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Quantification and Genotyping of Human Sapoviruses in the Llobregat River Catchment, Spain▽†

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Human sapoviruses (SaVs) were quantified and characterized in an 18-month survey conducted along the Llobregat river catchment area in Spain. Sample types included freshwater, untreated and treated wastewater, and drinking water. All genogroups were recovered, and a seasonal distribution was observed. This is the first report of SaV quantification and genotyping in the environment outside Japan.

Human enteric viruses occur in the water environment, even in developed communities (9, 20). Viruses causing gastroenteritis are acknowledged as a major health concern, and human sapoviruses (SaVs) are increasingly recognized as a major cause of acute diarrhea, mainly in children, although their medical significance is still poorly defined (8, 18, 21). SaVs are members of the family *Caliciviridae* and are nonenveloped, positive-strand RNA viruses (5). The prototype Sapporo strain was identified in an outbreak of diarrhea in an orphanage in Sapporo, Japan, in October 1977 (15), and since then, 15 genotypes in four genogroups (GI.1 to GI.8, GII.1 to GII.5, GIV, and GV) have been described as human SaVs (18). Because of the lack of cell cultures and animal models to replicate SaVs, information on the characteristics, such as tropism and virulence, of SaVs is scarce. SaVs have been detected by reverse transcription (RT)-PCR from a variety of epidemiological sources, including fecal specimens from symptomatic and asymptomatic individuals (1, 21), environmental water (6, 12, 13), and bivalves (7, 22) in Japan, indicating that SaVs can be transmitted via the fecal-oral route through water and contaminated foods, as well as through person-to-person contact.

A study of the occurrence of SaVs in the Llobregat river catchment area in Catalonia, northeastern Spain, was conducted monthly from November 2007 to April 2009. The Llobregat river is the second largest river in Catalonia, flowing 170 km from its source in the pre-Pyrenees mountains to the Mediterranean Sea, and is the source of drinking water for over 5 million inhabitants of municipalities around Barcelona (Fig. 1; see the supplemental material). The Llobregat river receives urban and industrial discharges from more than 30 sewage treatment plants (2). Since the drinking water treatment plant (DWTP) is located near the point where the river flows into the sea, pollution of river water represents a potential health risk for its consumers (14). Different types of samples were collected from 12 sites (Fig. 1; see the supplemental material): freshwater (S1, S2, S3, S4, S7, and S9), urban untreated sewage (S5, S8, and S12), urban treated wastewater (S6), and semitreated (prechlorination, flocculation, decanta-

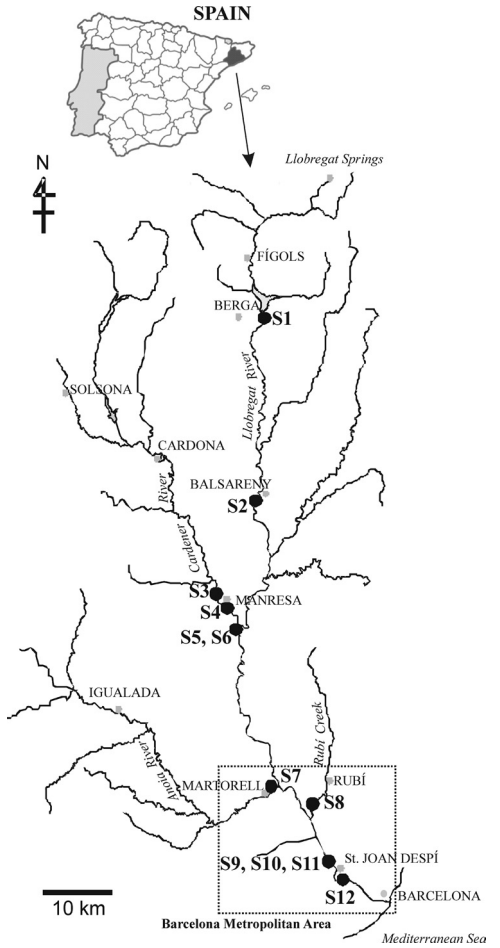

FIG. 1. Sampling points in the Llobregat river catchment area. Sampling points S5 and S6 are the inflow and outflow, respectively, of a WWTP, and S9, S10, and S11 correspond to different treatment steps in the DWTP (see the supplemental material).

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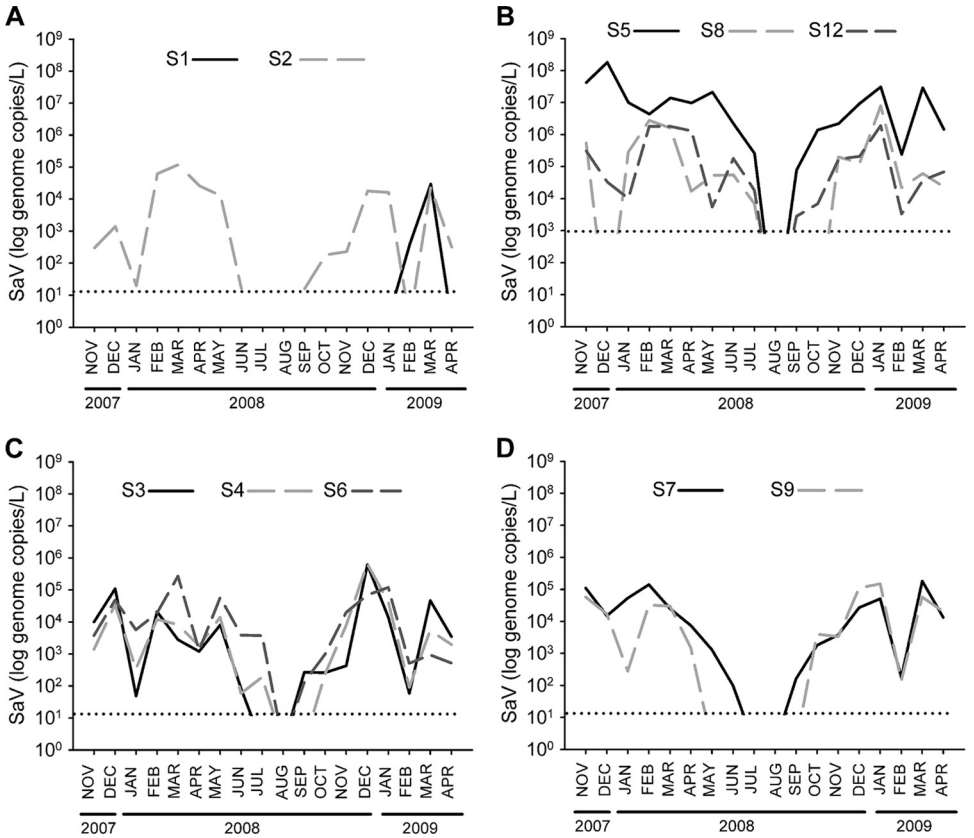

FIG. 2. Quantification of the numbers of SaV genome copies in water samples from the Llobregat river catchment area. A, numbers of SaV genome copies in samples from the beginning of the catchment area (S1 and S2); B, numbers of SaV genome copies in urban untreated sewage samples (S5, S8, and S12); C, numbers of SaV genome copies in a treated effluent from a wastewater treatment plant in Manresa (S6) and its neighboring river water samples (S3 and S4); D, numbers of SaV genome copies in downstream river water samples (S7 and S9). Dotted lines indicate the quantification limits of the SaV gene, which are 17 copies/liter for river water and treated effluent samples and 1,120 copies/liter for urban untreated sewage samples.

tion, sand filtration, ozonation, and carbon filtration; S10), and final (final chlorination, S11) drinking water. Viruses were concentrated from all types of water except raw sewage by filtration of 10-liter samples through positively charged glass wool (Ouest Isol, Alizay, France) and eluted twice with 50 ml glycine-beef extract buffer, pH 9.5 (13). The 100-ml eluate was further concentrated by polyethylene glycol (PEG) precipitation (23). The resulting pellet was resuspended in 20 ml of phosphate-buffered saline, pH 7.4, and stored at −80°C until further analysis. Viruses were recovered from 600-ml untreated sewage samples in a final volume of 24 ml by PEG precipitation. Viral RNA was extracted from the virus concentrates by using the NucliSens miniMAG magnetic system (bioMérieux) according to the manufacturer's instructions. SaVs were quantified by a one-step real-time quantitative RT-PCR (qRT-PCR) using previously described primers and probes (17) (see Table S1 in the supplemental material). Virus/nucleic acid extraction and enzyme efficiencies were monitored as described elsewhere (3, 19) and used to estimate actual genome copy numbers from the raw genome numbers measured by qRT-PCR. The nucleotide sequence of a 292- to 322-nucleotide fragment was obtained by nested RT-PCR amplification with primers targeting the RNA-dependent RNA polymerase/capsid junction region in open reading frame 1 (see Table S1 in the supplemental material) and the Thermo Sequenase II Dye Terminator Cycle Sequencing Premix kit (Amersham Pharmacia Biotech). Each nucleotide sequence was compared to those of reference strains using the BLAST program (National Center for Biotechnology Information) in order to assign a genotype, and a phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining approach using ClustalX software (version 2.0.10).

To our knowledge, this is the first report of the quantification and genotyping of human SaVs in the environment outside Japan. SaVs have also been qualitatively detected in river water in Kenya (13). All samples of semitreated (S10) and final (S11) drinking water taken at the DWTP were negative for SaVs. The advanced processes employed in the DWTP significantly reduce the concentration of SaVs present in the source water. However, information on issues such as statistical inference of virus concentration, infectivity, and exposure dose is required to ascertain the health risk of SaV infection through drinking water consumption.

At all other sampling sites, higher concentrations of human SaVs were observed from late autumn to spring, and concentrations sharply decreased in summer (Fig. 2). This seasonality is similar to that reported in Japan (10), although the quantified values in the Japanese study were not corrected using

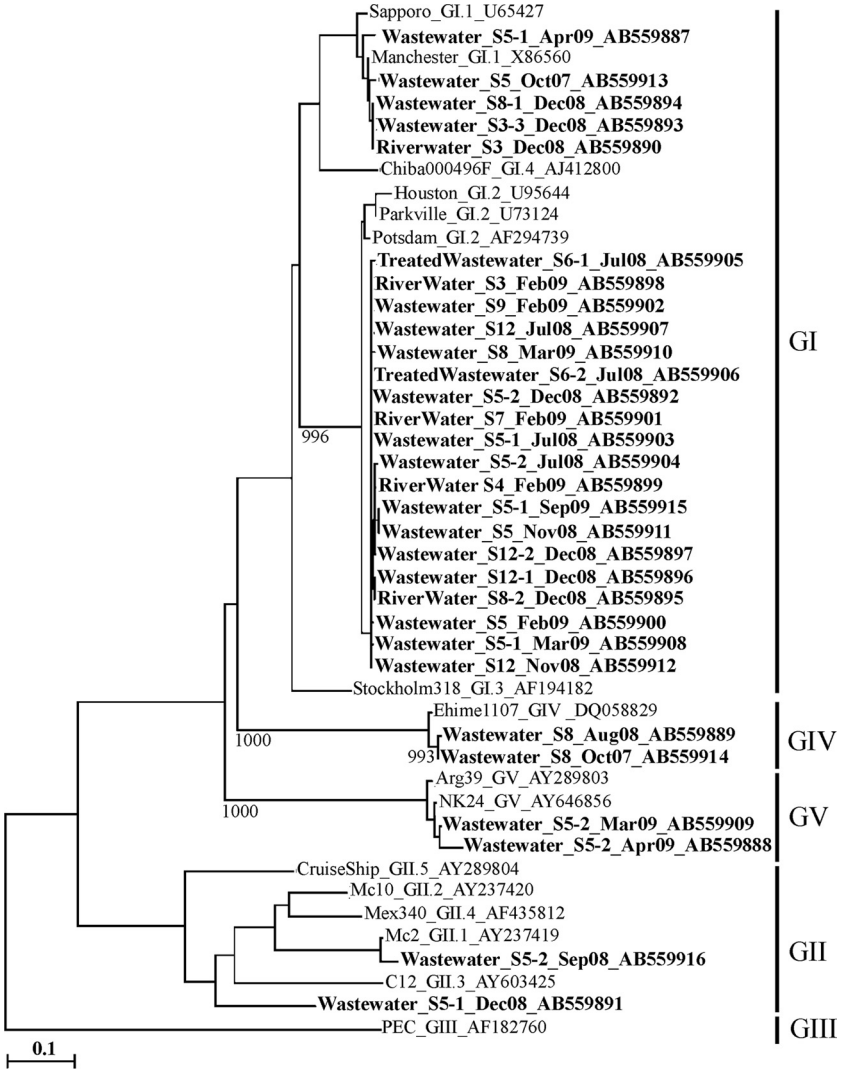

FIG. 3. Phylogenetic analysis of SaVs in water samples from the Llobregat river basin. This phylogenetic tree was created by the neighbor-joining approach with 1,000 bootstrap replicates using ClustalX software (version 2.0.10). The sequence from porcine SaV (GIII) was used as an outgroup. Only bootstrap values higher than 950 are displayed. The scale bar represents the number of substitutions per site. Bold type indicates the SaV sequences obtained in this study.

appropriate controls. The marked drop observed in summer is likely due to a decrease in SaV infections at this time of the year. However, the presence of such seasonality in the population remains to be elucidated. Very high virus titers (up to 1.8×10^8 genome copies/liter at sampling point S5) were observed in raw wastewater. Overall, the numbers of SaV genome copies/liter observed in this study are much higher than those reported in Japan (10), both in wastewater (1,000 times higher) and in freshwater (100 times higher). This increase could be due to the correction applied to the raw numbers of genome copies, after taking into consideration the virus/nucleic acid extraction and enzyme efficiencies, which provides a more accurate estimation of the actual genome copy numbers (3, 19).

The Manresa wastewater treatment plant (WWTP) receives raw sewage (S5) corresponding to 85,224 inhabitants. After primary sedimentation and activated sludge treatment (S6), the mean log reduction in the number of SaV genome copies/liter is 2.9 (maximum, 4.5; minimum, 1.7; standard deviation, 0.8), indicating that conventional wastewater treatment processes employed at this WWTP can reduce SaV levels by almost 3 logs. This apparent removal efficiency is significantly higher than that reported for norovirus (NoV); reductions of NoV genogroup I (GI) and GII, respectively, of 0.7 to 1.4 log and 1.2 log have been observed in a WWTP employing conventional wastewater treatment processes (11, 16). Although both genera belong to the same family, *Caliciviridae*, the capsids of SaV and NoV show different physicochemical properties, which may explain the different virus behaviors in WWTPs (4).

In this study, all human SaV genogroups (GI, GII, GIV, and GV) were detected. A total of 30 sequences were obtained (Fig. 3), including 5 GI.1, 19 GI.2, 2 GII, 2 GIV, and 2 GV sequences. The most abundant genotype was GI.2, which was isolated from July 2008 to March 2009. Multiple genotypes were observed in some wastewater samples from S5 (July,

September, and December 2008, and March 2009), and GI.2 was always the most prevalent sequence in these samples. On the clinical side, GI.2 has recently been detected quite frequently from gastroenteritis patients during a survey conducted in 2007 to 2009 in Netherlands, Sweden, Russia, and Slovenia (21), implying that GI.2 is widespread throughout Europe. Additionally, GI.2 shows a viral load in fecal samples that seems to be comparatively greater than that of other SaV types and possibly also shows increased virulence (24). Despite extensive testing, human SaVs were found only in sporadic cases of gastroenteritis before 2007 (21). Our data from environmental samples and clinical data from other parts of the world (21, 24) point to the emergence of SaVs as human pathogens with high environmental prevalence. A study is in progress to shed more light on the etiological role of these poorly understood viruses in gastroenteritis outbreaks in Catalonia.

Nucleotide sequence accession numbers. The nucleotide sequences of SaVs determined in this study were deposited in the DNA Data Bank of Japan under accession numbers AB559887 to AB559916.

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REFERENCES

1. **Akihara, S., et al.** 2005. Identification of sapovirus infection among Japanese infants in a day care center. J. Med. Virol. **77:**595–601.
2. **Céspedes, R., et al.** 2005. Distribution of endocrine disruptors in Llobregat River basin (Catalonia, NE Spain). Chemosphere **61:**1710–1719.
3. **Costafreda, M. I., A. Bosch, and R. M. Pintó.** 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl. Environ. Microbiol. **72:**3846–3855.
4. **Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.).** 2005. Eighth report of the International Committee on Taxonomy of Viruses, p. 843–851. Elsevier Academic Press, New York, NY.
5. **Hansman, G. S., et al.** 2007. Outbreak of gastroenteritis due to sapovirus. J. Clin. Microbiol. **45:**1347–1349.
6. **Hansman, G. S., et al.** 2007. Sapovirus in water, Japan. Emerg. Infect. Dis. **13:**133–135.
7. **Hansman, G. S., et al.** 2007. Human sapovirus in clams, Japan. Emerg. Infect. Dis. **13:**620–622.
8. **Harada, S., et al.** 2009. Surveillance of pathogens in outpatients with gastroenteritis and characterization of sapovirus strains between 2002 and 2007 in Kumamoto Prefecture, Japan. J. Med. Virol. **81:**1117–1127.
9. **Haramoto, E., H. Katayama, K. Oguma, and S. Ohgaki.** 2005. Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan. Appl. Environ. Microbiol. **71:**2403–2411.
10. **Haramoto, E., H. Katayama, C. Phanuwan, and S. Ohgaki.** 2008. Quantitative detection of sapoviruses in wastewater and river water in Japan. Lett. Appl. Microbiol. **46:**408–413.
11. **Haramoto, E., et al..** 2006. Seasonal profiles of human noroviruses and indicator bacteria in a wastewater treatment plant in Tokyo, Japan. Water Sci. Technol. **54:**301–308.
12. **Kitajima, M., et al.** 2010. Detection and genetic analysis of human sapovirus in river water in Japan. Appl. Environ. Microbiol. **76:**2461–2467.
13. **Kiulia, N. M., et al.** 2010. The detection of enteric viruses in selected urban and rural river water and sewage in Kenya, with special reference to rotavirus. J. Appl. Microbiol. **109:**818–828.
14. **Lucena, F., A. Bosch, J. Ripoll, and J. Jofre.** 1988. Fecal pollution in Llobregat river: interrelationships of viral, bacterial, and physico-chemical parameters. Water Air Soil Pollut. **39:**15–25.
15. **Nakata, S., et al.** 1983. Microtiter solid-phase radioimmunoassay for detection of human calicivirus in stools. J. Clin. Microbiol. **17:**198–201.
16. **Nordgren, J., A. Matussek, A. Mattsson, L. Svensson, and P.-E. Lindgren.** 2009. Prevalence of norovirus and factors influencing virus concentrations during one year in a full-scale wastewater treatment plant. Water Res. **43:**1117–1125.
17. **Oka, T., et al.** 2006. Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction. J. Med. Virol. **78:**1347–1353.
18. **Phan, T. G., et al.** 2006. Outbreak of sapovirus infection among infants and children with acute gastroenteritis in Osaka City, Japan during 2004–2005. J. Med. Virol. **78:**839–846.
19. **Pintó, R. M., M. I. Costafreda, and A. Bosch.** 2009. Risk assessment in shellfish-borne outbreaks of hepatitis A. Appl. Environ. Microbiol. **75:**7350–7355.
20. **Pusch, D., et al.** 2005. Detection of enteric viruses and bacterial indicators in German environmental waters. Arch. Virol. **150:**929–947.
21. **Svraka, S., et al.** 2010. Epidemiology and genotype analysis of emerging sapovirus-associated infections across Europe. J. Clin. Microbiol. **48:**2191–2198.
22. **Ueki, Y., et al.** 2010. Detection of sapovirus in oysters. Microbiol. Immunol. **54:**483–486.
23. **Ueki, Y., D. Sano, T. Watanabe, K. Akiyama, and T. Omura.** 2005. Norovirus pathway in water environment estimated by genetic analysis of strains from patients of gastroenteritis, sewage, treated wastewater, river water and oysters. Water Res. **39:**4271–4280.
24. **Wu, F.-T., et al.** 2008. Acute gastroenteritis caused by GI/2 sapovirus, Taiwan, 2007. Emerg. Infect. Dis. **14:**1169–1171.