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Etiological role and repeated infections of sapovirus among children aged less than two years in a cohort study in a peri-urban community of Peru

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Running title: Etiological role and repeated infections of sapovirus

Abstract

Human sapovirus has been shown to be one of the most important etiologies in pediatric patients with acute diarrhea. However, very limited data are available about the causative roles and epidemiology of sapovirus in community settings. A nested matched case-control study within a birth cohort study of acute diarrhea in a peri-urban community in Peru from 2007 to 2010 was conducted to investigate the attributable fraction (AF) and genetic diversity of sapovirus. By quantitative reverse transcription

real-time polymerase chain reaction (RT-qPCR), sapovirus was detected in 12.4% (37/299) of diarrheal and 5.7% (17/300) of non-diarrheal stools (p=0.004). Sapovirus AF (7.1%) was higher in the second (13.2%) than the first year (1.4%) of life of children. Ten known genotypes and one novel cluster (n=5) within four genogroups (GI, GII, GIV and GV) were identified by phylogenetic analysis of partial VP1 gene. Further sequence analysis of full VP1 gene revealed a possible novel genotype, tentatively named as GII.8. Notably, symptomatic reinfections with different genotypes within the same (n=3) or different genogroups (n=5) were observed in eight children. Sapovirus exhibited high attributable burden for acute gastroenteritis especially in the second year of life of children in a Peruvian community. Further large-scale studies are needed to understand better the global burden, genetic diversity, and repeated infections of sapovirus.

Key words: sapovirus; diarrhea; attributable fraction; genotype, case-control study

Introduction

Acute diarrhea is one of the most important causes of morbidity and mortality in

pediatric populations especially in developing countries. Rotavirus, norovirus and other viruses are common causative etiological agents and rotavirus accounts for about 440, 000 child deaths annually (1), whilst norovirus is a leading cause of epidemic and sporadic acute diarrhea (2). Currently a rotavirus vaccination program has been implemented in 80 countries as a part of national immunization programs (http://sites.path.org/rotavirusvaccine/rotavirus-vaccines/#global-intro). It successfully reduced the number of hospitalizations and deaths due to acute gastroenteritis (3, 4) and is cost-effective (5). Norovirus has now replaced rotavirus as the leading cause of medically attended acute diarrhea in pediatric populations (6, 7) and sapovirus, belonging to a separate genus of the Caliciviridae family, has been reported as the second most commonly detected virus after norovirus in children with acute diarrhea where rotavirus vaccination was implemented (8, 9). In addition, reports on sapovirus outbreaks across all age groups have increased in South Asia, Europe and North America recently (10-14).

The genome of sapovirus consists of a positive-sense, single-stranded RNA with two open reading frames (ORFs) (12). ORF1 encodes the nonstructural proteins and a

65 major capsid protein, VP1, and ORF2 encodes a protein whose function is still unknown
66 (12). Like norovirus, multiple genetic clusters of human sapovirus have been reported
67 including four genogroups (GI, GII, GIV, and GV) with 17 genotypes (GI.1–7, GII.1–7,
68 GIV.1, GV.1–2) (12). Mild acute diarrhea, severe and even fatal sapovirus infections
69 have been documented (15, 16). Viral shedding lasts about two weeks after onset of
70 symptoms (17, 18). Serological studies have identified that sapovirus infection are very
71 common during early childhood, however, epidemiology and protective immunity in
72 community settings have rarely been investigated. Sapovirus was identified as a
73 significant viral etiological agent in children in limited etiological studies of acute
74 diarrhea, which targeted multiple pathogens (19-21). To understand the etiological role
75 and genetic diversity of sapovirus for acute diarrhea, we conducted a nested matched
76 case-control study by analyzing selected samples from a birth cohort study of acute
77 diarrhea in a Peruvian peri-urban community conducted between June 2007 and May
78 2010 (22).
79
80 **Materials and Methods**

Stool sample selection

Stool samples collected during or one day after diarrhea episodes (n=862) were considered as diarrhea sample in this study. We stratified the samples previously tested for norovirus (22) by eight age groups (0–2, 3–5, 6–8, 9–11, 12–14, 15–17, 18–20, and 21–23 months) and four seasons (Dec-Feb, Mar-May, Jun-Aug and Sep-Nov). We assumed sapovirus should be detected in at least 5% of diarrheal episodes in children under two years based on detection rates of 5.4–16.6% by quantitative real-time PCR (qPCR) reported in two population based studies (9, 21). To ensure that at least 1 positive sample was detected in each age group, at least 20 samples were tested per group. Finally, 300 diarrheal stools (26–44 stools per age group) representing 34.2% of 862 episodes were randomly selected by using Stata 13 (StataCorp, College Station, TX, US). One non-diarrheal control specimen per diarrheal stool was selected at random, matched by age group and season using Stata 13. Non-diarrheal stools were defined as stools which were not collected within the diarrhea episode, nor within a week before the first day of diarrhea episodes, or within two weeks after the last day of diarrhea episodes, based on sapovirus incubation period (12) and duration of virus shedding (17,

18). Both diarrhea and non-diarrhea samples were selected from the samples previously tested for norovirus and included regardless of norovirus test results. Among 600 randomly selected samples, three samples (all negative for norovirus) were not available in the specimen bank and two of them were replaced with other samples in the same age-season category, therefore 599 samples were included in the analysis.

Quantitative reverse transcription real-time polymerase chain reaction (RT-qPCR) for sapovirus

Viral nucleic acid was extracted from 140 μl of 10% stool suspension using QIAamp viral RNA mini kits (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized by M-MLV reverse transcriptase kits with random primers (Life Technologies, Carlsbad, CA, USA). qPCR targeting polymerase-VP1 junction of sapovirus was performed on an ABI 7500 Fast Real-Time PCR system (Life Technologies), using previously described primers and probes (23). The detection limit of qPCR, which was determined by standard curves generated with 10-fold serial dilutions (10^7 to 10 copies/μl) of sapovirus GII.1 standard plasmid, was 100 copies/μl of cDNA corresponding to the cycle threshold (Ct) value of 38 at amplification of total 45

113 cycles. A sample with a Ct value <38 was considered to be positive for sapovirus and
114 three positive and one negative controls were included in each assay.

115 **Genotyping and sequence analysis**

116 Sapovirus positive samples were further amplified by hemi-nested PCR targeting
117 partial VP1 gene (720 nt, 5159–5878 nt corresponding of Manchester X86560) with the
118 external primers SV F13/F14 (24) and SaV 5857R-1/-2, and internal primers SaV
119 F1245 (23) and SaV 5857R-1/-2 (Supplementary Table 1). Cycling conditions for the
120 first and second rounds of hemi-nested PCR were 94°C for 5 min, 35 cycles of 94°C for
121 30 sec, 50°C for 30 sec and 72°C for 1 min, followed by 1 cycle of 72°C for 10 min.
122 For the possible new genotype of sapovirus, the full VP1 gene was amplified with
123 primer pair SV F13/F14 and SaV GIIR under same cycling conditions as hemi-nested
124 PCR. PCR products were sequenced using BigDye Terminator chemistries V1.1 (Life
125 Technologies) in a 3730 Genetic Analyzer (Life Technologies). Resulting sequences
126 were BLAST-verified and all unique sequences were deposited in GenBank (Accession
127 No. KT276516-KT276558, KU886206-KU886207, and KT306742). Genogroups and
128 genotypes were characterized by phylogenetic analysis of partial VP1 gene (606nt,

5236–5841 nt corresponding of Manchester X86560) (12) using the maximum likelihood method (Kimura 2-parameter model) in MEGA 5 (25). In addition, pairwise distances were calculated using Kimura 2-parameter method in MEGA 5 and the cut-off values to designate the same sapovirus genotypes and genogroups were ≤ 0.169 and ≤ 0.488, respectively (12, 26).

Statistical analysis

Unadjusted attributable fraction (AF) for estimating sapovirus burden of diarrhea was calculated by using the following formula (27);

$$AF=Pr(SaV \mid diarrhea)\left(1-\frac{1}{OR}\right)$$

where Pr(sapovirus/diarrhea) is the proportion of sapovirus in diarrhea stools and OR is the odds ratio of sapovirus for diarrhea and non-diarrhea. The median Ct value between diarrheal and non-diarrheal stools was compared by Mann-Whitney U test, using Stata 13 (StataCorp).

Ethics statement

143 Mothers provided written informed consent and the study was approved by the
144 Ethics Committees of Tohoku University Graduate School of Medicine, Asociación
145 Benéfica PRISMA, Universidad Peruana Cayetano Heredia (UPCH), Johns Hopkins
146 University, the Centers for Disease Control and Prevention (CDC), and the European
147 Union.
148
149 **Results**
150 Three hundred non-diarrheal and 299 diarrheal stools (one diarrheal stool in age
151 group 18–20 was not available) were tested for sapovirus. Sapovirus was detected in
152 9.0% (54/599) of stools from children aged under 24 months, with 12.4% in diarrheal
153 (37/299) and 5.7% in non-diarrheal stools (17/300) (p=0.004, Figure 1). In qPCR, the
154 median Ct values were significantly lower in diarrheal than non-diarrheal samples (24
155 vs. 29, p=0.024). Among the 37 sapovirus-associated episodes, the median duration of
156 diarrhea was 3 days (interquartile range [IQR], 2–5 days). The median maximum daily
157 number of diarrheal stools was four (IQR, 3.5–5.5). Fifteen episodes (40.5%) had
158 vomiting and only four episodes (10.8%) had fever. Detection rates of sapovirus in

159 diarrheal stools were higher in children aged 12–23 months (18.6%, 27/145) than
160 children aged <12 months (6.5%, 10/154) (p=0.002) and those in non-diarrheal stools
161 were similar between the two age groups (5.2% vs. 6.2%). The sapovirus AF (7.1%)
162 was higher in second (13.2%) than first year of life of children (1.4%). Sapovirus was
163 not detected in diarrheal stools of children aged 0–5 months (Figure 1), although only
164 68 diarrhea samples of 194 episodes were tested in this age group. Sapovirus was
165 detected together with norovirus in four diarrheal stools in the age group of 12–23
166 months.

167 Of 54 qPCR positive specimens, 45 specimens (83.3%) from 2007 to 2010 were
168 successfully sequenced. Genogroup I (n=21) and genogroup II (n=16) were detected
169 more frequently than genogroup IV (n=3) and genogroup V (n=5). Among these
170 genogroups, ten known genotypes (GI.1, GI.2, GI.6, GI.7, GII.1, GII.2 GII.4, GII.5,
171 GIV, and GV.1) were identified (Figure 2). Most genotypes were detected both in
172 diarrhea and non-diarrhea stools except two genotypes (GII.5 and GIV), which were
173 found only in one and three diarrhea stools, respectively. Interestingly, five Peruvian
174 sequences together with strains detected in USA (HM59058) and Taiwan (KM092511)

175 fell into a distinct cluster in GII with less than 78% similarity with other genotypes of
176 GII based on the partial VP1 gene. We further sequenced the full VP1 of one diarrhea
177 strain in this cluster and found that this Peruvian VP1 together with another full VP1
178 from Taiwan (KM092511) also formed a distinct cluster from other GII genotypes at the
179 phylogenetic tree of nucleotides (supplementary Figure 1) and amino acid sequences
180 (Figure 3). Moreover, the pairwise nucleotide distance of Peruvian VP1 from other
181 GII.1–7 genotypes (0.298–0.468) was larger than 0.169. Therefore, this cluster could be
182 tentatively classified as a novel genotype and the tentative genotype of GII.8 is
183 proposed.

184 Eight children had two sapovirus infections detected within short time (the interval
185 of two infections ranging from 20 days to 19 months, see Table 1). Five of these
186 children had repeated infections with a virus belonging to a different sapovirus
187 genogroup, and three children had repeated infections with a different genotype of the
188 same genogroup. All first and second infections of the eight children were symptomatic,
189 except in one child where the first infection with GII.1 was asymptomatic and the
190 second with GII.2 was symptomatic. None of repeated infections of sapovirus had

concurrent norovirus infection. The median Ct value of both the first (21, IQR, 17.5–25.5) and second infections (21, IQR, 15.8–27.6) were relatively low, suggesting that both infections had relatively large viral load. In addition, one child was considered to have shed sapovirus at 25 days after the onset of diarrhea since one diarrheal stool (Peru-406-D, Ct=24.2) and one non-diarrheal sample (Peru-426-ND, Ct=33.5) collected from the same child were found to contain the same GII.4 strain (Figure 2).

Discussion

We determined the positive rate and genotype distribution of sapovirus in a birth cohort study in Peru. Sapovirus had a considerable attributable burden of 7.1% for acute diarrhea in children under two years old in this community. In sapovirus infected children less than two years old, vomiting was frequently reported, while fever was rarely reported. The median duration of sapovirus diarrhea observed in this study was similar to the previously reported duration of 3–5 days (8, 17). When compared to other etiological studies of sapovirus, the overall detection rate of sapovirus in symptomatic children (12.4%) in this study was similar to that in UK (11.1%) (19) and Nicaragua (16.6%) (9), but higher than in the Netherlands (7.8%) (20) and USA (5.4%) (21). The detection rate of sapovirus in asymptomatic children

207 observed (5.7%) in this study was similar to that in USA (4.2%) (21), but higher than in the Netherlands
208 (1.8%) (20), UK (2.4%) (19) and Nicaragua (1.9%) (9). Our results are comparable with recent reports
209 from other continents, in which sapovirus was commonly detected in pediatric gastroenteritis (8, 28-31).
210 Possible reasons of increased detection rates of sapovirus include the emerging genotypes (10, 29, 31),
211 the implementation of rotavirus vaccination (9), and application of more sensitive molecular diagnostic
212 assays (32, 33). Limited data of sapovirus in South America is available (34) and more studies are needed
213 to confirm a clinical and public health significance of sapovirus in this region.

214 Our study found that sapovirus is more likely to be associated with acute diarrhea in the second year
215 of life of children, as described for norovirus in the same cohort (22). This finding was supported by
216 several recent reports, in which sapovirus was detected more commonly in symptomatic children aged
217 12–23 months than <1 year (9, 21, 35, 36). Lower detection rates of sapovirus in children aged <1 year
218 possibly reflect a protective effect of breastfeeding and/or transferred maternal antibodies (22, 37), as
219 supported by the fact that we did not detect sapovirus in symptomatic children under 6 months.

220 In this study, ten known genotypes and one novel cluster were detected, and none
221 of them appeared to be predominant, which differed from norovirus GII.4, having been
222 predominant in the same cohort (22) or worldwide for past two decades (38). The

emerging genotypes GI.2 and GIV, which caused outbreaks in various countries recently (10, 11, 14, 15), were detected, but not as dominant genotypes in this Peruvian community. Some uncommon genotypes of sapovirus such as GI.6, GI.7, GII.4 and GII.5 were observed. Five strains detected from 2008–2009 formed a distinct cluster and appear to belong to a novel genotype. BLAST searches from GenBank showed similar sequences deposited from South Africa (2010 and 2013), USA (2010), China (2011) and Taiwan (2014). Noteworthy, the strains in USA and Taiwan were associated with outbreaks of diarrhea, suggesting its wide distribution and pathogenicity. Currently, two complete VP1 sequences for this possible novel genotype are available from two countries (Peru and Taiwan), and it is tentatively proposed as GII.8, following both phylogenetic analysis of amino acid sequence (39) and pairwise distance classification proposed by Oka, T et al (12).

The cohort study design allowed us to identify repeated sapovirus infections in same child. Five children had symptomatic reinfections with different genogroups, and a similar observation was reported in two patients in Japan (40). Also, the occurrence of natural reinfections in pigs by different porcine sapovirus genogroups has been recently

239 reported (41). In addition, we found three children experienced a second diarrheal
240 episode caused by a different sapovirus genotype in a period of less than one month to
241 19 months. In the same cohort, children were found to commonly experience multiple
242 diarrheal episodes with different norovirus GII genotypes and therefore it was
243 speculated that children can develop genotype-specific immunity of norovirus with only
244 a modest level of cross-protection even within the same genogroup (22). In contrast,
245 very little information is available about protective immunity against sapovirus
246 infection. The serum collected from naturally infected human or immunized animals
247 with recombinant VP1 protein of sapovirus were shown to be moderately cross reactive
248 against the heterologous genotypes and weakly with heterologous genogroups of
249 sapovirus (42-44). Large-scale studies are needed to investigate whether protective
250 immunity for sapovirus is genotype specific or not since the number of children with
251 reinfections in this study was small due to limited sample size.

252 One of the limitations of this study was that samples were not tested for other
253 enteric pathogens except norovirus, therefore co-infections of sapovirus with other
254 pathogens was not analysed. The attribution of sapovirus in acute diarrhea should be

255 validated in the future study including other major enteric pathogens. Also, a small
256 proportion of symptomatic cases may shed sapovirus longer than two weeks after onset
257 of illness, as seen in the current and other studies (17, 18), therefore the prevalence of
258 sapovirus in asymptomatic children in this study could be slightly over-estimated and
259 the viral shedding pattern need to be furthered explored.

260 In conclusion, recent epidemiological reports have highlighted the importance of
261 sapovirus in acute diarrhea, especially after rotavirus vaccine implementation, but the
262 etiological role and epidemiology of sapovirus remains to be clarified. We found
263 sapovirus exhibited high attributable burden of acute diarrhea especially in the second
264 year of life in a peri-urban community in Peru. Co-circulation of multiple genotypes
265 with a possible novel genotype and symptomatic reinfection with different genotypes
266 within the same or different genogroups were observed. Further large-scale studies are
267 needed to define the global burden, incidence and the protective immunity of sapovirus
268 infection.

269

270 **Notes**

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Potential conflicts of interests

All authors report no potential conflict.

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454 **Figure legends**

455 **Figure 1. Prevalence of sapovirus in diarrheal and non-diarrheal stool samples by**

456 **age in the case-control study in Peru.**

457 The y-axis indicates the percentage of stools in which sapovirus was detected. The

458 x-axis shows age groups of infants with stools. The black bars and grey bars indicate

459 diarrheal and non-diarrheal stools. Error bars represent 95% confidence intervals. One

460 diarrheal sample was available in the age group of 18–23.

461 **Figure 2. Phylogenetic analysis of sapovirus based on partial VP1 region (606nt,**

462 **5236–5841 nt corresponding of Manchester X86560)** The phylogenetic tree was

463 inferred using the maximum likelihood method (Kimura 2-parameter model) using

464 MEGA5. The tree was drawn to scale, with branch lengths in the units of the number of

465 base substitutions per site. The percentage of trees (≥70%) in which the associated taxa

466 clustered together is shown next to the branches. Peru strains are marked with filled

467 triangles and are named as country-tested number-diarrheal or non-diarrheal.

468 **Figure 3. Phylogenetic analysis of full VP1 amino acid sequences of sapovirus.** The

469 phylogenetic tree was inferred using the maximum likelihood method (General Reverse

Transcriptase + Freq. model) using MEGA5. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The VP1 obtained in this study is marked with a filled triangle.

476 **Table 1. Characteristics of repeated infections of sapovirus observed in eight children in the birth cohort study in Peru**

477

Infant ID	Sampling date	Age (month)	Ct value	sapovirus Genotype	Accession Number	Symptoms	Duration of symptoms (day)
PX046	13-Jul-08	13	16.0	GI.6	KT276525	Diarrhea,vomiting	2
	16-Nov-08	17	15.5	GI.1	KT672521	Diarrhea	3
PX152	19-Jan-09	16	26.8	GI.6	KT276526	Diarrhea	5
	10-Feb-09	16	31.9	GI.7	KT276536	Diarrhea,vomiting	6
PX198	12-May-08	4	23.5	GII.1	KU886207	none	0
	15-Dec-09	23	31.4	GII.2	KT276555	Diarrhea	2
PX080	25-Aug-08	12	21.5	GIV	KT276558	Diarrhea,vomiting	2

	25-May-09	21	21.1	GII.2	KT276554	Diarrhea	2
PX135	17-Mar-08	6	34.4	GIV	KU886206*	Diarrhea, fever	12
	19-Jun-09	21	15.9	GV.1	KT276538	Diarrhea,vomiting	2
PX159	19-Jun-08	9	16	GII.4	KT276549	Diarrhea,vomiting	5
	27-Jan-09	16	15.7	GI.1	KT276520	Diarrhea	4
PX212	20-Apr-09	15	18.9	GI.1	KT276522	Diarrhea, fever	3
	30-Oct-09	21	23.9	GII.8	KT276546	Diarrhea	3
PX287	18-Jun-09	8	25.8	GIV	KT276557	Diarrhea,vomiting	6
	12-Oct-09	11	19.4	GI.1	KT276519	Diarrhea,vomiting	3

478 * This sequence has 450 bp and was not shown in Figure 2

479