Short-Form Paper

Quantification and genotyping of human sapoviruses

in the Llobregat River catchment, Spain.

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Running title: Human sapoviruses in environmental water

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Human sapoviruses (SaVs) were quantified and characterized in an 18-month survey conducted along the Llobregat river catchment in Spain. Sample types included fresh water, untreated and treated wastewater and drinking water. All genogroups were recovered and a seasonal distribution was observed. This is the first report of SaVs quantification and genotyping in the environment outside of 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 non-enveloped, 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 4 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 characteristics such as tropism and virulence of SaVs is scarce. SaVs have been detected by 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 on the occurrence of SaVs in the Llobregat river catchment in Catalonia, NE Spain, was conducted monthly from November 2007 to April 2009. The Llobregat river is the second largest river in Catalonia, flowing for 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 in...
municipalities around Barcelona (Fig 1 and 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, river water pollution represents a potential health risk for the consumers (14). Different types of samples were collected from 12 sites (Fig 1 and Supplemental Material): fresh water (S1, S2, S3, S4, S7 and S9), urban untreated sewage (S5, S8 and S12), urban treated wastewater (S6), and semitreated (pre-chlorination, flocculation, decantation, 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-L samples through positively-charged glass wool (Ouest Isol, Alizay, France), and were 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 PBS, pH 7.4, and stored at -80°C until further analysis. Viruses were recovered from 600-mL untreated sewage samples into a final volume of 24 mL by PEG precipitation. Viral RNA was extracted from the virus concentrates using the NucliSens miniMAG magnetic system (BioMérieux), following the manufacturer’s instructions. SaVs were quantified by a one-step real-time RT-PCR (qRT-PCR) using previously described primers and probes (17; Table 1S, 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 292–322nt fragment was obtained by using a nested RT-PCR amplification with primers targeting the RNA-dependent RNA polymerase /capsid junction region in ORF1 (Table S1, 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). The nucleotide sequences of SaVs determined in this study were deposited in DNA Data Bank of Japan under the accession numbers from AB559887 to AB559916. To our knowledge, this is the first report of quantification and genotyping of human SaVs in the environment outside of 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 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 $1.8 \times 10^8$ genome copies/L at sampling point S5) were observed in raw wastewater. Overall, concentrations of SaV genome copies/L observed in this study are much higher than those reported in Japan (10), both in wastewater (1000 times higher) and fresh water (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 of SaV genome copies / L is 2.9 (Max: 4.5, Min: 1.7, standard deviation SD: 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) for which reductions of 0.7 - 1.4 log and 1.2 log have been observed for NoV genogroup I (GI) and genogroup II (GII), respectively, in a WWTP employing conventional wastewater treatment processes (16). Although both genera belong to the same Caliciviridae family, the capsid of SaV and NoV show different physicochemical properties that may explain the different virus behavior 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 2007-2009 in The Netherlands, Sweden, Russia, and Slovenia (21), implying that GI.2 is widespread throughout Europe. Additionally, GI.2 viral load in fecal samples seems to be comparatively higher than 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.

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**FIGURE LEGENDS**

**Figure 1.** Sampling points in the Llobregat river catchment. 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 Supplemental Material).

**Figure 2.** Quantification of sapovirus (SaV) genome copies in water samples from the Llobregat river catchment. A, SaV genome copies in samples from the beginning of the catchment (S1 and S2); B, SaV genome copies in urban untreated sewage samples (S5, S8 and S12); C, 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, SaV genome copies in downstream river water samples (S7 and S9). Dotted lines indicate the quantification limits of SaV gene, which are 17 copies/L for river water and treated effluent samples, and 1120 copies/L for urban untreated sewage samples.

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