Quantification and molecular characterisation of human sapoviruses in water sources impacted by highly-polluted discharged wastewater in South Africa

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Abstract

Sapoviruses (SaVs) were detected and quantified in 8/10 water samples collected from wastewater treatment works (WWTW) and water sources impacted by these WWTWs in Limpopo Province, South Africa. The median SaV concentration was 2.45 x 10^6 copies/L and SaV genotypes I.2 and IV were characterised. This study provides new data on the high concentrations of clinically relevant SaVs in rivers and dams impacted by poor-performing WWTWs.

Keywords: Genotypes, sapovirus, South Africa, quantification, water

Introduction

Sapovirus (SaV), a genus in the Caliciviridae (CV) family, is increasingly being recognised as a cause of gastroenteritis outbreaks worldwide, infecting both children and adults (Lee et al., 2012,Miyoshi et al., 2010,Svraka et al., 2010). The genus is divided into at least five genogroups (GI-GV) based on the capsid gene sequence (Farkas et al., 2004). Sapoviruses that infect humans belong to GI, GII, GIV and GV (Farkas et al., 2004) and GI and GII are each further divided into at least seven genotypes (Oka et al., 2012). Sapovirus has been implicated in a waterborne outbreak of gastroenteritis in France, along with other human CVs (Bon et al., 2005) and has also been associated with foodborne outbreaks, where bivalve shellfish (Iizuka *et al.* 2010) and food handlers (Usuku et al., 2008,Kobayashi et al., 2012) were identified as suspected sources of infection. Few countries have reported the presence of SaVs in water sources. In Japan and Europe (France, Italy and Spain), SaVs have been documented in influent and effluent wastewater (Hansman et al., 2007,Sano et al., 2011,Sima et al., 2011,Di Bartolo et al., 2013). In South Africa (SA), SaVs have also been detected in discharged wastewater in several provinces of the country (Murray et al., 2013a).

Malfunctioning wastewater treatment works (WWTW) contribute significantly to the deteriorating water quality in SA (Mitchell et al., 2014). Consequently, in 2008 the Department of Water Affairs (DWA) introduced the Green Drop system which aims to improve WWTW performance by providing an incentive in the form of a scoring system. This system rates aspects of the WWTW's performance and those which perform properly are awarded Green Drop status (Mitchell et al., 2014). One of the

poorest-performing provinces is Limpopo (LP), which had the second lowest rating of 24% in the last Green Drop report (DWA, 2011). This score indicates that the province's wastewater treatment systems are in a critical state and need urgent attention. Green Drop certification criteria include a microbiological compliance score which is determined from faecal coliform counts of <1000 colony forming units (cfu) per 100 mL and is reported as the percentage of samples that have <1000 cfu/100 mL over the total number of samples tested. Over a one-year period from January to December 2012, which includes the duration of the study period, LP's average microbiological compliance was 5.75% (DWA 2012). This minimal functionality of WWTWs in the province is of particular concern for fresh produce farmers who use water sources downstream from the WWTWs to irrigate their crops. In this study, concentrations and genotypes of SaVs from water sources in a selected region of LP, including rivers and dams impacted by poor-performing WWTWs, are reported.

Methods

In January and March 2012, ten water samples (10 L) were collected from several locations in close proximity to two WWTWs in LP, SA. Samples were collected directly downstream from the two WWTWs (LP5 and LP23) as well as from two rivers (LP1 and LP7) into which the discharged wastewater flows. Samples were also collected from dams located nearby (LP9, LP16B and LP17B) and from the irrigation pivot (LP PVT) on a fresh produce farm in the close vicinity. Water samples were transported to the laboratory in cooler bags with cold packs and the temperature and pH were recorded upon arrival. Samples were stored at 4°C for no more than 24 hours, until processing.

Prior to virus recovery, mengovirus (5×10^5 genome copies) was added to each water sample as a process control (Saïd *et al.* 2014). Viruses were recovered from the water samples in a volume of 100 mL, using a glass wool adsorption-elution method which was developed by Vilaginès *et al.* (1993) and subsequently modified (Mans *et al.* 2013). Viruses were further concentrated to a final volume of 10 mL in phosphate-buffered saline (pH 7.4) (Sigma-Aldrich Co., St. Louis, MO, USA) by polyethylene glycol 8000 (Amresco, Solon, OH, USA) and sodium chloride (Merck Schuchardt OHC, Hohenbrunn, Germany) (PEG8000/NaCl) precipitation as described in the ISO/TS 15216-1:2013. Nucleic acid was extracted from 1 mL virus concentrate using the semi-automated Nucli-SENS® EasyMAG® instrument (BioMérieux, Marcy l'Etoile, France) as previously described (Saïd et al., 2014).

Mengovirus (5 µL extracted nucleic acid) was detected qualitatively by real-time reverse transcription-polymerase chain reaction (RT-PCR) using a commercial kit (mengo@ceeramTools™ Kit, Ceeram s.a.s, La Chappelle-Sur-Erdre, France) (Saïd et al., 2014). Norovirus GI and GII (5 µL extracted nucleic acid) were also detected qualitatively using commercial kits with internal amplification controls (IACs) (norovirusGI@ceeramTools™ Kit and norovirusGII@ceeramTools™ Kit). Sapovirus (2.5 µL extracted nucleic acid) was quantified in each sample using a real-time RT-PCR which targets 104 bp of the conserved RNA polymerase and capsid gene junction in ORF1. The assay included a SaV RNA standard, which contains only the 104 bp target region of the SaV genome, as the positive control in the assay and quantification standard. The assay also incorporates a competitive IAC (201 bp), constructed from non-target *Theileria parva* DNA flanked with the SaV-specific primers, in each sample to monitor inhibition (Murray et al., 2013a). All samples were tested once and if PCR inhibition was observed, the assay was repeated at a ten-fold dilution of the extracted nucleic acid. Detected SaVs were further characterised by genotyping a 5' region (approximately 300 bp) of the capsid gene using a nested PCR as previously described (Murray et al., 2013b). Sapovirus sequences were submitted to GenBank (accession numbers: KC904508-KC904513).

Standard quality control precautions were applied to reduce the possibility of cross-contamination and false-positive results. Separate laboratories were used for sample processing and preparation, nucleic acid extraction, reaction mix preparation and manipulation of amplicons. An extraction negative control and RT-PCR negative control were included in the real-time assay and PCR negative controls were included in both rounds of the genotyping nested PCR to monitor for false-positive results (Bosch et al., 2011).

Results and Discussion

Sapoviruses were detected in 80% (8/10) of the water samples (Table 1). Mengovirus was also detected in each sample (10/10), including samples from Dam 1 and Dam 2 that were SaV-negative, demonstrating successful virus recovery and nucleic acid extraction. Seven of the eight SaV-positive samples were also positive for hepatitis A virus (HAV) and norovirus (NoV) (Table 1; Saïd *et al.* 2014), indicating the presence of a variety of enteric viruses in the water sources. Sapovirus concentrations ranged from 1.11×10^5 copies/L to 1.62×10^7 copies/L (median 2.45×10^6 copies/L). The highest

Table 1. Water samples analysed for sapoviruses.

Sample ID	Collection date	Sample description	SaV concentration (copies/L)	SaV genotype	HAV[1]	NoV
LP1	31 Jan 2012	River A	5.88×10^6	GI.2	+	+
LP5	31 Jan 2012	WWTW (discharged wastewater)	1.59×10^7	GI.2	+	+
LP7	31 Jan 2012	River B	1.70×10^6	GI.2	+	+
LP9	31 Jan 2012	Dam 1 (borehole water)	-	-	-	-
LP16B	31 Jan 2012	Dam 2 (surface water)	-	-	-	-
LP17B	31 Jan 2012	Dam 3 (surface water)	1.11×10^5	ND	-	-
LP23	31 Jan 2012	WWTW (discharged wastewater)	1.62×10^7	GI.2	+	+
LP7	27 Mar 2012	River B	3.21×10^6	GI.2	+	+
LP17B	27 Mar 2012	Dam 3 (surface water)	1.42×10^6	ND	+	+
LP PVT	27 Mar 2012	Irrigation pivot	8.32×10^5	GIV	+	+

ND = genotype was not determined, WWTW = wastewater treatment works, LP5 and LP23 are two different WWTWs in the same municipality. [1]Data taken from Saïd *et al.* 2014.

concentrations were detected directly below the WWTWs (1.59 x 10^7 copies/L and 1.62 x 10^7 copies/L) and the lowest concentrations from Dam 3 (1.11 x 10^5 copies/L) and the irrigation pivot (8.32 x 10^5 copies/L) on the farm. The SaV concentrations from the WWTWs correlate to quantities previously reported in discharged wastewater in other provinces of SA (Murray et al., 2013a). The high concentrations also correlate to those reported in raw wastewater in Spain (Sano et al., 2011), suggesting the WWTWs are not successfully removing the virus prior to discharge. This reconfirms the poor performance of selected WWTWs in this area of SA, which impact water sources downstream from the WWTWs, as indicated by the low Green Drop score for the province.

Sapovirus strains were successfully genotyped from six of eight SaV-positive samples. Five of these strains clustered with GI.2 and one strain grouped with GIV. Genotype I.2 and GIV are two of the most prevalent genotypes implicated in SaV-associated outbreaks of gastroenteritis (Svraka et al., 2010,Kobayashi et al., 2012,Lee et al., 2012). Genotype I.2 is also frequently reported in water sources (Kitajima *et al.* 2011; Sano *et al.* 2011; Di Bartolo *et al.* 2013), including in SA (Kitajima et al., 2011,Sano et al., 2011,Di Bartolo et al., 2013,Murray et al., 2013b). The GI.2 strains shared high nucleotide identity (≥99%) to strains previously identified in water sources from other provinces of SA. In addition, the GI.2 strains were closely related (98-99% nucleotide identity) to strains found in water sources from Japan (Kitajima et al., 2011), Spain (Sano et al., 2011) and Italy (Di Bartolo et al., 2013). This is the first report of SaV GIV in the environment in SA. It was identified in a sample taken directly from an irrigation pivot on the farm, indicating the potential for direct contamination of fresh produce during irrigation. Sapovirus GIV has been reported in untreated sewage in Japan (Iwai et al., 2009) and Spain (Sano et al., 2011). The SA GIV strain shared 98% nucleotide identity over the typed region with strains identified from patients with gastroenteritis in Venezuela (HM214146) and the United States (HM800905). The strain also shared 97% nucleotide identity with a SaV genotyped from clams in Japan (Iizuka et al., 2013).

Conclusion

This study has provided new and informative data on the high concentration of SaVs present in water sources impacted by poor-performing WWTWs in a selected region of SA. In addition, it has shown that clinically relevant SaV GI.2 and GIV strains are circulating in these water sources. Enteric

viruses have a low infectious dose (10-100 infectious virions; Carter 2005; Teunis *et al.* 2008) and consequently, high titres of pathogens such as SaV in poorly-treated wastewater discharge may have a negative impact on the health of populations using the water sources for domestic, recreational or agricultural purposes.

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