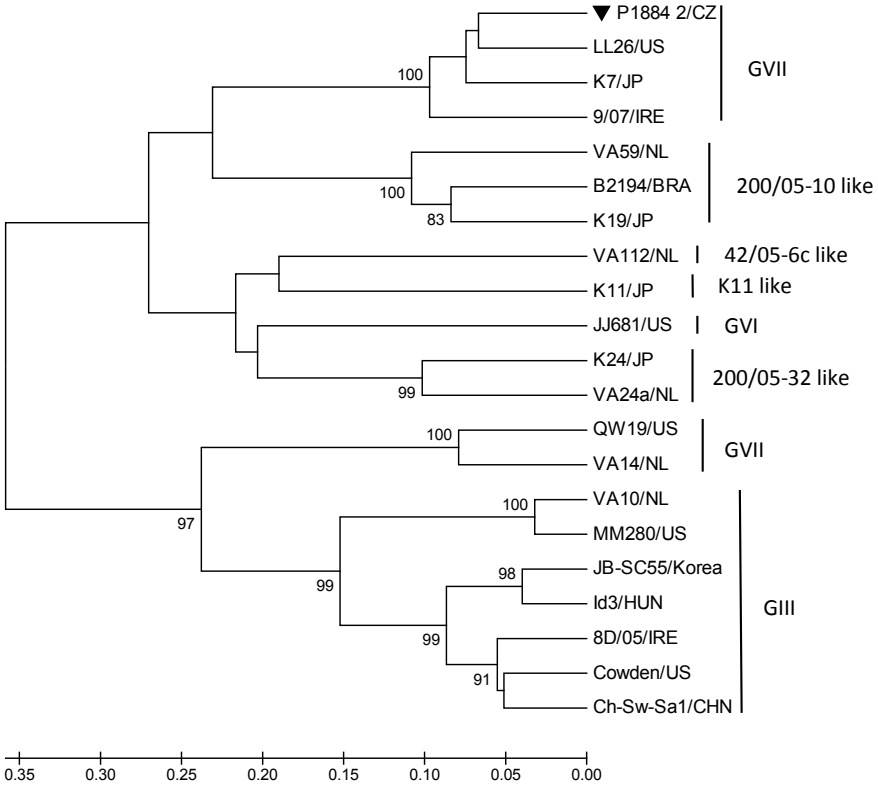

Figure 3. UPGMA phylogenetic tree based on a partial sequence of the RNA polymerase gene (330 nt; located at nucleotide positions 4180–4510 in the K7/JP virus genome) of porcine sapoviruses. The P1884/2 porcine sapovirus strain identified in this study is marked with a black triangle (▼). The scale represents genetic distances as units of expected nt substitutions per site

nogroup (Figure 3). The grouping was statistically supported by a 100% bootstrap value. Within the GVII genogroup, nt identity varied between 80% and 88% and strain P1884/2 was genetically more related to strain LL26/US than to other members of GVII (strains K7/JP and 9/07/IRE).

The sequences of the strain P1884/2 were submitted to the GenBank database and can be found under accessions numbers HQ423162 and HQ423163.

DISCUSSION AND CONCLUSIONS

The contradictory results of phylogenetic analysis of the RdRp and capsid genes indicate the detection of a recombinant strain (Wang et al., 2005; Jeong et al., 2007), although we cannot exclude the possibility that co-infection of the piglet occurred and that the two obtained sequences originated from two different strains. However, due to the low prevalence of sapoviruses in the Czech Republic (only one positive sample out of 251 tested in this study), co-infection is a less likely hypothesis and we believe it is more likely that we did indeed detect recombinant virus as has been reported by Wang et al. (2005).

To the best of our knowledge, this is the first report indicating the occurrence of porcine SaVs in pigs in the Czech Republic. Since the 251 samples were collected only from pigs showing clinical symptoms of enteritis and because only one sample tested positive, it seems that SaVs are not widespread in the Czech Republic. However, these data do not reveal the epidemiological significance of porcine SaVs on Czech pig farms since the detection of enteric viruses in faecal specimens using electron microscopy requires particle concentration of at least 10^6 per ml of faeces (Atmar and Estes, 2001). Although a negative result using electron microscopy is not an absolute diagnosis (Hazelton and Gelderblom, 2003), the low positivity of pigs was further corroborated using RT-PCR (data not shown), and we therefore assume that the prevalence of SaV enterits in pigs in the Czech Republic is indeed low.

REFERENCES

Atmar RL, Estes MK (2001): Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clinical Microbiology Reviews 14, 15–37.

Bank-Wolf BR, Konig M, Thiel HJ (2010): Zoonotic aspects of infections with noroviruses and sapoviruses. Veterinary Microbiology 140, 204–212.

Barry AF, Alfieri AF, Alfieri AA (2008): High genetic diversity in RdRp gene of Brazilian porcine sapovirus strains. Veterinary Microbiology 131, 185–191.

Collins PJ, Martella V, Buonavoglia C, O'Shea H (2009): Detection and characterizaton of porcine sapoviruses from asymptomatic animals in Irish farms. Veterinary Microbiology 139, 176–182.

Flynn WT, Saif LJ, Moorhead PD (1988): Pathogenesis of porcine enteric calicivirus-like virus in four-day-pld gnotobiotic pigs. American Journal of Veterinary Research 49, 819–825.

Green KY, Ando T, Balayan MS, Berke T, Clarke IN, Estes MK, Matson DO, Nakata S, Neill JD, Studdert MJ, Thiel HJ (2000): Taxonomy of the caliciviruses. Journal of Infection Diseases 181 (Suppl. 2), 322–330.

Guo M, Chang KO, Hardy ME, Zhang Q, Parwani AW, Saif LJ (1999): Molecular characterization of a porcine enteric calicivirus genetically related to Sapporo-like human caliciviruses. Journal of Virology 73, 9625–9631.

Guo M, Hayes J, Cho KO, Parwani AW, Lucas LM, Saif LJ (2001): Comparative pathogenesis of tissue culture-adapted and wild-type Cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhea by intravenous inoculation of wild-type PEC. Journal of Virology 75, 9239–9251.

Hall TA (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic Acids Symposium, Series 41, 95–98.

Hansman GS, Takeda N, Oka T, Oseto M, Hedlung KO, Katayama K (2005): Intergenogroup recombination in sapoviruses. Emerging Infectious Diseases 11, 1916–1920.

Hazelton PR, Gelderblom HR (2003): Electron microscopy for rapid diagnosis of infectious agents in emergent situations. Emerging Infectious Diseases 9, 294–303.

Jeong C, Park SI, Park SH, Kim HH, Park SJ, Jeong JH, Choy HE, Saif LJ, Kim SK, Kang MI, Hyun BH, Cho KO (2007): Genetic diversity of porcine sapoviruses. Veterinary Microbiology 122, 246–257.

Jiang X, Huang PW, Zhong WM, Farkas T, Cubbit DW, Matson DO (1999): Design and evaluation of a primer pair that detects both Norwalk-and Sapporo-like caliciviruses by RT-PCR. Journal of Virological Methods 83, 145–154.

Kim HJ, Cho HS, Cho KO, Park NY (2006): Detection and molecular characterization of porcine enteric calicivirus in Korea, genetically related ti sapoviruses. Journal of Veterinary Medicine B 53, 155–159.

L'Homme Y, Sansregret R, Plante-Fortier E, Lamontagne AM, Lacroix G, Ouardani M, Deschamps J, Simard G, Simard C (2009): Genetic diversity of porcine norovirus and sapovirus: Canada, 2005–2007. Archives of Virology 154, 581–593.

Martella V, Banyai K, Lorusso E, Bellacicco AL, Decaro N, Mari V, Saif L, Constantini V, De Grazia S, Pezzotti G, Lavazza A, Buonavoglia C (2008): Genetic heterogeneity of porcine enteric caliciviruses identified from diarrhoeic piglets. Virus Genes 36, 365–373.

Martinez MA, Alcala AC, Carruyo G, Botero L, Liprandi F, Ludert JE (2006): Molecular detection of porcine enteric caliciviruses in Venezuelan farms. Veterinary Microbiology 116, 77–84.

Mauroy A, Scipioni A, Mathijs E, Miry C, Ziant D, Thys C, Thiry E (2008): Norovires and sapoviruses in pigs in Belgium. Archives of Virology 153, 1927–1931.

Oliver SL, Asobayire E, Dastjerdi AM, Bridger JC (2006): Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury 1) endorses a new genus in the family Caliciviridae. Virology 350, 240–250.

Reuter G, Biro H, Szucs G (2007): Enteric caliciviruses in domestic pigs in Hungary. Archives of Virology 152, 611–614.

Reuter G, Zimsek-Mijovski J, Poljsak-Prijatelj M, Di Bartolo I, Ruggeri FM, Kantala T, Maunula L, Kiss I, Kecskemeti S, Halaihel N, Buesa J, Johnsen C, Hjulsager CK, Larsen LE, Koopmans M, Bottiger B (2010): Incidence, diversity and molecular epidemiology of sapoviruses in swine across Europe. Journal of Clinical Microbiology 48, 363–368.

Saif LJ, Bohl EH, Theil KW, Cross RF, House JA (1980): Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. Journal of Clinical Microbiology 12, 105–111.

Shuffenecker I, Ando T, Thouvenot D, Lina B, Aymard M (2001): Genetic classification of "Sapporo-like viruses". Archives of Virology 146, 2115–2132.

Tamura K, Dudley J, Nei M, Kumar S (2007): MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Wang QH, Han MG, Funk JA, Bowman G, Janies DA, Saif LJ (2005): Genetic diversity and recombination of porcine sapoviruses. Journal of Clinical Microbiology 43, 5963–5972.

Wang QH, Souza M, Funk JA, Zhang W, Saif LJ (2006): Prevalence of noroviruses and sapoviruses in swine of various ages determined by reverse transcription-PCR and microwell hybridization assay. Journal of Clinical Microbiology 44, 2057–2062.

Wang QH, Constantini V, Saif LJ (2007): Porcine enteric caliciviruses: Genetic and antigenic relatedness to hu-

man caliciviruses, diagnosis and epidemiology. Vaccine 25, 5453–5466.

Yang S, Zhang W, Shen Q, Huang F, Wang Y, Zhu J, Cui L, Yang Z, Hua X (2009): Molecular characterization and phylogenetic analysis of the complete genome of a porcine sapovirus from Chinese swine. Virology Journal 6, 216–226. http://www.virologyj.com/content/6/1/216

Yin Y, Tohya Y, Ogawa Y, Numazawa D, Kato K, Akashi H (2006): Genetic analysis of calicivirus genomes detected in intestinal content of piglets in Japan. Archives of Virology 151, 1749–1759.

Zhang W, Shen Q, Hua X, Cui L, Junfeng L, Yang S (2008): The first chinese porcine sapovirus strain that contributed to an outbreak of gastroenteritis in piglets. Journal of Virology 82, 8239–8240.

Received: 2010–12–17
Accepted after corrections: 2011–08–13

Corresponding Author:

Jana Prodelalova, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 101, Fax +420 541 211 229, E-mail: prodelalova@vri.cz