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# Incidence, Diversity, and Molecular Epidemiology of Sapoviruses in Swine across Europe[▽]

Gábor Reuter,[1]* Janet Zimšek-Mijovski,[2] Mateja Poljšak-Prijatelj,[2] Ilaria Di Bartolo,[3]
Franco Maria Ruggeri,[3] Tuija Kantala,[4] Leena Maunula,[4] István Kiss,[5]
Sándor Kecskeméti,[5] Nabil Halaihel,[6] Javier Buesa,[7] Christina Johnsen,[8]
Charlotte K. Hjulsager,[9] Lars E. Larsen,[9] Marion Koopmans,[10]
and Blenda Böttiger[8]

*ÁNTSZ Regional Institute of State Public Health Service, Pécs, Hungary[1]; University of Ljubljana, Ljubljana, Slovenia[2];
Istituto Superiore di Sanità, Rome, Italy[3]; University of Helsinki, Helsinki, Finland[4]; Central Agricultural Office,
Veterinary Diagnostic Directorate, Debrecen, Hungary[5]; University of Zaragoza, Zaragoza, Spain[6];
University of Valencia, Valencia, Spain[7]; Statens Serum Institut, Copenhagen, Denmark[8];
Technical University of Denmark, National Veterinary Institute, Copenhagen,
Denmark[9]; and National Institute for Public Health and the Environment,
Bilthoven, The Netherlands[10]*

**Porcine sapovirus is an enteric calicivirus in domestic pigs that belongs to the family *Caliciviridae*. Some porcine sapoviruses are genetically related to human caliciviruses, which has raised public health concerns over animal reservoirs and the potential cross-species transmission of sapoviruses. We report on the incidence, genetic diversity, and molecular epidemiology of sapoviruses detected in domestic pigs in a comprehensive study conducted in six European countries (Denmark, Finland, Hungary, Italy, Slovenia, and Spain) between 2004 and 2007. A total of 1,050 swine fecal samples from 88 pig farms were collected and tested by reverse transcription-PCR for sapoviruses, and positive findings were confirmed by sequencing. Sapoviruses were detected in 80 (7.6%) samples collected on 39 (44.3%) farms and in every country. The highest prevalence was seen among piglets aged 2 to 8 weeks, and there was no significant difference in the proportion of sapovirus-positive findings for healthy animals and animals with diarrhea in Spain and Denmark (the only countries where both healthy animals and animals with diarrhea were tested). On the basis of the sequence of the RNA polymerase region, highly heterogeneous populations of viruses representing six different genogroups (genogroups III, VI, VII, and VIII, including potential new genogroups IX and X) were identified, with a predominance of genogroup GIII (50.6%). Genogroup VIII, found in five of the six countries, had the highest degree of homology (up to 66% at the amino acid level) to human sapovirus strains. Sapoviruses are commonly circulating and endemic agents in swine herds throughout Europe. Highly heterogeneous and potential new genogroups of sapoviruses were found in pigs; however, no "human-like" sapoviruses were detected.**

Caliciviruses (family *Caliciviridae*) are small, nonenveloped viruses with single-stranded, positive-sense genomic RNA which are classified at present into five genera: *Vesivirus*, *Lagovirus*, *Norovirus*, *Nebovirus*, and *Sapovirus* (18; http://talk.ictvonline.org/media/p/1203.aspx). The viruses within one calicivirus genus are phylogenetically related and have the same genomic organization (5). The sapovirus genome is 7.3 to 7.5 kb in length and contains two main open reading frames (ORFs). Sapoviruses are important enteric pathogens that can cause diarrhea in humans, pigs, and minks (5, 6, 15). On the basis of phylogenetic clustering of the capsid gene (ORF1) and protein sequences, sapoviruses have been classified into five distinct genogroups (genogroups GI to GV) (3). Human sapoviruses belong to genogroups GI, GII, GIV, and GV, whereas porcine sapovirus belongs to genogroup GIII. Recently, new porcine sapovirus genogroups (genogroups GVI, GVII, and GVIII)

were proposed (14, 25, 27). Recombinant sapoviruses have also been described in both human and swine hosts (8, 24). Each genogroup can be further divided into genetically diverse genotypes.

Porcine sapovirus (historically called porcine enteric calicivirus [PEC]) was first identified by electron microscopy in the United States in 1980 (21) and was genetically characterized as a sapovirus in 1999 (5, 24). Porcine sapoviruses have been reported in only a few additional countries: The Netherlands (R. van der Heide et al.; available only in the GenBank database), South Korea (11), Venezuela (16), and Hungary (20) and recently in Italy (15), Brazil (1), and Canada (13). The porcine sapovirus strains Cowden and LL14/02/US, representatives of sapovirus genogroup GIII, were detected in diarrheic piglets and have been shown to induce enteric diseases and lesions in experimentally infected pigs (4, 7).

Public health concerns over potential cross-species transmission and animal reservoirs for sapoviruses have been raised. Here we report on the incidence, genetic diversity, and molecular epidemiology of sapoviruses detected in domestic pigs in six European countries.

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* Corresponding author. Mailing address: Regional Laboratory of Virology, ÁNTSZ Regional Institute of State Public Health Service, Szabadság út 7, Pécs H-7623, Hungary. Phone: 36 06 (72) 514 979. Fax: 36 06 (72) 514 949. E-mail: reuter.gabor@ddr.antsz.hu.

TABLE 1. Incidence of sapoviruses in fecal samples collected from domestic pigs from 88 randomly selected swine farms in six European countries between January 2004 and December 2007[a]

| Country | Sampling period | Age group | Names of primers for RNA polymerase region (reference) | No. (%) of farms | | No. (%) of fecal samples | | | Prevalence (%) of sapovirus in feces by age group | No. of sapovirus sequence(s) confirmed by sequencing (genogroup distribution) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Tested | Positive | Tested | Positive[b] | Positive by age group | | |
| Denmark | July and Dec. 2007 | 2–8 wk | p290 and p289 (10), 290D and p289D (3) | 31 | 21 (68) | 57 | 25 (43.8) | 20 | 66.7 | 35, including double infections in 7 samples (III, VI, VII, VIII, IX, X) |
| | | 9–12 wk | SR80 and JV33 (23) | 31 | 21 (68) | 57 | 18 (31.6) | 7 | 50 | |
| | | 13–22 wk | | | | | 28[c] (49.1) | 1 | 7.7 | |
| Finland | April and June 2007 | 2–3 mo | p290 and p289 | 11 | 2 (18) | 52 | 5 (9.6) | 3 | 17.6 | 5 (III, VIII, IX) |
| | | 3–4 mo | | | | | | 0 | 0 | |
| | | 5–6 mo | | | | | | 2 | 7.7 | |
| Hungary | March 2005, Feb. and March 2007 | <10 days | p290 and p289 | 9 | 3 (33) | 436 | 7 (1.6) | 4 | 3.3 | 7 (III, VIII) |
| | | 3–4 wk | | | | | | 0 | 0 | |
| | | 3 mo | | | | | | 3 | 2.8 | |
| | | 6 mo | | | | | | 0 | 0 | |
| Italy | Jan.–June 2006, Nov.–Dec. 2007 | 1–3 mo | p290 and p289 | 15 | 5 (33) | 201 | 14 (6.9) | 0 | 0 | 6 (VIII, IX, X) |
| | | 3–4 mo | | | | | | 13 | 27.6 | |
| | | 6–10 mo | | | | | | 0 | 0 | |
| | | 11–12 mo | | | | | | 1 | 7.1 | |
| | | 1 year | | | | | | 0 | 0 | |
| Slovenia | Jan.–April 2004, Oct. 2004 and Jan. 2005, May 2005, Dec. 2005 | <3 wk | SR80 and JV33 | 8 | 7 (87.5) | 83 | 36 (43.4) | 10 | 35.7 | 33 (III, VII, VIII, X) |
| | | 4–10 wk | | | | | | 18 | 66.7 | |
| | | >11 wk | | | | | | 8 | 28.5 | |
| Spain | 2005 and 2006 | <4 wk | p290 and p289 | 14 | 1 (7) | 221 | 27 (12.2) | 6 | 13.3 | 1 (III) |
| | | 4–8 wk | | | | | | 2 | 10.5 | |
| | | 9–16 wk | | | | | | 10 | 12.0 | |
| | | 17–24 wk | | | | | | 8 | 12.7 | |
| | | >25 wk | | | | | | 1 | 9.1 | |
| Total | Jan. 2004–Dec. 2007 | | p290 and p289, SR80 and JV33 | 88 | 39 (44.3) | 1,050 | 117 (11.1) | | | 87 in 80 animals (7.6) |

[a] Information on sampling periods, age groups, primers, the number of tested and positive farms, and the number of animals per country are shown. Samples from healthy animals were collected in Finland, Hungary, Italy, and Slovenia, whereas samples from both healthy and diarrheic animals were collected in Denmark and Spain.
[b] Bands with the expected size were detected.
[c] Total number of sapovirus-positive specimens.

## MATERIALS AND METHODS

**Stool samples.** A total of 1,050 fecal samples from domestic pigs (*Sus scrofa domestica*) collected at 88 swine farms in six European countries (Denmark, Finland, Hungary, Italy, Slovenia, and Spain) from January 2004 to December 2007 were tested for sapoviruses (Table 1). Detailed information on the sampling periods, age groups, the number of farms tested, and the number of animals per country is summarized in Table 1. The farms and sampled animals were randomly selected. Clinically healthy animals, i.e., animals with no signs of gastroenteritis, were tested in Finland, Hungary, Italy, and Slovenia; and both healthy and diarrheic animals were tested in Denmark and Spain. Fresh fecal samples were placed into sterile containers and were stored frozen at $-20°C$ until they were tested.

**RNA extraction and RT-PCR.** The RNA was extracted from fecal suspensions according to the standard RNA extraction methods used by each laboratory. Reverse transcription-PCR (RT-PCR) was also performed by the participating laboratories with the generic calicivirus-specific primer pair (primers p290 and p289) (10), a degenerated version of these primers (primers p290D and p289D) (3), or a sapovirus-specific primer pair (primers SR80 and JV33) (23). All primers were designed to amplify the RNA-dependent RNA polymerase region (Table 1). The PCR products obtained with primer pairs p289 and p290, p290D and p289D, and SR80 and JV33 were 331 nucleotides (nt; 95 amino acids [aa]), 331 nt, and 320 nt, respectively, for sapovirus.

**Sequence and phylogenetic analysis.** The PCR products were sequenced directly in both directions by using the PCR primers. The sequences were analyzed by using the GeneDoc (version 2.6) program (19). A dendrogram was constructed by the unweighted-pair group method using average linkages (UPGMA) with the MEGA (version 3.1) program (12). Maintaining the continuity of the

current nomenclature for sapovirus genogrouping, we used the previously proposed taxonomic names (25).

**Statistics.** To test the differences in the proportions of double and single infections in animals with diarrhea, Fischer's exact test was used, and a single-tailed $P$ value of $<0.5$ was considered a significant difference. The association between the incidence of sapovirus infection and diarrhea was tested by chi-square analysis.

## RESULTS

A total of 117 (11.1%) samples from 1,050 pigs yielded a PCR fragment with the expected size for sapoviruses following RT-PCR and gel electrophoresis. Two-thirds ($n = 80$) of these (68% of gel electrophoresis-positive samples, 7.6% of samples tested) were confirmed to be sapovirus specific by sequence analysis of the PCR products (Table 1). The small amount of DNA obtained from some samples did not allow sequence reactions to be performed for all samples. In addition, bacterial and host DNA sequences with the expected size (false-positive
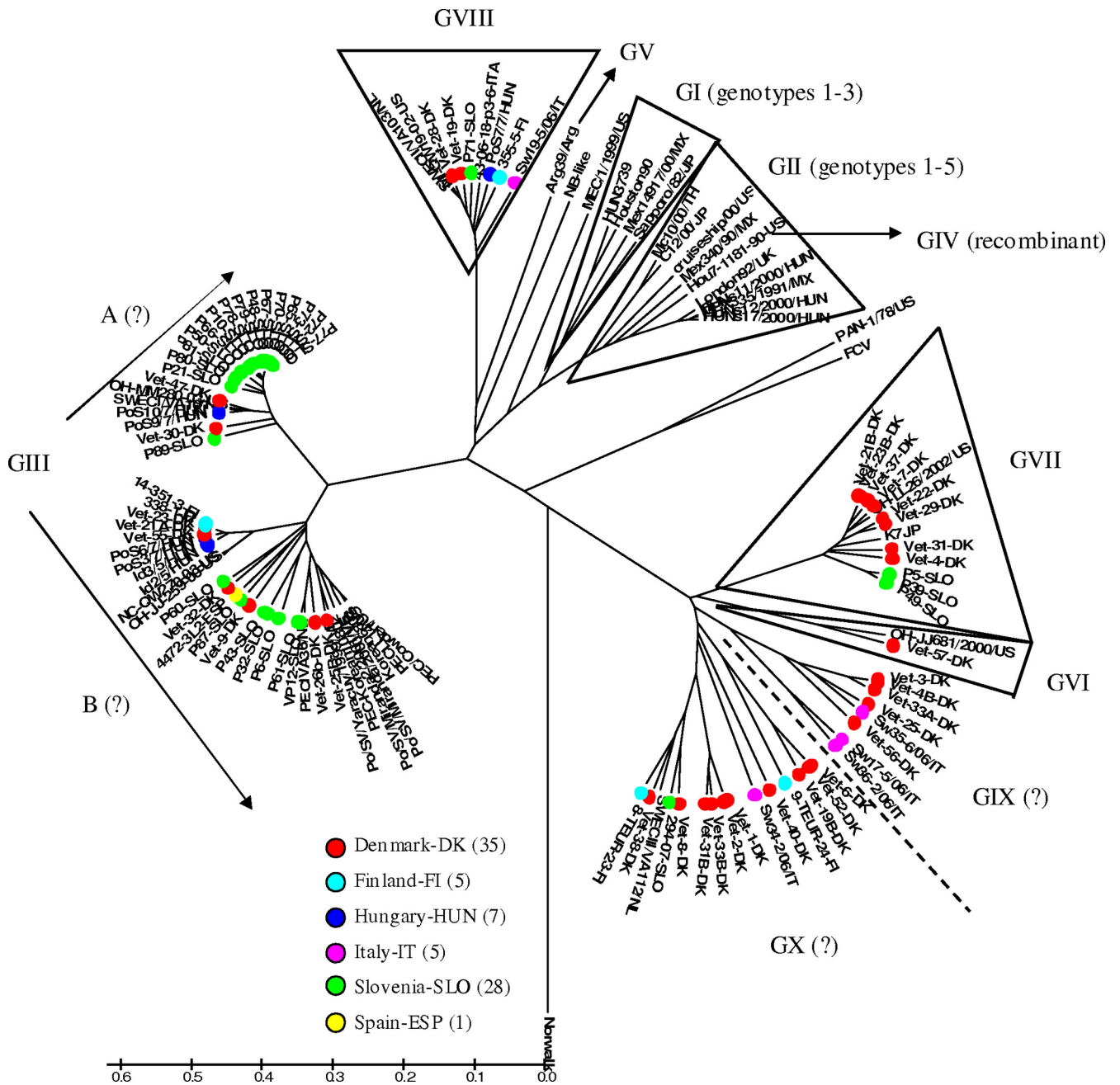
FIG. 1. Phylogenetic analysis of sapoviruses, including porcine sapoviruses, detected in this study in Europe on the basis of the 286-nt RNA-dependent RNA polymerase region of caliciviruses. A dendrogram was constructed by the UPGMA method with the MEGA (version 3.1) program. The country origin of the strains is indicated by color coding (Denmark, red; Finland, light blue; Hungary, blue; Italy, purple; Slovenia, green; Spain, yellow). To maintain the continuity of the current nomenclature for sapovirus genogrouping, we used the previously proposed taxonomic names as a basis (14, 25). Sapoviruses in genogroups GI, GII, GIV, and GV are detected in humans. Sapoviruses of swine origin belong to genogroups GIII, GVI, GVII, GVIII, GIX, and GX. The following reference strains were obtained from the GenBank database (the GenBank accession numbers are provided in parentheses): PEC/Cowden/US (AF182760), PECLL14/US (AY425671), PECIVA36/NL (AY615805), Korean6802 (AY289186), London/92/UK (U95645), HUNs11/2000/HUN (AF488717), HUNs12/2000/HUN (AF488718), HUNs17/2000/HUN (AF488720), HUN3739/2008/HUN (FJ844411), Sapporo/82/JP (U77903), Houston/90/US (U95644), Po/SV/Yaracuy/1999/VE (AY633966), Po/SV/Miranda/2000/VE (AY633963), Po/SV/Miranda2/2001/VE (AY633965), PEC-Korean10802 (AY289188), OH-JJ259-00-US (AY826423), NC-QW270-03-US (AY826426), SWECI/VA10/NL (AY615807), OH-MM280-03-US (AY823308), MEX335/1991/MX (AY157869), PAN-1/78/US (AF091736), 43-06-18-p-3-6-ITA (AB221477), SWECIII/VA112/NL (AY615814), OH-LL26/2002/US (AY974195), OH-JJ681/2000/US (AY974192), Norwalk (M87661), Mex14917/00/MX (AF435810), Mex340/90/MX (AF435809), cruiseship/00/US (AY157863), Mc10/00/TH (AY237420), C12/00/JP (AY603425), Hou7-1181-90-US (AF435814), Arg39/Arg (AF405715), MEC/1/1999/US (AF338404), FCV (M86379), NB-like (AY082891), K7JP (AB221130), SWECII/VA103/NL (AY615811), and MI-QW19-02-US (AY826424).

results) were also amplified with primers p289 and p290. Double infections were detected in seven pigs, increasing the total number of sapovirus sequences available for study to 87. All these sequences contained the conserved amino acid motif GLPSG of the calicivirus RNA-dependent RNA polymerase. The incidence of the sapovirus-positive samples ranged from 1.6% (Hungary) to 49.1% (Denmark). Samples from 39 (44.3%) of the 88 farms were sapovirus PCR positive, with the proportions of positive farms ranging from 7% in Spain to 87.5% in Slovenia.

The number of the fecal samples positive by age group and the incidence of sapovirus-positive animals by age group are summarized in Table 1. The sapovirus incidence ranged from 0% to 66.7% within each age group (Table 1). In Finland, Hungary, Italy, and Slovenia all samples—both sapovirus positive and sapovirus negative—were obtained from clinically healthy pigs, while in Denmark and Spain, samples from both pigs with diarrhea and pigs without diarrhea were tested. In Denmark, sapoviruses were detected in 14/30 (47%) and 14/27 (52%) samples from animals with and without diarrhea, respectively. The corresponding proportions in Spain were 13/113 (12%) and 14/108 (13%), respectively. No significant differences in the proportion of sapovirus-positive samples among pigs with or without diarrhea were seen.

A total of 81 of 87 sapovirus polymerase sequences could be used for further analysis: 35 (43.2%) sequences from Denmark, 5 (6.2%) from Finland, 7 (8.6%) from Hungary, 5 (6.2%) from Italy, 28 (34.6%) from Slovenia, and 1 (1.2%) from Spain. The phylogenetic analysis divided the sapovirus sequences into six different genogroups (Fig. 1). Most sapoviruses belonged to genogroup GIII ($n = 41$; 50.6%), but the presence of genogroups GVI ($n = 1$; 1.2%), GVII ($n = 11$; 13.6%), and GVIII ($n = 6$; 7.4%) was also confirmed. Furthermore, strains belonging to two potentially new sapovirus genogroups, tentatively classified as genogroups GIX (N = 8; 9.9%) and GX ($n = 14$; 17.3%), were also detected (Fig. 1). By phylogenetic analysis, two lineages of genogroup GIII strains (lineages A and B) were found (Fig. 1). All six genogroups were identified in Denmark. Three genogroups (genogroups GIII, GVIII, and GX) were found in at least four of the countries studied, whereas genogroups GVI, GVII, and GIX were detected in only one or two countries.

For comparison of the sapovirus strains found in pigs and in humans, we compared the nucleotide and amino acid sequence identities of the RNA polymerase gene between the study strains from pigs and the prototype sapovirus genogroup strains from humans (Table 2). The highest levels of sequence identity (up to 66% at the amino acid level) were found between genogroup GVIII and all human sapovirus genogroups. Genogroup GVIII strains in pigs are related to each other with 63 to 87% nucleotide sequence identity and 76 to 98% amino acid identity. The lowest level of sequence identity was seen between genogroup IX strains and human sapoviruses. Between the two genogroup GIII lineages, GIIIA and GIIIB, the nucleotide and amino acid identities were 68 to 84% and 75 to 91%, respectively.

The distributions of sapovirus genogroups over time (data not shown) and by age group (Fig. 2) were further analyzed. In the first 2 years of the study, genogroup GIII strains were the most commonly detected (86%), but these data were from

TABLE 2. Nucleotide and amino acid sequence identities based on the RNA polymerase region between the study strains detected in pigs and the sapovirus genogroup prototype of human origin[a]

| Genogroup and identity parameter[b] | % Identity with the following human sapoviruses: | | | |
|---|---|---|---|---|
| | GI Sapporo/82/JP (U77903)[c] | GII London/92/UK (U95645) | GIV Hou-7-1181-90-US (AF435814) | GV Arg39/Arg (AF405715) |
| GIIIA ($n^d = 20$) | | | | |
| Nucleotide | 51–55 | 52–57 | 54–58 | 52–56 |
| Amino acid | 51–55 | 50–54 | 50–54 | 49–52 |
| GIIIB ($n = 21$) | | | | |
| Nucleotide | 50–59 | 54–59 | 55–60 | 53–55 |
| Amino acid | 52–62 | 50–60 | 53–57 | 46–49 |
| GVI ($n = 1$) | | | | |
| Nucleotide | 49 | 50 | 52 | 45 |
| Amino acid | 41 | 42 | 43 | 32 |
| GVII ($n = 11$) | | | | |
| Nucleotide | 42–46 | 44–48 | 44–47 | 41–45 |
| Amino acid | 36–38 | 35–41 | 36–42 | 33–36 |
| GVIII ($n = 6$) | | | | |
| Nucleotide | 50–**62** | 53–**64** | 54–**65** | 54–**60** |
| Amino acid | 52–**63** | 53–**65** | 55–**66** | 52–**55** |
| GIX ($n = 8$) | | | | |
| Nucleotide | 40–42 | 39–46 | 40–47 | 41–44 |
| Amino acid | 33–36 | 33–37 | 36–41 | 29–30 |
| GX ($n = 14$) | | | | |
| Nucleotide | 47–52 | 44–52 | 44–56 | 41–48 |
| Amino acid | 37–41 | 37–43 | 38–43 | 30–35 |

[a] Range of nucleotide and amino acid sequence identities for all strains sequenced on the basis of the sequence of the RNA polymerase region between the study strains detected in pigs (rows) and the sapovirus genogroup prototype strains of human origin (columns). Boldface numbers indicate the highest levels of nucleotide and amino acid sequence identity.

[b] All study strains ($n = 81$) in each genogroup, as separated in Fig. 1, were included for sequence analysis. For homology comparison, the results for the lowest and the highest nucleotide and amino acid strain identities are indicated in each cell as a percentage range for each genogroup.

[c] GenBank accession numbers are given in parentheses.

[d] $n$ = number of sequences analyzed.

Hungary and Slovenia only, whereas strains belonging to six genogroups (genogroups GIIIA and GIIIB, GVI, GVII, GVIII, GIX, and GX) were found in five countries in the last 2 years (2006 and 2007). Clustering of the genotypes by age showed that strains of dominant genogroup GIII were primarily detected in young pigs (78%, 49%, and 0% of strains from pigs in age groups less than 1 month, 1 to 3 months, and more than 3 months, respectively). Genogroup GVI and GVI strains were identified only in pigs ages 1 to 3 months.

Double infections with two sapoviruses from different genogroups were identified in seven animals under 12 weeks of age (strains Vet4 and Vet4B, GVII and GIX, respectively; strains VET19 and Vet19B, GVIII and GX, respectively; strains Vet21A and Vet21B, GIII and GVII, respectively; strains Vet23 and Vet23B, GIII and GVII, respectively; strains Vet25 and Vet25B, GIX and GIII, respectively; strains Vet31 and Vet31B, GVII and GX, respectively; strains Vet33A and Vet33B, GIX and GX, respectively). The different virus types were detected by the use of different diagnostic primer pairs. Six of the 7 animals with double
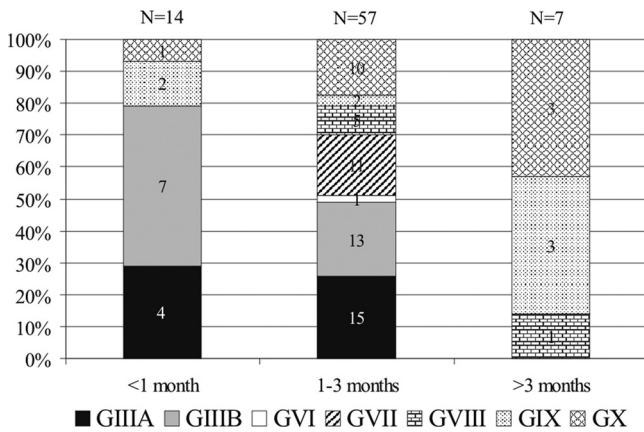
FIG. 2. Prevalence of sapovirus genogroups by age group on the basis of the findings for 78 sapovirus strains. The *y* axis represents the prevalence of genogroups (in percent). The count numbers in each bar slice represent the sample size for the genogroup. The total sample size for each age group is provided at the top of the bars.

infections had diarrhea, which was a significantly greater rate than that seen in the 21 animals infected with a single sapovirus, and only 8 animals in Denmark had diarrhea. Furthermore, identical sapovirus sequences were detected within the same herds in six animal pairs in Denmark (*n* = 4) and Hungary (*n* = 2).

## DISCUSSION

Only a few studies have been done to investigate the molecular epidemiology of sapoviruses in swine, since the discovery of porcine sapovirus in 1980 (21). In this report, we describe a large, integrated analysis related to the incidence, genetic diversity, and molecular epidemiology of porcine sapoviruses in six European countries.

On the basis of our data, approximately 8% of the animals tested were infected with sapoviruses and were shedding the virus in feces, and sapoviruses were present on more than 40% of the selected swine farms in Europe, indicating that sapoviruses are circulating as endemic agents in swine herds. However, the variation in the incidence of sapoviruses in individual pigs, as well as among farms, in the participating countries was considerable (data not shown). This could be a reflection of the different abilities to obtain samples in each of the participating countries. The overall sapovirus positivity rate in our study was lower than that in some other studies, which showed a prevalence of sapoviruses of at least 62% in 621 pigs from 7 different farms in the United States (26), 30% in 113 piglets from 34 farms in Brazil (1), and 23% in 53 postweaning pigs from 5 farms in South Korea (28); but considerable ranges have been published as well (25). The primers used and the selection of samples tested by the age and the state of health of the pigs also differed in those studies, making direct comparisons of the results of the different studies difficult, but it can be concluded that sapovirus infection in pigs is common. However, our data confirm that pigs are infected with sapoviruses early in life, a finding that is in line with the findings of studies performed in Venezuela, Brazil, South Korea, and the United States (1, 9, 16, 26). As also described in the papers from Venezuela, Brazil, and Belgium (17), we found sapoviruses in nearly equal

numbers in pigs with and without diarrhea as well. However, diarrhea was significantly associated with coinfection with different sapovirus strains. This does not necessarily imply that the double infection itself causes diarrhea; but it might reflect lower hygienic standards in the pens of these pigs, and other pathogens could therefore also be present.

It should be also noted that double infections were detected only by the use of different primer sets, indicating that there is a diagnostic gap in sapovirus detection. Against this background, extremely diverse groups of sapoviruses were detected among the swine evaluated in this study. The porcine strains clustered into a total of four known genogroups and two previously unknown potential new genogroups (genogroups GIX and GX). On the other hand, genotype III strains were detected the most frequently. Their predominance over a limited time period and in young animals suggests that the pigs were sampled during a GIII epidemic, but coordinated studies across regions or countries are needed to better understand the dynamics of the genogroup(s) changes and the possibilities of the drift and international spread of sapoviruses among pigs.

It was recently shown that in some cases there was no sharp demarcation of the host species for some calicivirus infections; some caliciviruses may have zoonotic potential, and animals such as domestic pigs may act as a reservoir for noroviruses or vesiviruses (2, 22, 24). By phylogenetic analysis, a higher degree of genetic diversity was seen among porcine sapoviruses than among the known human sapoviruses, indicating that the coevolution of sapoviruses in swine was longer. One sapovirus genogroup, genogroup GVIII, occupies a special position among sapoviruses. Genogroup GVIII is genetically more closely related to human sapoviruses (especially those of genogroups GV and GI) than to other sapovirus genogroups in swine (15, 25). A previous study suggested that this strain circulates infrequently and has low numbers of virus copies in the feces of pigs; therefore, it probably originates from a nonporcine host (25). Interestingly, in our study, these unique sapovirus strains were found in five of the six countries and constituted 7.4% of the sapovirus-positive samples. This indicates the circulation of sapoviruses throughout Europe and the relatively frequent incidence of this strain in pigs. Until now, genogroup GVIII or GVIII-like strains have not been detected in humans. Among the known sapoviruses of swine, however, the genogroup GVIII strains have the closest evolutionary crossing-point between human and porcine sapoviruses, as shown by sequence and phylogenetic analyses.

On the basis of the findings of this comprehensive study, no evidence for zoonotic sapovirus could be found in pigs, but a surprisingly diverse profile of sapoviruses was observed in European swine herds. While the clinical relevance remains to be determined, it reflects how little we know about the viral pathogens present in these food animals. Knowledge about their potential for spread, including their contribution to recombination events, as has been described for sapoviruses belonging to different genogroups, is important to obtaining an understanding of the role of swine as a potential sapovirus reservoir.

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