



Published in final edited form as:

Arch Virol. 2014 June ; 159(6): 1313–1319. doi:10.1007/s00705-013-1942-x.

Molecular detection and genetic characterization of kobuviruses and astroviruses in asymptomatic local pigs in East Africa

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Conflict of interest None.

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Abstract

In this study, swine fecal specimens (n = 251) collected from nursing and weaned piglets raised under smallholder production systems were screened for the presence of kobuviruses by RT-PCR. Porcine kobuviruses were detected in 13.1 % (33/251) of the samples. We demonstrated that porcine kobuvirus infections exist in indigenous pigs in Kenya and Uganda and that the prevalence was higher in young piglets than older pigs: nursing piglets (15 %), post-weaning (3-month-old) pigs (17 %), 4-month-old pigs (10 %). Genetic analysis of the partial RNA-dependent RNA polymerase (RdRp) region (690 nt) revealed that kobuviruses circulating in East Africa are diverse, sharing nucleotide sequence identities ranging from 89.7 to 99.1 % and 88 to 92.3 % among them and with known porcine kobuviruses, respectively. The nucleotide sequence identities between our kobuvirus strains and those of human, bovine and canine kobuviruses were 69.4-70.7 %, 73.1-74.4 % and 67-70.7 %, respectively. Additionally, upon sequencing selected samples that showed consistent 720-bp RT-PCR bands while using the same primer set, we detected porcine astroviruses in our samples belonging to type 2 and type 3 mamastroviruses. To our knowledge, this study reports the first detection and molecular analysis of both porcine kobuviruses and astroviruses in an African region. Further studies are required to determine the role of these viruses in gastrointestinal infections of pigs in this region and to determine the genetic diversity of the circulating strains to develop accurate diagnostic tools and implement appropriate control strategies.

Introduction

Small-scale pig production (free range or small backyard herds) constitutes >70 % of the total pig farms in East Africa. Pigs in this region often live in close proximity to humans, causing public health concerns and a need for increasing health standards. East Africa is also known for the large population of wild (bush) pigs and warthogs that are in close contact with domestic pigs, creating favorable conditions for intermixing and spread of viral strains. Emerging viruses represent a threat to human and food animal health, as evidenced by sporadic outbreaks of influenza and coronavirus infection. Hence, knowledge about the diversity of viruses present in reservoir animals can lead to a better understanding of the origin of emerging pathogens. Kobuviruses, which are members of the genus *Kobuvirus*, family *Picornaviridae*, are small, non-enveloped viruses with single-stranded, positive-sense genomic RNA. The genus *Kobuvirus* includes three officially recognized species: *Aichivirus A*, *Aichivirus B* (bovine kobuvirus), and *Aichivirus C* (porcine kobuvirus) [1]. The kobuvirus genome is approximately 8.3 kb long and is organized into three structural (VP0, VP3 and VP1) and seven non-structural (2A-2C and 3A-3D) regions with a leader protein (L). The 3D gene region encodes a viral RNA-dependent RNA polymerase (RdRp) and represents a region that is conserved among kobuviruses [2]. Nucleotide and amino acid sequence identities of the 3D RdRp coding region among porcine kobuvirus, bovine kobuvirus and Aichi virus vary from 74.0 % to 81.0 % [3]. Aichi virus was first detected in Japan in 1989

from a human patient with acute gastroenteritis [4]. Since then, Aichi virus has been detected in Asia, Europe, South America and Tunisia [5-8]. Bovine kobuvirus was first recognized in 2003 as a cytopathic contaminant in cell culture medium derived from bovine serum in Japan. Later, it was found in fecal samples of clinically healthy cattle [9]. Bovine kobuvirus has also been detected in domestic sheep in Hungary [10, 11]. Porcine kobuvirus (S-1-HUN/2007: EU787450) was first identified from fecal samples of domestic pigs in 2008 in Hungary [1]. Thereafter, porcine kobuvirus was reported in additional countries, including Asian countries [12, 13], the Netherlands and Brazil [14], and the USA [15]. The prevalence of kobuvirus infection in pigs ranges from 30 % to 99 %. This large variation could be a result of different ages within the populations evaluated, diarrheal status, regional differences, and other factors. Studies showing association of porcine kobuvirus infection with clinical disease are limited; however, a recent study in Korea found an association between porcine kobuvirus detection and diarrhea in pigs [13]. An association between kobuvirus infection and age has been reported in cattle and in pigs [16].

Astroviruses belong to the family *Astroviridae*, which consists of two genera: *Avastrovirus* and *Mamastrovirus*, whose members are associated with gastroenteritis in avian and mammalian hosts, respectively. Astroviruses are generally associated with either mild or severe enteric disease symptoms such as diarrhea and vomiting in a number of mammalian species [17]. Bridger in the UK and Saif et al. [18, 19] in the USA reported the first porcine astroviruses (PAstV) detected in pigs by electron microscopy. Later in 1990, PAstV was isolated in Japan [20]. Recently, Mor and co-workers also reported PAstV in pigs in the USA with a prevalence of 62 % [21]. To date, five PAstV types (PAstV1–PAstV5) have been identified from different countries, including the Czech Republic, Colombia, Canada, the USA, China and Hungary [22-27]. Among the five types, PAstV1, PAstV2 and PAstV3 were identified in fecal samples from healthy pigs.

Epidemiological information on the geographical distribution, incidence and genetic diversity of kobuviruses in African swine populations is not available. In this study, we report the first detection of porcine kobuviruses in samples collected from nursing and weaned pigs in East Africa and analyze the phylogenetic relationships between the East African porcine kobuvirus strains and known kobuvirus strains as well as other representative picornaviruses. This study also reports the first detection of porcine astrovirus in this region.

Accession numbers of nucleotide sequences strains described in this study were deposited in GenBank under the following accession numbers: kobuviruses, KF494340-KF494343 and KF597279; astroviruses, KF597280-KF597282.

Materials and methods

Sample collection

A total of 251 fecal samples were obtained from nursing and post-weaning piglets raised in small-scale farms (n = 1 to 25 pigs per farm) with different management systems in western Kenya (n = 140) and eastern Uganda (n = 111). The management system consisted of pigs being housed, housed and tethered, housed and free-range, tethered, tethered and free-range

and free-range. The pigs were mostly fed swill from hotels and leftover household food. The sample collection consisted of 41 samples from nursing pigs (less than 4 weeks of age), 90 samples from post-weaning pigs (12 weeks of age) and 120 samples from growers (16 weeks of age). All pigs appeared clinically healthy at the time of sampling. Fresh samples were collected from individual pigs, placed into a sterile specimen container and stored on dry ice before being transported to the International Livestock Research Institute (ILRI) laboratory in Busia, where the samples were stored at -70°C until use. The samples were then shipped to the BecA-ILRI laboratories in Nairobi for analysis.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Fecal samples were prepared as 10 % (v/v) fecal suspensions in minimum essential medium (MEM). The prepared sample suspensions were vortexed and centrifuged for 30 min at $1,800\times g$, 4°C . RNA was extracted from a 250- μl starting volume of centrifuged 10 % fecal suspensions using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The total RNA recovered was suspended in 40 μl of nuclease-free water and stored at -70°C until used. cDNA was synthesised using a Maxima First Strand cDNA Synthesis Kit (Thermo scientific[®]) according to manufacturer's instructions, and the cDNA was immediately used for amplification or stored at -20°C .

Previous studies using the calicivirus primer pair p110/p290 [28, 29] targeting the calicivirus RdRp gene (317 nt for norovirus and 329 nt for sapovirus) also amplified the 3D RdRp regions of porcine kobuvirus (~ 1100 nt) [15], which was confirmed by sequence analysis of five representative suspect samples in the present report. This same primer pair was used for the detection of porcine kobuviruses from East African pigs. RT-PCR was carried out using Accupower[®] PCR premix (Bioneer Co, S. Korea) according to manufacturer's instructions. Accupower[®] PCR premix is a ready-to-use lyophilized premix of dNTPs, Taq DNA Polymerase, reaction buffer, a tracking dye, and a stabilizer. Briefly, in each 20- μl reaction premix tube, 2 μl (5-50 ng) of cDNA template was mixed with 1 μl (10 pMol) each of forward and reverse primers, and 16 μl of nuclease-free water. PCR was performed at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 48°C of 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The temperature was then held at 4°C . The RT-PCR products were analyzed by 1.5 % agarose gel electrophoresis and visualized by ultraviolet illumination after staining with Gel Red[™] nucleic acid gel stain (Biotium, Hayward, CA). When bands were found in the region of 1,100 bp on the agarose gel, we tentatively considered the results kobuvirus positive. The samples with the correct band size were purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA USA) according to the manufacturer's instructions for sequencing. In other fecal samples, an approximately 720-nt-long weak, nonspecific PCR product was also observed. The nucleotide (nt) sequence of the PCR products of three selected samples from three different farms were determined by direct sequencing.

Sequencing and molecular analysis

To confirm the RT-PCR results and to obtain genetic information on virus diversity, the partial 3D RdRp amplicons of five kobuvirus-positive samples were sequenced directly using forward (p110) and reverse (p290) primers using BigDye Terminator cycle chemistry

(Applied Biosystems, Foster City, CA, USA). The resulting RdRP gene sequences were edited using Bioedit version 7.1.9 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and compared with sequences of kobuvirus reference strains in the GenBank database by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence similarity analysis was performed for the aligned nucleotide and amino acid sequences using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Phylogenetic analysis of the East African porcine kobuviruses with kobuvirus reference strains based on the nucleotide and amino acid sequence alignments was conducted using the neighbor-joining method supported with a bootstrap test of 1000 replicates in MEGA 5 software [30]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in units of base substitutions per site.

To analyze porcine kobuvirus infection in pigs of different ages with different management systems, the piglet fecal samples were divided according to the age of the animals into three groups: nursing, 1-month-old (n = 47), weaned, (3-month-old, n = 90) and growers (>4-month-old, n = 120). Statistical analysis was performed with SAS[®] to compare the proportions of kobuvirus-positive samples among the four districts, the two countries, the three age groups evaluated and the six different management systems. The analysis was performed using the chi-square (χ^2) test or Fisher's exact test. The confidence limit for the statistical tests was set at 95 % (P < 0.05).

Results and discussion

Incidence of kobuvirus infection

Kobuvirus RNA was detected in 13.1 % (33/251) of the pig fecal samples analyzed using primer pair p110/p290 [28, 29]. Kobuvirus was detected in both Kenya (14.9 %) and Uganda (15.5 %) in equal proportions, and in all four districts sampled (Table 1), an indication of a general circulation and endemicity of the virus on the tested farms. Among the age groups, younger piglets shed more virus than older pigs, and a higher incidence (15 of 90, 16.7 %) of kobuvirus was reported in post-weaning (3-month-old) piglets compared to nursing piglets (6 of 41, 14.6 %) and grower pigs (12 of 120, 10 %). This could possibly be due to an inefficient immune response, diminishing maternal immunity post-weaning, or other intrinsic age-related factors. Higher rates of infection in young piglets has also been reported in other studies [3, 13, 14]. Based on management systems in the study area, confinement of pigs presented a higher risk of kobuvirus infection, with housed pigs shedding more virus than free-ranging pigs (Table 1). This could be the result of viral accumulation in the pig houses or the places where pigs are tethered. Additionally, kobuvirus was more prevalent in the farms with larger herd size (>10 pigs, 20 %) than farms with small herds (<10 pigs, 12.6 %).

The incidence of porcine kobuvirus infection reported in this study (13.1 %) was lower than that reported in other countries, which ranged between 30.1 % in China and 99 % in Thailand [3, 31-33]. A recent study in Korea detected porcine kobuvirus in 84.5 % of diarrheic pigs and 19.3 % of healthy pigs [13]. Similarly, a study in Japan using samples from healthy pigs reported 45.4 % prevalence of porcine kobuvirus [12]. This difference

may be attributable to variations in the samples due to factors like sampling time, sampling place (distribution of sampling farms), sample size, age of pigs, and clinical background of the tested animal population (diarrhea or clinically healthy). The association of this agent with enteric diseases in pigs in the study area remains unclear, since we only analyzed samples from asymptomatic pigs, and no data were available from tests for kobuvirus in pigs with gastroenteritis from the same farms; however, some of the positive samples came from farms with a history of diarrhea. This study confirmed that infection with kobuvirus in asymptomatic pigs is common, as has been reported in other studies [14, 32, 34]. Further studies on risk factors for this infection and the association of this virus with gastroenteritis are necessary, especially in developing countries where a majority of pigs are raised in smallholder production systems with varied management.

Genetic and phylogenetic analysis of East African porcine kobuviruses

To gain more information on the genetic heterogeneity of porcine kobuvirus strains circulating in swine in the East African farms, five kobuvirus-positive samples were selected, and their partial 3D RdRP region was sequenced. Genetic analysis of the partial sequence revealed that the kobuviruses circulating in the East African region are more variable, sharing nucleotide sequence identity ranging from 89.7 to 99.1 % among them. At the nucleotide and amino acid level, they were 69.4–70.7 % and 70.7–71.9 % identical, respectively, to Aichi virus, 73.1–74.4 % and 78.8–80.5 % to bovine kobuvirus, 70.8–71.9 % and 78.5–79.5 % to kobuvirus in sheep, 67–70.7 % and 67.8–69.1 % to canine kobuviruses and 88–92.3 % and 90.4–97.5 % to other porcine kobuviruses (Table 2).

Phylogenetic analysis of partial 3D RdRp nucleotide and amino acid sequences of porcine kobuvirus strains detected in this study, together with published sequences of kobuvirus reference strains (porcine kobuviruses, aichi viruses and bovine kobuvirus) and representatives of other picornaviruses, revealed that three of the detected strains (Damaris-3-KF494340, Kuoba-3-KF494341 and K-1033-KF597279) grouped together and formed their own cluster, while one of the strains (K-118-KF494342) clustered with Chinese strains, and another strain (K-460-KF494343) clustered with Korean strains (Fig. 1). This indicates that porcine kobuviruses circulating in the East African region are genetically diverse. Damaris-3-KF494340, Kuoba-3-KF494341 and K-1033-KF597279 samples were collected in the same geographical location, indicating that porcine kobuviruses in the East African region may be geographically restricted; however, more strains from different parts of East Africa need to be sequenced to ascertain the geographical distribution of this virus. Genetic diversity among geographically separated porcine kobuviruses have been reported in other studies [13].

Phylogenetic analysis also confirmed that the five strains detected were more closely related to the kobuviruses, notably porcine kobuvirus, than to any other picornaviruses, and this was supported by the 100 % bootstrap value (Fig. 1). Evidence of a close genetic relationship or interspecies transmission among different viruses in the genus *Kobuvirus* has been documented. A sheep kobuvirus strain has high nucleotide sequence identity to bovine kobuvirus [10], and one porcine kobuvirus is more closely related to bovine kobuvirus than

to porcine strains [12]. However, in this study, we did not find kobuvirus strains that were similar to members of kobuviruses from species other than pigs.

Genetic and phylogenetic analysis of porcine astroviruses

In other fecal samples, an approximately 720-nt-long weak, nonspecific RT-PCR product was also observed. The nucleotide (nt) sequences of the RT-PCR products of three selected samples were determined by direct sequencing, and the result showed that one of the strains (K-268-KF597282) had 86.1 % nt sequence identity to porcine astrovirus type 2 in the RdRp gene. The other two strains (K-006-KF597280 and K-332-KF597281) had 96.7 % identity to porcine astrovirus type 3. This was confirmed by phylogenetic analysis, in which K-006-KF597280 and K-332-KF597281 strains clustered with astrovirus type 3 (PAstV-3-US-MO123-JX556691) detected in the USA, while the K-268-KF597282 strain clustered with astrovirus type 2 (PAstV-2/2007/HUN-GU562296) detected in Hungary (Fig. 2). Recent studies in the USA revealed high genetic heterogeneity of PAstV strains [35]. The importance of co-circulation of PAstVs and the role pigs may play in the transmission and recombination of this virus need further investigation.

In conclusion, the findings of this study demonstrate that porcine kobuviruses and astroviruses are present in the swine population in East Africa, and to our knowledge, this study reports the first detection of porcine kobuvirus and astrovirus in this African region and the first molecular analysis of the detected strains. The presence of these gastroenteritis-producing viruses in clinically healthy pigs represents a source of infection of pigs, and possibly to humans, and hence, further studies are required to determine their role in gastrointestinal infections of pigs in this region and to determine their genetic diversity in order to develop accurate diagnostic tools and implement appropriate control strategies.

Acknowledgments

We gratefully acknowledge support of this project to A.J.O. through the African Bioscience Challenge Fund (ABCF) Fellowship of Biosciences of East and Central Africa (BecA). We also thank VPH-Biotech East Africa Consortium and University of Nairobi for supporting A.J.O. We would like to acknowledge the co-operation and assistance of the Africa Swine Fever Project team (International Livestock Research Institute) for their support in sample collection. We thank smallholder pig farmers in Kenya and Uganda for allowing us to sample their pigs for this research study. The samples discussed in this study were sequenced by the Segolip unit of ILRI-BecA hub in Kenya.

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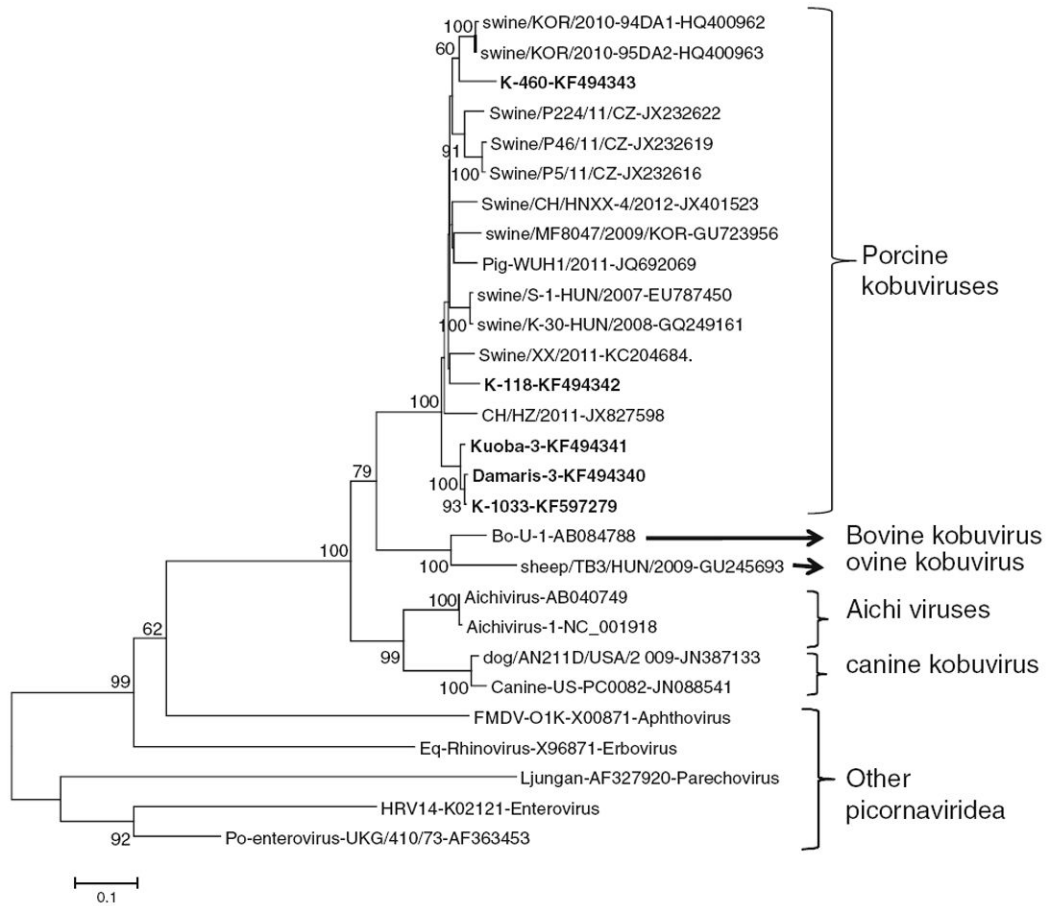
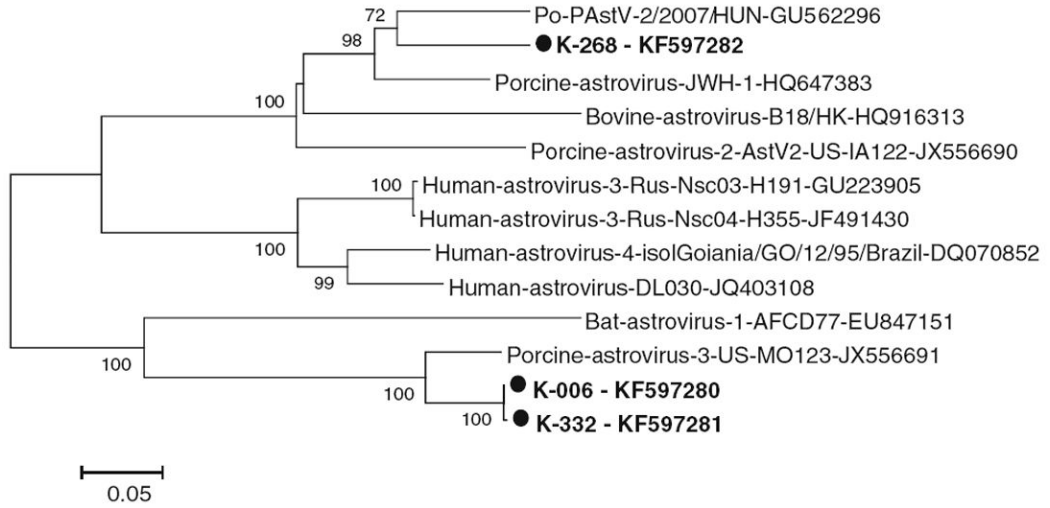


Fig. 1.

Phylogenetic dendrogram of the partial (690-bp) nucleotide sequence of the 3D RdRp gene of field kobuvirus strains (**bold**) compared with similar gene sequences for known kobuviruses and other picornaviruses in the GenBank database. The horizontal branch lengths are proportional to the genetic distance calculated by the neighbor-joining method. The evolutionary distances were computed using the Tamura-Nei method [36] and are in the units of base substitutions per site

**Fig. 2.**

Phylogenetic dendrogram of the partial nucleotide sequence of 3D RdRp gene (541 nt) of field astrovirus strains (**bold**) compared with similar gene sequences for known astroviruses in the GenBank database. The horizontal branch lengths are proportional to the genetic distance calculated by the neighbor-joining method. The evolutionary distances were computed using the Tamura 3-parameter method [36] and are in the units of base substitutions per site

Table 1

Porcine kobuvirus infection status in pigs of different ages, raised under different management systems in East Africa

Details	Groups	N	Positive (%)
	Overall	251	33(13.1)
Country	Kenya	139	18 (14.9)
	Uganda	112	15 (15.5)
Districts	Busia_Ke	69	9 (13.0)
	Teso	70	9 (12.9)
	Busia_Ug	64	10 (15.6)
	Tororo	48	5 (10.4)
Age group	Nursing-<1 mo	41	6 (14.6)
	Weaner-3 mo	90	15 (16.7)
	Grower-4 mo	120	12 (10.0)
Management systems	Free range	8	1 (12.5)
	Housed	6	2 (33.3)
	Housed & free range	17	3 (17.6)
	Housed & tethered	3	0 (0)
	Tethered	115	14 (12.2)
	Tethered & free range	102	13 (12.7)
No. of pigs per household	1 to 5	215	27 (12.6)
	6 to 10	16	2 (12.5)
	>10	20	4 (20.0)

Table 2

Nucleotide and amino acid sequence identities (%) of partial 3D RdRP genes of selected field strains compared with similar gene sequences for known kobuviruses and other picornaviruses in the GenBank database

Strain	Nucleotide sequence identity (%)				Amino acid sequence identity (%)				
	Damaris-3	K-118	Kuoba-3	K-460	Damaris-3	K-118	Kuoba-3	K-460	K-1033
Swine/S-1-HUN/2007-EU787450	92.0	91.9	92.0	89.9	92.3	97.5	96.5	97.2	95.3
Swine/K-30-HUN/2008-GQ249161	91.9	91.7	91.9	89.7	92.2	97.5	96.5	97.2	95.3
Swine/CH/HNXX-4/2012-JX401523	91.1	90.2	91.7	88.5	91.1	97.2	95.1	96.8	94.5
Swine/CH/HZ/2011-JX827598	90.9	90.2	91.4	88.0	90.9	97.2	96.5	96.8	95.3
swine/MF8047/2009/KOR-GU723956	91.2	91.2	91.2	88.9	90.9	96.8	95.8	96.5	95.3
Swine-WUH1/2011-CHN-JQ692069	91.1	92.0	91.4	89.4	91.1	97.2	96.8	96.8	95.3
Swine/XX/CHN/2011-KC204684	90.5	92.2	90.8	88.6	90.5	97.2	96.8	93.2	94.9
Swine/KOR/2010-94DA1-HQ400962	90.6	91.6	91.6	91.8	91.0	92.6	91.4	92.6	96.9
Swine/KOR/2010-95DA2-HQ400963	91.0	91.6	92.0	92.2	91.4	92.6	91.4	92.6	96.9
Swine/P224/11/CZ-JX232622	89.8	89.4	90.4	89.2	90.2	92.2	90.4	92.2	97.0
Swine/P46/11/CZ-JX232619	90.6	90.0	91.2	89.6	91.0	92.2	90.4	92.2	97.0
Swine/P5/11/CZ-JX232616	90.6	90.6	91.2	89.6	91.0	92.2	90.4	92.2	97.0
Aichivirus-AB040749	70.7	70.4	70.5	69.7	70.5	71.4	70.7	71.0	71.9
Aichivirus-1-NC_001918	70.4	70.1	70.2	69.4	70.2	71.4	70.7	71.0	71.9
Bo-U-1-AB084788	74.0	73.9	74.0	73.1	74.4	80.2	78.8	79.9	80.5
Sheep/TB3/HUN/2009-GU245693	71.3	71.9	71.4	70.8	71.3	79.5	78.5	79.2	79.3
Dog/AN211D/USA/2009-JN387133	69.7	69.0	69.6	67.7	70.1	68.6	67.8	68.2	69.1
Canine-US-PC0082-JN088541	70.4	69.4	70.2	67.0	70.7	68.6	67.8	68.2	69.1
Ljungan-87-012-AF327920-Parechovirus	39.3	39.5	39.2	40.0	39.2	25.6	26.3	25.3	25.2
Eq-Rhinovirus-X96871-Erbovirus	49.4	49.2	48.3	48.6	48.9	37.3	37.3	37.3	36.5
FMDV-O1K-X00871-Aphthovirus	49.8	49.1	50.2	49.4	50.0	34.5	34.2	34.2	35.0
HRV14-K02121-Enterovirus	41.4	41.1	41.6	41.6	41.1	34.6	34.6	34.6	33.1
Po-UKG/410/73-AF363453-enterovirus	43.4	43.6	43.9	44.2	43.2	34.1	34.1	34.1	32.5
Damaris-3 - KF494340	100.0	92.0	98.2	89.9	99.1	100.0	96.8	99.7	96.1
K-118 - KF494342		100.0	91.7	89.7	92.0		100.0	96.5	94.9
Kuoba-3 - KF494341			100.0	90.9	98.5		100.0	95.4	95.7

Strain	Nucleotide sequence identity (%)				Amino acid sequence identity (%)				
	Damaris-3	K-118	Kuoba-3	K-460	Damaris-3	K-118	Kuoba-3	K-460	K-1033
K-460 - KF494343			100.0	89.9			100.0	100.0	91.7
K-1033 - KF597279				100.0					100.0

The strains that were detected and discussed in this study are in bold