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Molecular characterization of norovirus and sapovirus detected in animals and humans in Costa Rica: Zoo-anthropozoonotic potential of human norovirus GII.4

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Abstract

Background: Noroviruses (NoV) and sapoviruses (SaV) are major causes of acute viral gastroenteritis in humans worldwide, as well as gastrointestinal infections in animals. However, it has not been determined whether these viruses are zoonotic pathogens.

Aim: In this study, we investigated the presence of NoV and SaV in stool samples from dogs, pigs, cows, and humans to determine some aspects of the molecular epidemiology and the genetic relationship of several strains present in these species.

Methods: Polymerase chain reaction and sequencing of NoV and SaV strains present in stool samples from humans and dogs with diarrhea, pigs, and cattle with and without diarrhea were carried out during fragmented periods from 2002 to 2012.

Results: Of all samples analyzed, 11.6% (123/1,061) of the samples were positive for NoV and 0.88% (9/1,023) were positive for SaV. The phylogenetic analysis confirmed 16 human strains of NoV (HuNoV) belonging to HuNoV G?/GII.P2 (1), GII.4/GII.P4 (5), G?/GII.P4 (9), and GII.6/GII.P6 (1) and allowed us to verify and assign three strains of human SaV to genotypes GI.2 (1) and GII.5 (2). In dogs, eight strains of NoV [HuNoV G?/GII.P4 (4) and canine G?/GVI.P1 (4)] and two strains of canine SaV were determined. In pigs, six strains were assigned to HuNoV G?/GII.P4 and four strains to porcine SaV were assigned to genogroup GIII (2), GVIII (1), and GXI (1). In bovines, five strains were characterized as HuNoV G?/GII.P4.

Conclusions: This study showed that NoV and SaV prototype strains have been present in humans and dogs in Costa Rica. Additionally, it revealed that the zoonotic potential of SaV is very limited, while the zoonotic implications for HuNoV GII.4 are stronger due to the simultaneous circulation of strains related to HuNoV GII.4 in four species, which suggests a zoo-anthropozoonosis.

Keywords: Costa Rica, Norovirus, Phylogeny, Sapovirus, Zoonosis.

Introduction

Norovirus (NoV) and Sapovirus (SaV) belong to the Caliciviridae family and are emerging infectious agents of global distribution. NoV are the leading epidemiological cause of foodborne nonbacterial diarrhea outbreaks and acute viral gastroenteritis in humans (Hall *et al.*, 2014; Mans, 2019). On a smaller scale, SaV are related to outbreaks and sporadic gastroenteritis cases in humans (Magwalivha *et al.*, 2018; Varela *et al.*, 2019). NoV also causes diarrhea in cattle (Di Felice *et al.*, 2016), pigs (Wang *et al.*, 2005), and dogs (Martella *et al.*, 2008c), while SaV causes diarrhea in pigs (Wang *et al.*, 2007) and have been identified in dogs with diarrhea (Li *et al.*, 2011).

Given that caliciviruses are +ssRNA viruses, mutations and genetic recombination to occurs very frequently

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(White, 2014; Ludwig-Begall *et al.*, 2018). As a result, serious difficulties arise when assigning the same genotype to a strain when the phylogenetic information is obtained from the gene encoding the polymerase and capsid protein VP1. However, this classification system based on both genes (polymerase/capsid) is very appropriate to characterize strains and assign them to a known genotype and P-type or identify recombinant strains and new genotypes (Farkas *et al.*, 2004; Katayama *et al.*, 2004; Bull *et al.*, 2007; Dey *et al.*, 2018; Lun *et al.*, 2018; Cavicchio *et al.*, 2022). Basically, NoV and SaV are classified based on the genetic similarity of these genes in three levels: genogroups, genotypes within each genogroup, and viral strains or variants within each genotype (Katayama *et al.*, 2002; Zheng *et al.*, 2006; Oka *et al.*, 2015; Cavicchio *et al.*, 2022).

A more updated classification has been recently proposed suggesting expanding the number of NoV genogroups to 10 and the number of genotypes to 49 (Chhabra *et al.*, 2019). Therefore, human NoV strains (HuNoV) are included within genogroups GI, GII, GIV, GVIII, and GIX (Chhabra *et al.*, 2019; Parra, 2019; Ludwig-Begall *et al.*, 2021). The sequence analysis based on VP1 recognizes nine genotypes GI (GI.1-GI.9) and 27 genotypes GII (GII.1-GII.10, and GII.12-GII.17) in humans (Katayama *et al.*, 2002; Zheng *et al.*, 2006; Mathijs *et al.*, 2011; Chhabra *et al.*, 2019), while genotypes GII.11, GII.18, and GII.19 are prototype strains in pigs (Wang *et al.*, 2005; Nakamura *et al.*, 2010). Genogroup GIII includes genotypes GIII.1 (Günther and Otto, 1987), GIII.2, and GIII.3 in cattle (Woode and Bridger, 1978; Dastjerdi *et al.*, 1999; Chhabra *et al.*, 2019). Genogroup GIV includes human genotype GIV.1 (Vinjé and Koopmans, 2000) and canine genotype GIV.2 (Martella *et al.*, 2008c). In addition, genogroup GVI includes genotypes GVI.1 and GVI.2 in canine NoV (Chhabra *et al.*, 2019).

Human SaV (HuSaV) strains are included within genogroups GI (genotypes GI.1-GI.8), GII (genotypes GII.1-GII.8), GIV (genotype GIV.1), and GV (genotypes GV.1 and GV.2) (Farkas *et al.*, 2004; Katayama *et al.*, 2004; Reuter *et al.*, 2010; Dufkova *et al.*, 2011; Shibata *et al.*, 2015; Diez-Valcarce *et al.*, 2018; Xue *et al.*, 2019). Pigs are infected by genogroups GIII, GV, GVI, GVII, GVIII, GIX, GX, and GXI (Wang *et al.*, 2005; Nakamura *et al.*, 2010; Reuter *et al.*, 2010; Dufkova *et al.*, 2011; Kuroda *et al.*, 2017; Diez-Valcarce *et al.*, 2018) and dogs by genogroup GXIII (Kuroda *et al.*, 2017; Diez-Valcarce *et al.*, 2018).

The zoonotic potential of NoV and SaV has been described in many studies. Porcine GII NoV are genetically and antigenically related to HuNoV (Wang *et al.*, 2005, 2007), and the presence of HuNoV GII.3, GII.13 (Nakamura *et al.*, 2010), and GII.4 has been demonstrated in naturally infected pigs (Mattison *et al.*, 2007; Nakamura *et al.*, 2010). The close genetic similarity between GII.4 strains identified in dogs that have been in direct contact with humans with HuNoV genetic variants GII.4-2006b (98.6% similarity) and GII.4-2008 (100% similarity) suggests that HuNoV GII.4 caused an infection in the gastrointestinal tract (Summa *et al.*, 2012). That observation has also been supported by serological evidence found in dogs exposed to virus-like particles similar to HuNoV GI and GII attached to gastrointestinal tissue and by HuNoV, the latter indicated a productive infection in dogs (Caddy *et al.*, 2014, 2015). HuNoV GII.4 has also been reported in cattle (Mattison *et al.*, 2007), and HuNoV GII.4-HS66 has been shown to infect and induce an immune response in pigs (Cheetham *et al.*, 2006) and gnotobiotic calves (Souza *et al.*, 2008). Strains of HuNoV GII.4 are a more dominant cause of outbreaks and epidemics of gastroenteritis in humans due to their ability to infect and circulate in domestic animals (dogs, pigs, and cattle) and their ability to evolve faster than other NoV genotypes given the frequent mutations and recombinations in the polymerase region and capsid protein VP1 (Bull *et al.*, 2007; Bull and White, 2011; White, 2014; Parra, 2019). However, more studies are needed to elucidate the zoonotic potential of NoV.

The strain of porcine SaV (PoSaV) GVIII/MI-QW19 is related to the strains of HuSaV GII/Mc10, GII/C12, and GII/Cruise Ship, with which it shares a 66.3% identity in a sequence fragment of 95 amino acids in the polymerase (Wang *et al.*, 2007). PoSaV GVIII is also genetically related to the HuSaV GIV/Hou7-1181 strain, with which it shares a 66% identity in the polymerase (Reuter *et al.*, 2010). In addition, PoSaV GVIII strain 43/06-18p3 is 50% genetically similar to HuSaV GI and GV in the polymerase gene (Martella *et al.*, 2008b), and HuSaV GV has been detected in pig feces (Nakamura *et al.*, 2010). Consequently, the possibility of co-infection with PoSaV GVIII and HuSaV (GI, GII, GIV, or GV) suggests the occurrence of zoonotic transmission or pig/human recombinant strains (Bank-Wolf *et al.*, 2010).

As of today, the presence of strains associated with the NoV or SaV that cause diarrhea in humans in Costa Rica has not been well documented. There is no evidence of common strains between humans and animals. Available studies do not include an analysis of the four species, and it has not been confirmed that animals in direct contact with humans are reservoirs of viral strains with public health impact. Therefore, knowing about the reservoirs and molecular epidemiology of these viruses is necessary to reduce the impact of gastroenteritis outbreaks and to clarify whether HuNoV GII.4 strains have zoonotic potential. The objective of this study was to identify NoV and SaV strains in human, canine, porcine, and cattle stool samples to establish the genetic similarity between circulating strains in the metropolitan area of Costa Rica.

Materials and Methods

Stool samples

This research included an ambispective descriptive analysis. A total of 1,061 fecal samples were collected in Costa Rica during the 2002–2012 period. Residual human samples (240 stool samples) were obtained from adults and children under 5 years of age during acute gastroenteritis outbreaks. The samples were previously evaluated for rotavirus and other pathogens by hospitals in the Costa Rican metropolitan area and the National Bacteriological Reference Center of the Costa Rican Institute for Teaching and Research in Health and Nutrition as described in the literature (Bourdett-Stanziola *et al.*, 2008). As far as animal samples, canine diarrheal fecal samples belonged to dogs (278 samples) that had been in direct contact with humans from the greater metropolitan area of Costa Rica and were sent to the Veterinary Virology Diagnostics and Research Unit (UNDIVE) through a commercial microbiological laboratory (ACOPSA, Heredia, Costa Rica). Fecal samples from pigs (194 samples) of all ages with and without diarrhea were obtained from intensive breeding farms. Bovine stool samples (349 samples) were collected from specialized dairy farms from calves and cows with and without diarrhea. Samples were stored at −70°C until processing.

RNA extraction and retro-transcription

Stool samples were suspended in 20% (w/v) PBS at pH 7.2, and once cleared, 250 and 50 µl aliquots were collected. Five 50 µl aliquots were combined to extract viral RNA based on a final volume of 250 µl. Nucleic acid was extracted from each group and each individual sample (when necessary) using Trizol (Ambion, USA) following the manufacturer's instructions. Subsequently, RNA was diluted with 25 µl RNase free-H_2O (Fermentas, Lithuania) and was immediately subjected to a reverse transcription reaction using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania) following the manufacturer's instructions and using the 2720 Thermal Cycler equipment (Applied Biosystems, USA). The cDNA obtained was stored at −70°C until use.

Detection of NoV and SaV by RT-PCR

The presence of NoV and SaV was initially evaluated in a mix of five samples and then in each individual sample if found positive. The screening was performed to amplify the RdRp region by PCR using p290hijk(+)/p289hi(−)(Invitrogen, USA) primer pairs to detect NoV and SaV in humans and animals, while the SR80(+)/JV33(−) (Invitrogen, USA) primer pairs were used to detect SaV in the four species (Table 1). The PCR reaction mix was prepared based on a final volume of 50 µl using 25 µl PCR Master Mix (2X), #K0171 (Fermen tas, Lithuania), 1 µl of each sense/antisense primer, 2–5 µl cDNA, and RNAase-free water (Fermentas, Lithuania) to complete the volume. PCR conditions were performed as described in the literature for NoV (Jiang *et al.*, 1999; Reuter *et al.*, 2010) and SaV (Vinjé *et al.*, 2000; Reuter *et al.*, 2010). The expected product was 319 bp and 320 bp for NoV and SaV, respectively.

The cDNA polymerase-based RT-PCR of positive samples was analyzed using a second RT-PCR to amplify the ORF1-ORF2 junction in NoV and the NS segment of the VP1 protein in SaV. The PCR reaction mix was prepared similarly to that described above. HuNoV

Table 1. Primers used to detect NoV and SaV by RT-PCR.

Primer	Sequence (5' - 3')	Polarity	Specificity	Target	Position (nt)
p289h	TGA CGA TTT CAT CAT CAC CAT A	Negative	NoV, SaV	RdRp	4886–4865
p289i	TGA CGA TTT CAT CAT CCC CGT A	Negative	NoV, SaV	RdRp	4886–4865
p290h	GAT TAC TCC AGG TGG GAC TCC AC	Positive	NoV, SaV	RdRp	4568–4590
p290i	GAT TAC TCC AGG TGG GAC TCA AC	Positive	NoV, SaV	RdRp	4568–4590
p290j	GAT TAC TCC ACC TGG GAT TCA AC	Positive	NoV, SaV	RdRp	4568–4590
p290k	GAT TAC TCC ACC TGG GAT TCC AC	Positive	NoV, SaV	RdRp	4568–4590
G1SKR	CCA ACC CAR CCA TTR TAC A	Negative	GI NoV	ORF1-ORF2	5653–5671
NVGIFIb	CGY TGG ATG CGN TTC CAT GA	Positive	GI NoV	ORF1-ORF2	5311–5330
G2SKR	CCR CCN GCA TRH CCR TTR TAC AT	Negative	GII NoV	ORF1-ORF2	5379–5401
NVG2flux1	ATG TTY AGR TGG ATG AGR TTY TC	Positive	GII NoV	ORF1-ORF2	5012–5033
JV33	GTG TAN ATG CAR TCA TCA CC	Negative	SaV	RdRp	4666–4685
SR80	TGG GAT TCT ACA CAA AAC CC	Positive	SaV	RdRp	4366–4385
SLV5749	CGG RCY TCA AAV STA CCB CCC CA	Negative	SaV	NS	5494–5516
SLV5317	CTC GCC ACC TAC RAW GCB TGG TT	Positive	SaV	NS	5083–5105

genotyping was performed using specific genogroup degenerate primers NVG1F1b(+)/G1SKR(−) for HuNoV GI and NVG2flux1(+)/G2SKR(−) for HuNoV GII (Invitrogen, USA) in separate tubes. Reaction conditions were determined as described in the literature (Kojima *et al.*, 2002; Nordgren *et al.*, 2008; Nakamura *et al.*, 2010). Expected products for GI NoV and GII NoV were 381 bp and 390 bp, respectively. Regarding SaV, SLV-5317(+) and SLV-5749(−) primer pairs (Invitrogen, USA) (Table 1) were used, and the PCR cycle was partially modified from Hansman *et al.* (2004) as follows: 94°C × 3 minutes, 40 cycles of 94°C× 1 minute, 48°C× 1.5 minutes, and 72°C × 1 minute and a final extension of 72°C × 10 minutes. The expected amplicon size was 434 bp.

The strain of HuNoV LU201335I-IID00/2003/CR (GenBank accession number: KM057715) was used as a positive control in each round of PCR to detect NoV, while the strain of HuSaV SJ164-D11/2007/CR (GenBank accession number: KM001680) was used as a positive control in each round of PCR to detect SaV. In addition, RNAase-free water (Fermentas, Lithuania) was used as a negative control in each round of PCR. Amplified products by PCR were separated in 2% agarose gel containing 2 µl GelRed Nucleic Acid Stain 10,000X Water (Biotium, USA) and displayed using image capture and UV illuminator (UVP BioDoc-ItTM, USA) equipment. The DNA products obtained were stored at −70°C until sequencing.

Sequencing and sequence analysis

A total of 110 positive samples directly from the 1% agarose gel were purified using the QIAquick gel extraction kit (QIAGEN, Inc) following the manufacturer's instructions, and 46 samples positive to NoV and 10 positives to SaV were selected as a representation for sequencing according to period and species, band quality on the agarose gel, and DNA concentration (ng/µl). The partial sequences of DNA nucleotides corresponding to the polymerase, NS segment, and ORF1-ORF2 junction were determined by direct cycles in both directions using the strains described above for each genus and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), following the manufacturer's instructions. Amplified fragments were purified using the QIAquick PCR Purification Kit (QIAGEN, Inc), and the nucleotide sequence was obtained using the 3130 Genetic Analyzer automated sequencer (Applied Biosystems, USA). DNA sequences were analyzed and edited with the MEGA v6.06 software and aligned using ClustalW 1.6 (Tamura *et al.*, 2013).

Phylogenetic analysis

The strain's genotype was assigned taking into consideration the genetic classification based on the range of nucleotide similarity determined for the partial sequence of the gene encoding polymerase or the capsid region, the international classification and nomenclature, and reference strains for each genotype reported in different studies for humans, dogs, pigs, and cattle (Katayama *et al.*, 2002; Zheng *et al.*, 2006; Wang *et al.*, 2007; Martella *et al.*, 2008a, 2008c; Nakamura *et al.*, 2010; Reuter *et al.*, 2010; Kuroda *et al.*, 2017; Chhabra *et al.*, 2019). Phylogenetic trees were built using MEGA X (Kumar *et al.*, 2018), including for each genus the reference genotypes found with the Basic Local Alignment Search Tool (Altschul *et al.*, 1990). The genetic distance between different genotypes was analyzed using the Kimura 2-parameter model as a method for nucleotide substitution (Kimura, 1980). Phylogenetic trees were created using the Neighbor-Joining method (Saitou and Nei, 1987), and the statistical significance was obtained by 2,000 bootstrap repetitions (Efron and Gong, 1983).

Accession numbers

The NoV and SaV sequences obtained in this study were added to the GenBank with the following accession numbers: canine GVI.P1 NoV from KM057711 to KM057714, human GII NoV from KM057715 to KM057730, canine GII.P4 NoV from KM057731 to KM057734, porcine GII.P4 NoV from KM057735 to KM057740, bovine GII.P4 NoV from KM057741 to KM057745, recombinant strain of human GII NoV KP067788, human GI.2 SaV KM001680, human GII.5 SaV KJ418887 and KM001679, canine SaV KM001673 and KM001674, and PoSaV from KM001675 to KM001678.

Ethical approval

The residual human diarrheal stool samples were sent from hospitals and public health reference centers located in the Costa Rican metropolitan area to the UNDIVE, School of Veterinary Medicine of National University, as part of the cooperation plan to monitor outbreaks of diarrhea in the country to detect the presence of *Calicivirus* by ensuring the privacy of patients and in accordance with Article 42 of the current Health Surveillance Regulation (Decree No. 40556-S, La Gaceta, Scope No. 2016, August 23rd. 2017). Meanwhile, the feces samples of animals were obtained from local diagnostic centers, cattle or pig farms with the prior consent of the producer and considering the Canadian Council on Animal Care guidelines on: the care and use of farm animals in research, teaching, and testing (Canadian Council on Animal Care, 2009).

Results

Detection of NoV and SaV in humans and animals by RT-PCR

The use of RT-PCR with the degenerate primer pair p290hijk(+)/p289hi(−) targeting the NoV and SaV RdRp region allowed the identification of 123 positive samples for NoV from 1,061 stool samples analyzed, and 2 HuSaV strains and 2 PoSaV strains were characterized by sequencing. In addition, the use of RT-PCR with a specific primer pair SR80(+)/JV33(−)

targeting the SaV polymerase allowed the identification of 5 positive samples from the 1,023 stool samples analyzed.

The 123 samples positive for NoV were genotyped with a second RT-PCR using degenerate primers NVG1f1b(+)/G1SKR(−) and NVG2flux1(+)/G2SKR(−) targeting the ORF1-ORF2 junction of genogroups GI and GII, respectively. This additional assay identified one human sample as GI NoV and six human samples as GII NoV; however, no animal samples were classified as GI or GII. The presence of SaV by RT-PCR targeting the NS segment of capsid protein VP1 with primers SLV-5317(+)/SLV-5749(−) was confirmed only in one human sample. A complete summary of these results is shown in Tables 2 and 3.

NoV and SaV genotypes characterized in humans and animals

To determine the nucleotide sequence similarity (ntS) between the NoV and SaV identified in humans and animals, 56 positive samples were selected from the products obtained with the primers used in this study to confirm their presence and assign the genotype. High-quality nucleotide (nt) sequences were obtained from 50 samples, while 36 samples were associated with NoV (17 in humans, 8 in dogs, 6 in pigs, and 5 in cattle) and 9 with SaV (3 in humans, 2 in dogs, and 4 in pigs). No sequence was determined in five of the samples.

The sequence analysis was based on a 274 nt fragment of the polymerase for the sequences obtained with primers p290hijk(+)/p289hi(−) and a 332 nt fragment of the ORF1-ORF2 junction of the VP1 protein for sequences obtained with primers NVG1f1b(+)/G1SKR(−) and NVG2flux1(+)/G2SKR(−). On the other hand, in the case of SaV, the comparative sequence analysis was based on a 280 nt fragment of the polymerase for sequences obtained with primers SR80(+)/JV33(−), a 286 nt fragment of the polymerase for sequences obtained with primers p290hijk(+)/p289hi(−), and 388 nt of the NS subregion for sequences obtained with primers SLV-5317(+)/SLV-5749(−).

A total of 17 human samples were verified as HuNoV using sequence analysis. Using the phylogenetic analysis, strain LZ075-D/07/CR detected in the study was classified within P-type GII.P2 because of its 86.7% ntS with the GII.2/Melksham/94 UK strain in the polymerase region; meanwhile, strain INC002-D10/10/CR showed a 94.3% ntS with the GII.6/SaitamaU3 Japanese strain in the polymerase region and was confirmed as a HuNoV GII.6 strain according to the phylogenetic information obtained from the ORF1-ORF2 junction, as it showed a greater association with reference strains GII.6/Seacroft/90/UK (95.2% ntS) and GII.6/SaitamaU3/JP (94.7% ntS), so it is a GII.6/GII.P6 strain. A total of 14

Table 2. Presence of NoV in human, canine, swine, and bovine fecal samples in Costa Rica during the 2002–2012 period.

Species	Period of sample collection	Primers p290hijk/p289hi (RdRp) No. (%) of samples		Characterization by RT-PCR (ORF1-ORF2) No. (%) of samples			No. of samples confirmed Sequencing Genogroup (Capsid/RdRp)
				Analyzed	Positive		
		Analyzed	Positive		GI	GII	
Human	2002–2003	76	16 (21.0)	16	1 (6.2)	2 (12.5)	2 (ND/GII.P4) 1 (GII.4/GII.P4) 1 (GII.4/GII.P4-R)
	2006–2007	103	25 (24.2)	25	0	0	1 (ND/GII.P2) 2 (ND/GII.P4)
	2009–2010	44	25 (56.8)	25	0	1 (4.0)	4 (ND/GII.P4) 1 (GII.6/GII.P6)
	2012	17	6 (35.3)	8	0	3 (37.5)	2 (GII.4/GII.P4) 2 (ND/GII.P4) 1 (ND/GII.P4-R)
Canine	2009–2010	106	2 (1.8)	2	0	0	4 (ND/GII.P4) 4 (ND/GVI.P1)
	2012	172	7 (4.0)	7	0	0	
Swine	2006–2007	21	2 (9.5)	5	0	0	6 (ND/GII.P4)
	2009–2010	39	2 (5.1)	2	0	0	
	2012	134	6 (4.4)	6	0	0	
Bovine	2006–2007	125	14 (11.2)	14	0	0	5 (ND/GII.P4)
	2012	224	18 (8.0)	18	0	0	
		1,061	123	128	1	6	36

(ND): The sequence was not determined; (R)- Recombinant.

Table 3. Presence of SaV in human, canine, swine, and bovine fecal samples in Costa Rica during the 2002–2012 period.

Species	Period of sample collection	Primers SR80/JV33 (RdRp) No. (%) of samples		Primers SLV5317/ SLV5749 (NS) No. (%) of samples		Primers p290hijk/ p289hi (RdRp) No. (%) of samples		No. of samples confirmed Sequencing Genogroup
		Analyzed	Positive	Analyzed	Positive	Analyzed	Positive	(Capsid/RdRp)
Human	2002–2003	72	0	1	0			0
	2006–2007	103	1 (0.9)	1	1			1 (GI.2/GI.2)
	2009–2010	42	0	0	0			0
	2012	17	0	0	0	17	2 (11.7)	2 (ND/GII.5)
Canine	2009–2010	74	1 (1.3)	1	0			1 (ND/GXIII)
	2012	172	1 (0.6)	2	0			1 (ND/GXIII)
Swine	2006–2007	21	0	0	0	21	2 (9.5)	2 (ND/GIII)
	2009–2010	39	0	0	0			0
	2012	134	2 (1.5)	2	0			1 (ND/GVIII) 1 (ND/GXI)
Bovine	2006–2007	125	0	0	0			0
	2012	224	0	0	0			0
		1,023	**5**	**7**	**1**	**38**	**4**	**9**

(ND): The sequence was not determined.

sequences were grouped within P-type GII.P4 based on polymerase phylogenetic information. This subgroup showed 95.9% ntS among groups when compared to the representative strains of GII.P4 reported in different countries (Farmington Hills/02/USA, Toronto/SK/02/CAN, AlbertaEI513/06/CAN, AlbertaEI003/12/CAN, Tokyo/10-1443/10/JP, Norwalk/10378/10/VNM, and Hunter504D/04O/AU). In addition, the presence of five strains was confirmed in genotype GII.4 according to the phylogenetic information obtained from the ORF1-ORF2 junction, all of which were highly associated with reference strains GII.4 AlbertaEI388/CAN (97.2% ntS), Norwalk10386/10/VNM (96.2%–97.2% ntS), and Farmington Hills/02/USA (95.7%–99.5% ntS) (Table 4, Fig. 1a and b). On the other hand, a recombinant strain of HuNoV GII.4 (INC313846-D/2012/CR) was determined to be present in the gene encoding the polymerase (GenBank accession number: KP067788).

In animals, the sequence analysis was based solely on phylogenetic information obtained from the gene encoding the polymerase. Eight nt sequences obtained from canine samples were associated with NoV, of which strains CS-C011D/09/CR, C005-D00/12/CR, C010-D00/12/CR, and C107-D00/12/CR were grouped with reference strains of HuNoV GII.P4 (92.4% ntS between groups); meanwhile, the other four strains (CS-C022D/09/CR, C003-D00/12/CR, C006-D00/12/CR, and C057-D00/12/CR) showed a closer association with the representative strains of the canine P-types GVI.P1 (86.8% ntS between groups). In addition, six nt sequences obtained from pig fecal samples and five from cattle samples showed to be very similar to HuNoV GII.P4, with 96.4% and 95.5% ntS among groups with HuNoV GII.P4 reference strains, respectively (Table 4, Fig. 1a).

Strain HuSaV SJ164-D11/2007/CR identified in this study was very similar to the GI.2/VIG-236/PA strain from Brazil (98.7% ntS) (Aragão *et al.*, 2010) and the GI.2/Houston/90 strain from the USA (88.9% ntS). The canine strains identified here (C014-D10/2009/CR and C104-D00/2012/CR) were genetically similar to the AN196 (83.7% ntS) and AN210D (86.4% ntS) SaV strains recorded in the USA, respectively (Li *et al.*, 2011). In addition, porcine strain CP12-SD21/2012/CR was closely associated with USA PoSaV strains GVIII/WGP180 (91.3% ntS) and GVIII/MI-QW19 (86.2% ntS) (Wang *et al.*, 2006). Porcine strain CP20-SD21/2012/CR showed greater genetic similarity to unknown genogroup (G?) of Canada's porcine strain F5-3 (84.2% ntS) (L'Homme *et al.*, 2009) and, together with Brazil's BRA21-RS strain (Barry *et al.*, 2008), formed a new genogroup (GXI) (Fig. 2a and detailed information in Table 5). The nt sequence obtained with the SLV5317(+)/SLV5749(-) primers allowed us to verify that the HuSaV SJ164-D11/07/CR strain was human GI.2 SaV according to the phylogenetic information of the NS segment of the VP1 protein. However, it showed greater genetic similarity to Japan's GI.2/Oshima1/09 reference strain (99.3% ntS) (Miyoshi *et al.*, 2010) (Table 5, Fig. 2b).

From the four sequences obtained with primers p290hijk(+)/p289hi(−) (Table 3), two were classified as PoSaV GIII (strains BOP007-D10/2007/CR and BOP017-D10/2007/CR) because they exhibited

Table 4. Genetic similarity between NoV strains detected in humans, canines, swine, and cattle with prototype NoV strains of the four species.

Genotype Reference NoV (Accession No.) Region analyzed	Nt. similarity (%)						
	Species Genotype (Sequences analyzed)						
	Human GII.2 (*n* = 1)	Human GII.4 (*n* = 14)	Human GII.6 (*n* = 1)	Canine GII.4 (*n* = 4)	Canine GVI.P1 (*n* = 4)	Swine GII.4 (*n* = 6)	Cattle GII.4 (*n* = 5)
GI.1 Hu/Norwalk/68/US (M87661) RdRp ORF1-ORF2	38.8 -	41.5–45.3 26.5–29.3	55.3 41.5	46.0–53.2 -	60.7–64.0 -	43.0–49.2 -	45.1–49.0 -
GII.2 Hu/Melksham/94/UK (X81879) RdRp ORF1-ORF2	86.7 -	76.0–78.3 55.5–59.6	60.8 66.6	63.5–75.0 -	52.3–55.0 -	70.7–76.7 -	71.8–75.7 -
GII.4 Hu/Farmington Hills/02/US (AY502023) RdRp ORF1-ORF2	72.1 -	94.4–99.5 95.7–99.5	57.1 60.6	78.4–94.4 -	45.1–50.8 -	94.4–97.2 -	90.7–99.3 -
GII.4 Hu/Norwalk10386/10/VNM (KC175387) RdRp ORF1-ORF2	70.3 -	94.3–99.5 96.2–97.2	55.2 61.4	80.2–98.6 -	40.3–43.3 -	91.4–98.6 -	93.6–98.6 -
GII.4 Hu/AlbertaEI388/11/CAN (JX445168) RdRp ORF1-ORF2	72.1 -	93.7–98.9 97.2	53.1 60.6	80.2–98.6 -	42.0–44.9 -	91.4–98.6 -	93.6–98.6 -
GII.6 Hu/SaitamaU3/JP (AB039776) RdRp ORF1-ORF2	55.9 -	53.1–56.2 63.7–64.4	94.3 94.7	48.0–52.2 -	52.4–61.1 -	50.9–57.4 -	51.0–54.9 -
GII.6 Hu/Seacroft/90/UK (AJ277620) ORF1-ORF2	-	62.1–63.7	95.2	-	-	-	-
GII.11 Sw/MI-QW48/02/US (AY823303) RdRp ORF1-ORF2	59.2 -	65.1–70.3 57.5–60.2	68.2 65.8	63.0–67.3 -	48.6–56.3 -	64.1–69.3 -	67.3–70.3 -
GII.18 Sw/OH-QW101/03/US (AY823304) RdRp ORF1-ORF2	59.9 -	60.5–65.0 53.3–58.5	66.1 65.3	59.7–65.0 -	43.4–53.5 -	60.5–65.0 -	61.7–67.2 -
GII.19 Sw/OH-QW170/03/US (AY823306) RdRp ORF1-ORF2	64.0 -	60.7–63.0 60.1–62.8	68.3 64.6	51.2–60.7 -	45.7–51.3 -	60.7–61.9 -	60.7–61.8 -
GIII.2 Bo/Newbury2/76/UK (AF097917) RdRp ORF1-ORF2	51.7 -	52.9–58.0 30.5–39.1	48.7 35.6	56.9–57.3 -	51.0–59.7 -	51.7–56.9 -	55.6–56.9 -
GVI.P1 Dog/Bari/170/07/ITA (EU224456) RdRp ORF1-ORF2	52.3 -	40.6–47.0 54.6–56.1	55.0 70.9	42.2–67.9 -	92.9–95.9 -	40.9–45.5 -	40.6–46.9 -

(-): Uncalculated data.

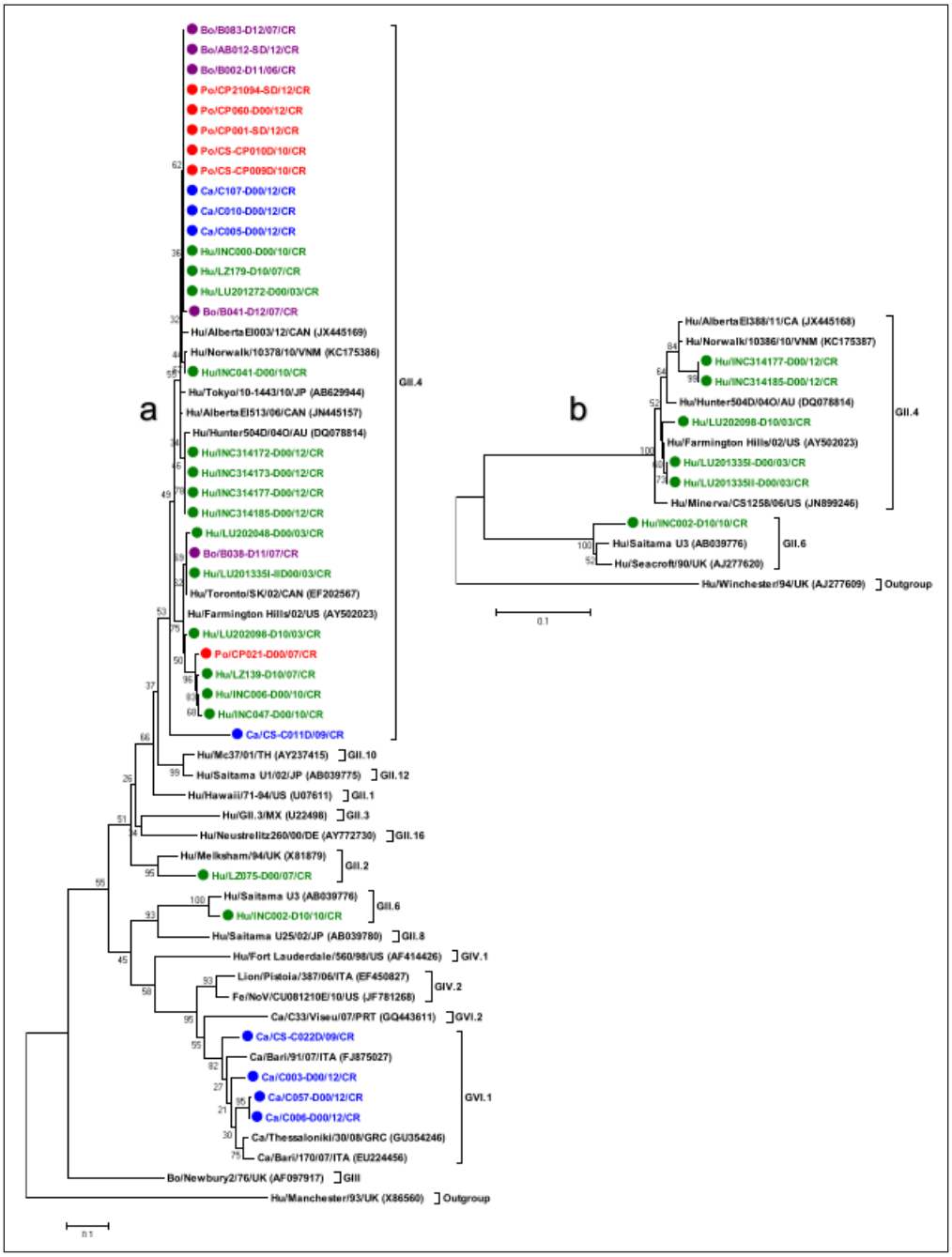

Fig. 1. Phylogenetic analysis of *NoV*. (a): The tree was constructed using the 274 nt partial sequence of the polymerase. (b): The tree was constructed using the 332 nt partial sequence of the ORF1-ORF2 junction. Statistical significance was determined using the bootstrap method from 2,000 resamples; values are displayed above the node. The GenBank accession number of the reference strains is shown in parentheses. The Hu/SaV/Manchester/93/UK (X86560) and Hu/Winchester/94/UK (AJ277609) strains were used as an outgroup for trees (a) and (b), respectively. The strains detected in the study are color-coded (human strains are green, canine blue, swine red, and bovine purple).

a high degree of genetic similarity with reference strains from Canada GIII/F4-6/06 (96.6% ntS) and from the USA GIII/Cowden/80 (78.4% ntS) (Wang *et al.*, 2005); meanwhile, the other two are associated mostly with HuSaV (strains INC313871-D00/12/CR and INC313934-D00/12/CR), grouping with genotype GII.5 strains and showing greater genetic relationship with the GII.5/Hokkaido/Nay1/05 strain from Japan

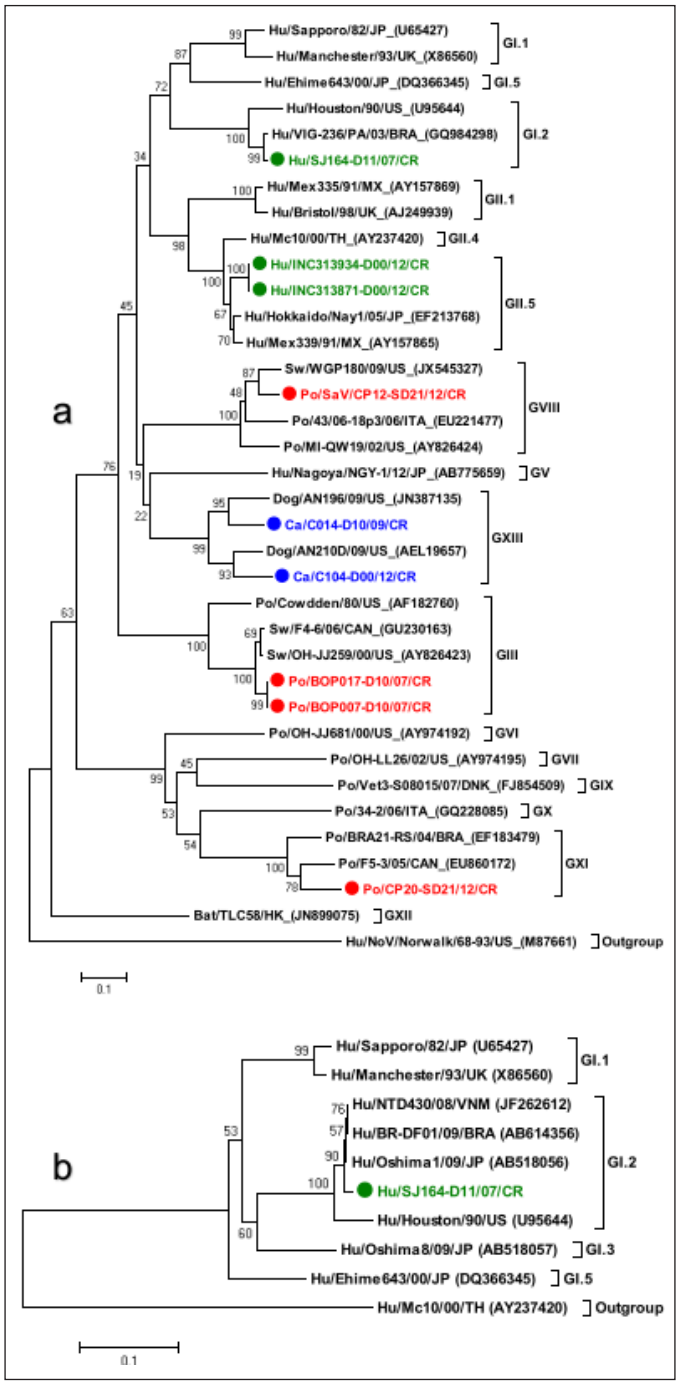

Fig. 2. Phylogenetic analysis of *SaV*. (a): The tree was constructed using the 280 nt partial sequence of the polymerase. (b): The tree was constructed using the 388 nt partial sequence of the NS subregion. Statistical significance was determined using the bootstrap method from 2,000 resamples; values are displayed above the node. The GenBank accession number of the reference strains is shown in parentheses. The Hu/NoV/Norwalk/68-93/US (M87661) and Mc10/00/TH (AY237420) strains are used as an outgroup for trees (a) and (b), respectively. The strains detected in the study are color-coded (human strains are green, swine red, and canine blue).

Table 5. Genetic similarity between SaV strains detected in humans, canines, and swine with prototype SaV strains of the three species.

Genotype Reference SaV (Accession No.) Region analyzed	Nt. Similarity (%) Species Genotype (Sequences analyzed)						
	Human GI.2 (*n* = 1)	Human GII.5 (*n* = 2)	Canine AN196 (*n* = 1)	Canine AN210D (*n* = 1)	Swine GIII.2 (*n* = 2)	Swine GVIII (*n* = 1)	Swine GXI (*n* = 1)
GI.1 Hu/Sapporo/82/JP (U65427) RdRp NS	 59.5 84.6	 53.7 -	 53.5 -	 52.7 -	 31.2 -	 39.9 -	 0 -
GI.2 Hu/Houston/90/US (U95644) RdRp NS	 88.9 93.4	 46.1 -	 42.6 -	 41.7 -	 45.8 -	 34.9 -	 6.0 -
GI.2 Hu/VIG-236/PA/03/BRA (GQ984298) RdRp	 98.7	 60.4	 46.2	 45.3	 44.9	 30.5	 2.1
GI.2 Hu/Oshima1/09/JP (AB518056) NS	 99.3	 -	 -	 -	 -	 -	 -
GII.5 Hu/Hokkaido/Nay1/05/JP (EF213768) RdRp NS	 61.8 28.5	 94.1 -	 54.7 -	 45.1 -	 43.1 -	 52.9 -	 12.9 -
GIII.2 Sw/Cowden/80/US (AF182760) RdRp NS	 43.7 4.4	 43.3 -	 30.7 -	 34.5 -	 78.4 -	 43.5 -	 16.7 -
GIII.2 Sw/F4-6/06/CAN (GU230163) RdRp	 44.1	 42.0	 37.3	 39.7	 96.6	 38.0	 4.9
GVIII Sw/MI-QW19/02/US (AY826424) RdRp	 28.4	 48.0	 46.1	 47.9	 36.6	 86.2	 8.5
GVIII Sw/WGP180/09/US (JX545327) RdRp	 35.9	 50.3	 47.1	 47.9	 42.3	 91.3	 6.8
G? Sw/F5-3/05/CAN (EU860172) RdRp	 0.9	 13.3	 0	 0	 7.0	 11.4	 84.2
G? Dog/AN210D/09/US (AEL19657) RdRp NS	 48.7 17.1	 47.5 -	 72.8 -	 86.4 -	 42.1 -	 39.9 -	 1.9 -
G? Dog/AN196/09/US (JN387135) (RdRp) NS	 40.5 19.7	 48.9 -	 83.7 -	 75.5 _	 36.1 -	 38.8 -	 3.4 -

(-): Uncalculated data.

(94.1% ntS) associated with gastroenteritis outbreaks (Hansman *et al.*, 2007) (Table 5, Fig. 2a).

Phylogenetic comparison of NoV and SaV in humans and animals

All strains identified in humans were compared with those detected in animals, and the high prevalence of the HuNoV GII.P4 in Costa Rican species was noticeable. The strains detected in dogs, pigs, and cattle showed a high degree of genetic association with the HuNoV GII.P4 strains characterized in humans, showing 91.9%, 96.0%, and 95.1% ntS between groups, respectively. On the other hand, the ntS identified in the study in human HuNoV GII.P4 strains was 89.8%–100%, 79.4%–100% in canine HuNoV GII.P4 strains, 91.4%–100% in porcine HuNoV GII.P4 strains, 87.6%–100% in cattle HuNoV GII.P4 strains, and 83.3%–100% in CaNoV GVI.P1 strains.

Regarding the similarity between human, canine, and PoSaV, the HuSaV GII.5 strains detected here showed 51% ntS in the RdRp region with the porcine GVIII/CP12-SD21/12/CR strain. In comparison, swine GIII strains showed greater similarity to human strain GI.2/Houston/90 from the USA (45.8% ntS), and canine strain C014-D10/2009/CR showed 53.5% ntS with the HuSaV GI.1/Sapporo/82 strain from Japan.

Discussion

Few studies have sought to explore the molecular epidemiology of NoV and SaV in humans and animals simultaneously. This article describes a comprehensive and integrated analysis of the detection, molecular characterization, and phylogeny between NoV and SaV identified in human, canine, porcine, and cattle fecal samples from 2002 to 2012 fragmented periods. Different studies have documented the HuNoV genetic diversity in the pediatric population as a cause of acute diarrhea and vomiting, being genogroup GII more prevalent than GI and genotype GII.4 is the most prevalent within genogroup GII (Bull and White, 2011; Hasing *et al.*, 2019; Mans, 2019). In this study, the sequence analysis confirmed the presence of HuNoV G?/GII.P2, G?/GII.P4, GII.4/GII.P4, and GII.6/GII.P6 in human diarrheal samples, dominated by HuNoV G?/GII.P4 and GII.4/GII.P4 during the periods evaluated, which being consistent with other reports in which HuNoV GII.4 is the main cause of human gastroenteritis (Bucardo *et al.*, 2008; Gómez-Santiago *et al.*, 2012).

Canine P-types GVI of NoV strains were found in less than seven-month-old dogs with diarrhea. Our findings were similar to those reported in puppies with diarrhea for the Bari/91/07 strain in Italy (Martella *et al.*, 2009), the C33/Viseu/07 strain in Portugal (Mesquita *et al.*, 2010), and the Thessaloniki/30/08 strain in Greece (Ntafis *et al.*, 2010). Canine strains CS-C011D/09/CR, C005-D00/12/CR, C010-D00/12/CR, and C107-D00/12/CR were mostly associated with reference strain HuNoV GII.P4/Farmington Hills/02/USA. The circulation of HuNoV GII in dogs in Brazil (Sokel and Kale, 2019), canine strains IC-09, 261-10, and 3-09 in Finland were related to genetic variants GII.4-2006b and GII.4-2008, which were associated with the human GII.4/Bristol/93/UK reference strain (Summa *et al.*, 2012). Like so, the transmission of HuNoV GII.e-GII.4 Sydney from human to canine have also been reported (Charoenkul *et al.*, 2020). On the other hand, knowledge about the ability of HuNoV to attach to *in vitro* canine tissues and the presence of anti-HuNoV antibodies in dogs suggest the possibility of productive infection (Caddy *et al.*, 2014, 2015). Therefore, given that this research study has shown that HuNoV GII.4 strains have circulated in domestic dogs, HuNoV zoo-anthropozoonotic implications are very strong, and the risk to generate recombinant strains between HuNoV and CaNoV present in dogs is high, while the zoonotic potential for GIV and GVI NoV has been reported to be limited (Ford-Siltz *et al.*, 2019).

Regarding pigs, direct contact with HuNoV has been demonstrated by serological findings in the pig population of Nicaragua (Bucardo *et al.*, 2016), and the circulation of strains related to HuNoV GII.4, GII.3, and GII.13 in Japan (Nakamura *et al.*, 2010) has been described. In this study, strains associated with the HuNoV GII.4 genotype were found in diarrheal samples. Strains CP021-D00/07, CS-CP009D/10, CS-CP010D/10, CP001-SD/12, CP060-D00/12, and CP21094-SD/12 showed great ntS with human strain GII.P4/Farmington Hills/02/USA. In addition, porcine strains very similar to the reference strain GII.P4/Farmington Hills/02/USA were reported in Canada (Mattison *et al.*, 2007). Although it has been shown that the HuNoV GII.4/HS66/01/USA and variant GII.4-2006b can be replicated and cause acute disease in gnotobiotic pigs (Cheetham *et al.*, 2006; Bui *et al.*, 2013), in this study, the evidence found in the field confirms that HuNoV GII.P4 infects pigs and that the absence of PoNoV prototypes could be associated with the circulation of an unknown strain common between humans and animals in Costa Rica, unlike the study described by Cavicchio *et al.* (2020). This study also demonstrates the presence of HuNoV GII.P4 in cow feces. Previously, the circulation of the GII.4/CE-M-06-0509/07 strain has been reported in Canada, which is closely associated with the reference strain Farmington Hills/02/USA (Mattison *et al.*, 2007). In addition, it has been proven that the HuNoV GII.4/HS66 can cause infections and disease in cattle (Souza *et al.*, 2008), although the evidence found in this study suggests that cattle could spread the virus, Oliver *et al.* (2003) did not obtain evidence to support that HuNoV circulates between these two species.

Genetically variable NoV strains related to HuNoV have been reported present in animals, which may indicate that animals are NoV reservoirs or are being infected by humans directly or indirectly (Wang *et al.*, 2007; Scipioni *et al.*, 2008; Martella *et al.*, 2009; Bank-Wolf *et al.*, 2010; Caddy *et al.*, 2015). In this sense, this

study shows the circulation of strains associated with HuNoV GII.P4 in the four species evaluated, which suggests that humans facilitate animal infection by indirect contact through the consumption of water and food contaminated with human waste, resulting in zoo-anthropozoonosis caused by recombinant strains G?/GII.P4, which are capable of infecting dogs, pigs, and cattle due to the occurrence of essential and necessary changes in the VP1 region of genotype GII.4 that allowed it to stabilize as described by Sato *et al.* (2017) and with an impressive capacity to infect other species. The foregoing could also be explained by a co-infection between human and animal strains along with the ability of RNA polymerase to switch templates and promote the development of recombinant RNA viruses (White, 2014). Given that recombination is an evolutionary mechanism used by NoV (Ludwing-Begall *et al.*, 2018) that causes major changes in the viral genome and that intergenogroup recombinations have been recorded between GII.9/GI.7 (Reuter *et al.*, 2010) and GI.3/GII.4 (Nayak *et al.*, 2008), it is likely that the undetermined phylogenetic information from the capsid region in most strains characterized in this study as HuNoV GII.P4 in humans and animals is associated with recombinant virus polymerase of prototype genotypes of each animal, or it is a common strain with G?/GII.P4. Consequently, we would agree with the hypothesis described by Villabruna *et al.* (2019), which states that NoV may not be host restricted and might be able to jump the species barrier, especially HuNoV GII.4 due to their ample capacity to generate high replication rates (Ludwing-Begall *et al.*, 2021), resulting in the zoo-anthropozoonotic events that have long occurred in Costa Rica. Therefore, further research is essential to determine the phylogenetic information of the VP1 region and clarify the significance of these findings.

Regarding SaVs, the low genetic diversity of HuSaV observed in humans in this study is comparable to what was reported in Mexico (Gómez-Santiago *et al.*, 2012), Argentina (Gomes *et al.*, 2008), Brazil (Aragão *et al.*, 2010), and Nicaragua (Bucardo *et al.*, 2012) where genotypes GI.1, GI.2, and GII.1 HuSaV were reported in under 5-year-old children with diarrhea. Unlike those records, this study describes the presence of genotype GII.5.

In pigs, the identified genetic diversity of PoSaV (genogroups GIII, GVIII, and GXI) was broader than the one reported in Brazil (genogroups GIII and GXI) (Barry *et al.*, 2008), while in dogs, Li *et al.* (2011) proposed that the AN210D and AN196 CaSaV strains found in the USA should be classified into a new genogroup (GIX). In addition, this study identified two CaSaV strains (C014-D10/09 and C104-D00/12), which were very similar to the strains described above and, like those strains, were grouped within the same genogroup (Fig. 2a). Soma *et al.* (2015) reported the presence of CaSaV that were very similar to the USA strains (72.5%–86.5% ntS), and Bodnar *et al.* (2016) also described two CaSaV strains that were closely related to strain AN210D (90.4% ntS) in the polymerase. Therefore, the evidence found in this and other studies demonstrates the clear segregation of CaSaV as a new genogroup GXIII (Kuroda *et al.*, 2017). Due to the phylogenetic information obtained from the polymerase in other studies, considering GIX as a CaSaV genogroup is very controversial because pigs are infected by genogroup GIX (Wang *et al.*, 2005; Martella *et al.*, 2008a; Nakamura *et al.*, 2010; Reuter *et al.*, 2010; Dufkova *et al.*, 2011; Diez-Valcarce *et al.*, 2018).

No SaV were detected in cattle in this investigation. Similarly, Smiley *et al.* (2003) and Mijovski *et al.* (2010) reported the absence of SaV in cattle. On the other hand, there was not a strong genetic similarity of polymerase among the human, canine, and PoSaV identified in this research or among the host species, unlike Oka *et al.* (2015), who suggested not using the polymerase region for genotyping because it is less diverse. So, unlike NoV, the zoonotic potential of SaV is limited because the SaV in animals were distant from human strains, as indicated by Eu Lim *et al.* (2020) for PoSaV.

Overall, the results of this study suggest that NoV and SaV should be included in epidemiological surveillance programs. Since the prevalence of genotypes varies geographically, active monitoring of NoV and SaV circulating strains in humans and animals will provide evidence to optimize prevention measures for gastroenteritis cases and promote vaccine formulation.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author's contributions

Designed the project and wrote the article, D.P., and C.J.; Ran the stool sample analysis, D.P., C.S. and R.C.; Performed sequencing and phylogenetic analysis of the data, D.P.; critically reviewed the scientific content of the manuscript, C.S., H.B., and E.C. All authors read and approved the final manuscript.

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