Application of a competitive internal amplification control for the detection of sapoviruses in wastewater

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Abstract

In this study, a competitive internal amplification control (IAC) was constructed for application in the real-time reverse transcription-polymerase chain reaction detection of sapoviruses (SaVs). A SaV RNA standard was also created for quantification of the virus. The IAC was included in the screening of environmental samples for SaVs. From August 2010 to December 2011, 51 wastewater samples were collected from five provinces in South Africa. Sapoviruses were found in 72.5% (37/51) of samples, including four samples where detection was initially inhibited. Sapovirus concentrations ranged from 4.24×10^3 to 1.31×10^6 copies/ml. The IAC successfully identified samples which contained inhibitors and inclusion of an IAC is necessary to ensure the prevalence of SaVs is accurately determined. Sapoviruses are present at high concentrations in wastewater in several provinces of South Africa. This widespread occurrence indicates that SaV circulation in the South African population may be underestimated.

Keywords *Calicivirus, enteric virus, sapovirus, sewage, South Africa*

Introduction

Caliciviruses (CVs) are small, non-enveloped, single-stranded (ss) RNA viruses that cause gastroenteritis in humans. Sapoviruses (SaVs) and noroviruses (NoVs) are the two genera in the CV family that contain strains which infect humans (Green 2007). The SaV genus is divided into five genogroups, of which four infect humans (GI, GII, GIV and GV) (Farkas et al. 2004). Sapoviruses are transmitted via the faecal-oral route, either through person-to-person contact or from contaminated food and water (Hansman et al. 2007a; Kitajima et al. 2011). The infectious dose for SaVs has not been established, but other human CVs,

namely NoVs, have shown very low infectious dose (10 to 100 infectious virions) (Teunis et al. 2008) and relatively high stability in the environment (Green 2007). Sapoviruses have been quantitatively detected and characterised in various food and environmental samples including river water (Kitajima et al. 2010; Sano et al. 2011), wastewater (Haramoto et al. 2008; Kitajima et al. 2011) and shellfish (Hansman et al. 2007b; Iizuka et al. 2010; Ueki et al. 2010); predominantly in Japan and Spain. However, when compared with NoVs, there is limited information available on SaVs circulating in the environment worldwide.

Sapoviruses are most frequently detected in environmental samples using real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Haramoto et al. 2008; Kitajima et al. 2010; Sano et al. 2011). This molecular method allows for sensitive and specific detection and quantification of SaVs (Chan et al. 2006) but its application can be hampered by the presence of inhibitory compounds in the sample. Inhibitors are often co-concentrated and extracted with the target nucleic acid and can interfere with the RT and/or PCR amplification processes (Gibson et al. 2012). Inhibition of the assay can either result in a shift in the real-time PCR cycle threshold (Ct) value, thus adversely affecting quantification, or inhibit amplification completely. Virus concentrations in environmental samples are typically low and inhibition can result in an underestimation of the presence or quantity of virus in the sample (Gregory et al. 2011). This can potentially underestimate health risk, particularly since enteric viruses have a low infectious dose.

Identifying inhibition in a reaction forms an integral part of the strict quality control/quality assurance (QC/QA) procedures which are necessary in the molecular detection of viruses in food and environmental samples (Bosch et al. 2011). Several controls are recommended to ensure correct interpretation of the results (Rodríguez-Lázaro et al. 2007). These include a process control to monitor extraction efficiency of nucleic acid from the sample and an amplification control (AC) to indicate the success of the RT-qPCR reaction (Bosch et al. 2011). Mengovirus is a non-enveloped, positive sense ss RNA virus (Racaniello 2007) which has been used as a process control for nucleic acid extraction from food and environmental samples (Costafreda et al. 2006; da Silva et al. 2007). Various

ACs have been developed to validate amplification in a RT-qPCR assay (Costafreda et al. 2006; Diez-Valcarce et al. 2011; Gibson et al. 2012) and these can be classified as external amplification controls (EACs) or internal amplification controls (IACs). The EAC approach involves performing two separate reactions for each sample. One reaction contains only the sample nucleic acid while the second is seeded with the amplification control (Costafreda et al. 2006). The EAC approach is time-consuming and expensive as each sample is tested twice. An alternative approach is an IAC which is simultaneously amplified in the same reaction with the target nucleic acid (Hoorfar et al. 2004). Many different IACs have been developed for real-time RT-qPCR assays (Parshionikar et al. 2004; Müller et al. 2007; Rolfe et al. 2007; Gregory et al. 2011). These include endogenous and exogenous IACs and the exogenous controls can either be competitive or non-competitive. Endogenous controls utilise a 'house-keeping gene' which is naturally present in the sample with the target and is amplified using a set of primers different to those of the target (Hoffmann et al. 2009). Selecting a 'house-keeping gene' that would be present in all environmental samples is challenging and the exogenous IAC approach is therefore more suitable for these samples. An exogenous IAC is added to the reaction prior to real-time RT-qPCR screening for the target virus. A competitive IAC is synthetically modified nucleic acid that is amplified using the same primers and PCR conditions as the target in a single reaction tube. A non-competitive control uses a different primer set to the target (Hoorfar et al. 2004). In both the IAC and EAC methods, if the AC is amplified but the target is not, the sample is a true negative for the target virus. However, if neither the target nor the AC is amplified, the reaction has been inhibited (Diez-Valcarce et al. 2011). Competitive IACs are increasingly being used to monitor inhibition during the detection of enteric viruses in environmental samples (Parshionikar et al. 2004; Gregory et al. 2011; Gibson et al. 2012).

For the purpose of this study, mengovirus was used as a process control for nucleic acid extraction and a competitive exogenous IAC was constructed, referred to as the "competitive IAC" in this article, to monitor inhibition of the detection assay. To our knowledge, the detection of SaVs in the environment has not yet included a competitive IAC to monitor inhibition and this study aimed to

design and implement an IAC specifically for the detection of SaVs. The IAC was applied in the detection and quantification of SaVs in wastewater in several provinces of South Africa (SA).

Materials and methods

Environmental samples

From August 2010 to December 2011 wastewater samples, which included effluent and sludge, were collected from wastewater treatment plants in several provinces of SA. Of these, 51 samples with high faecal coli counts [$>10^6$ colony forming units (cfu)/100ml] were selected for the study (Fig 1). Twenty samples were from Mpumalanga province (MP), 16 from North West province (NW) and

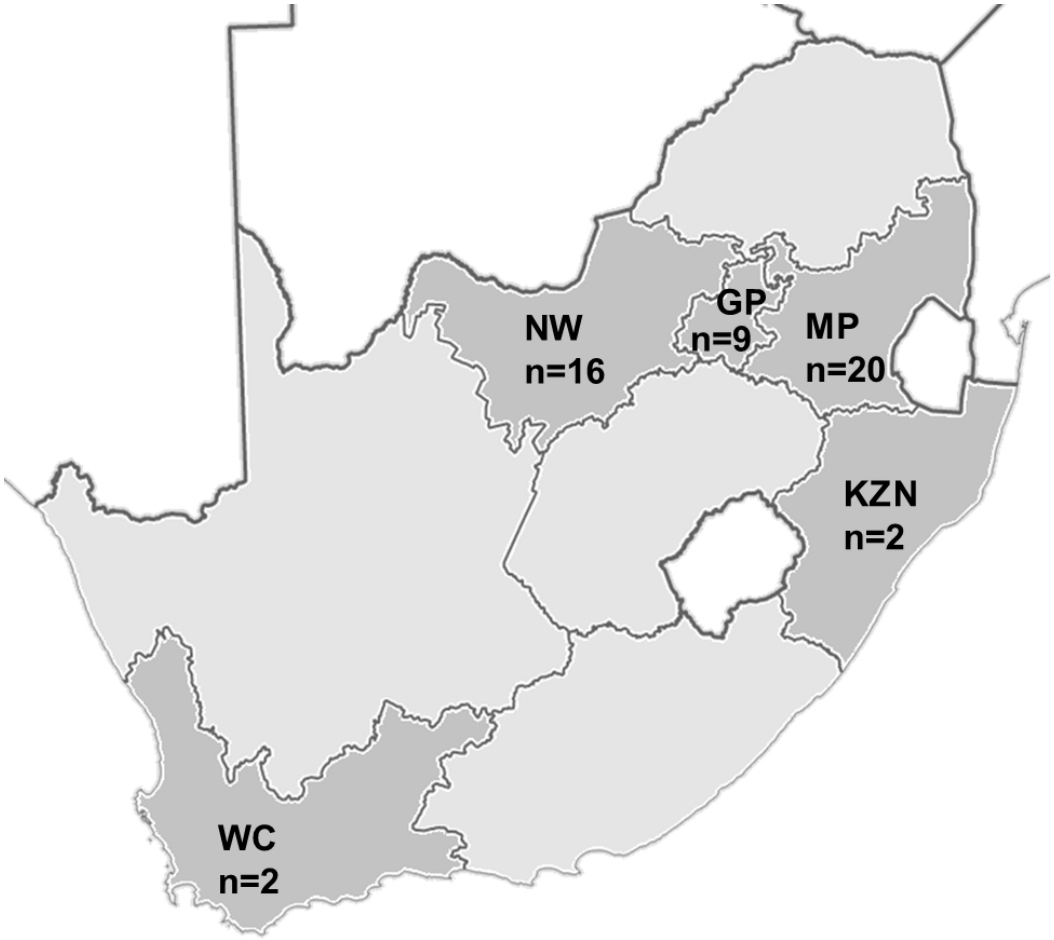

Fig 1 A map of South Africa indicating from which provinces wastewater samples were received (provinces are shaded dark grey) and the number of samples received (n). A total of 51 samples were collected, including 2 of unknown provincial location. GP = Gauteng province, KZN = KwaZulu-Natal province, MP = Mpumalanga province, NW = North West province and WC = Western Cape province

nine from Gauteng province (GP). Two samples each were selected from KwaZulu-Natal province (KZN) and the Western Cape province (WC) and two samples had unknown provincial origin.

Virus concentration and nucleic acid extraction

Viruses were concentrated from 100 ml (2010) or 75 ml (2011) wastewater samples to a volume of 2 ml in phosphate-buffered saline (pH 7.4) (PBS; Sigma-Aldrich Co., St. Louis, MO) by polyethylene glycol/sodium chloride precipitation (Minor 1985; European Committee for Standardization (CEN) Technical Committee 275).

Total nucleic acid was extracted from 1 ml virus concentrate using the MagNA Pure LC Total Nucleic Acid Isolation kit (large volume) on the automated MagNA Pure LC instrument (Roche Diagnostics, Mannheim, Germany). An extraction negative control (nuclease-free water) was included for every 15 samples. Extracted nucleic acid was eluted in 100 µl and stored in 5 µl or 10 µl aliquots at -70°C until use.

Samples that tested negative for mengovirus and SaV were re-extracted manually using the QIAamp® UltraSens® RNA extraction kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Extracted nucleic acid was eluted and stored as described above.

Reverse transcription and real-time qPCR

Human SaVs were detected and quantified using a two-step real-time RT-qPCR assay that targets the highly conserved RNA polymerase-capsid gene junction of the SaV genome, producing a 104 base pair (bp) product. Firstly, cDNA was synthesised from the extracted RNA using RevertAid^{TM} Premium Reverse Transcriptase (Thermo Scientific, Waltham, MA). The 20 µl reaction contained 10 µl RNA, 30 µM random hexamer primers and 50 U RevertAid^{TM} Premium Reverse Transcriptase. Five µl of cDNA was added to a 20 µl reaction mix

consisting of 0.9 µM of each primer (CU-SV-F1, CU-SV-F2 and SaV1245R) and 0.2 µM TaqMan probe (CU-SV-probe) in the LightCycler TaqMan Master Mix (Roche Diagnostics) (Table 1). The following cycling parameters were used in the Roche LightCycler 2.0 (Roche Diagnostics): 95°C for 15 min and 45 cycles of 95°C for 15 sec, 56°C for 1 min and 65°C for 1 min.

Table 1 Primers and probes used in this study

Primer/Probe name	Sequence (5'-3')	Position	Reference
ThPF	TCG CTG TGT CCC TTC G	623-638 [a]	This study
ThPR	CC AAC AAA ATA GAA CCA AAG	764-784 [a]	This study
CU-SV-F1-ThPF	GAC CAG GCT CTC GCT ACC TAC TCG CTG TGT CCC TTC G	N/A	This study
SaV1245R-ThPR	CCC TCC ATT TCA AAC ACT ACC AAC AAA ATA GAA CCA AAG	N/A	This study
CU-SV-F1	GAC CAG GCT CTC GCY ACC TAC	5074-5094[b]	(Chan et al. 2006)
CU-SV-F2	TTG GCC CTC GCC ACC TAC	786-803 [c]	(Chan et al. 2006)
SaV1245R	CCC TCC ATY TCA AAC ACT A	5159-5177[b]	(Oka et al. 2006)
ThPP	VIC-TCG GAC GGA GTT CGC TTT GTC-MGBNFQ	662-682 [a]	This study
CU-SV-Probe	FAM-TGG TTY ATA GGY GGT AC-MGBNFQ	5101-5117[b]	(Chan et al. 2006)

[a]L02366
[b]AY237422
[c]U95644
Y = C/T

Construction of SaV RNA standard

A stool specimen that previously tested positive for SaV was provided by the virology diagnostic laboratory, Tshwane Academic Division, National Health Laboratory Service. RNA extracted from the specimen was used as template in the construction of the SaV RNA standard. The SaV target region was amplified using the RT-PCR conditions previously described for the detection of SaV. The 104 bp amplicon was cloned into a pGEM®-T Easy vector (Promega Corp, Madison, WI) at an insert:vector ratio of 2:1 and ligated overnight at 4°C. The ligation mixture (2µl) was transformed into E.cloni® Chemically Competent Cells (Lucigen Corp, Middleton, WI), according to the manufacturer's instructions. To produce the RNA transcript, the SaV insert and SP6 promoter were amplified

from the plasmid and *in vitro* transcribed using the Riboprobe SP6 *in vitro* transcription system (Promega Corp), according to the manufacturer's instructions. Template DNA was digested using RQ1 DNase (Promega Corp) and RNA was purified using the Zymoclean RNA clean up kit (Zymo Research, Irvine, CA). Absence of DNA in the RNA stock was confirmed by PCR amplification of the RNA without the RT step. RNA was quantified using the Quant-iT^{TM} RiboGreen RNA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A SaV standard curve was created using ten-fold serial dilutions of the SaV RNA transcript in triplicate in the SaV real-time assay. During the detection of SaV in environmental samples, a SaV RNA standard was included in each real-time RT-qPCR run to quantify the amount of SaV present in SaV-positive samples.

Construction and optimisation of IAC

Highly conserved *Theileria parva* DNA encoding the 18S rRNA gene was selected as non-target DNA for the IAC (Allsopp et al. 1993). *Theileria parva* is a tick-borne parasite that infects cattle and buffalo and was therefore not likely to be present in the wastewater samples. First, the *T. parva* target region (161 bp) was amplified using the ThPF and ThPR primers (Table 1). The 50 µl reaction mix contained 5 µl DNA and 0.3 µM of each primer, 0.2 mM dNTPs and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The thermocycling parameters were as follows: 95°C for 10 min, 45 cycles of 95°C for 30 sec, 52°C for 30 sec and 72°C for 40 sec, followed by 72°C for 10 min. The *T. parva* amplicon was then tagged with the SaV primers using 0.4 µM hybrid oligonucleotide primers (Table 1) and the same cycling parameters as previously described. The 201 bp IAC amplicon was cloned into a pGEM®-T easy vector at an insert:vector ratio of 2:1 and the IAC RNA was *in vitro* transcribed and quantified as described for the SaV RNA standard.

The optimum working concentration of IAC RNA to be added to the extracted nucleic acid from each environmental sample to monitor inhibition was determined. The RNA transcript was ten-fold serially diluted and amplified using the SaV real-time RT-PCR assay. Using the highest dilution at which the IAC produced a positive signal and the next higher and previous lower dilutions, the

dilution series was repeated three more times, in triplicate. The dilution which consistently produced a positive signal from separate reaction mixes and dilution series was considered the working concentration of the IAC to be included in the SaV assay. To confirm that the IAC would not out-compete SaV present in a sample, the IAC was added to each dilution of SaV RNA standard in the SaV standard curve at the optimum IAC concentration and subjected to the real-time assay. The assay remained the same as previously described, with the addition of the IAC-specific probe (ThPP) at a concentration of 0.1μM. During the detection of SaVs in environmental samples, the working concentration of IAC was included in each RT reaction mix prior to reverse transcription. A sample was considered to be inhibited if amplification of the IAC was unsuccessful or if the IAC Ct value was over 3.3 cycles higher than expected.

Process control

Mengovirus was included as a process control to monitor the nucleic acid extraction efficiency. All samples were seeded with a known concentration of mengovirus before extraction (3.6×10^4 copies for automated extraction; 7.6×10^4 copies for manual extraction). Following extraction, all samples were screened for mengovirus using a published real-time RT-PCR assay (Pinto et al. 2009). Samples that tested negative for SaV and mengovirus were re-extracted manually and detection of both viruses was repeated. Samples that still tested negative for mengovirus were re-tested at a ten-fold dilution of the RNA to overcome potential PCR inhibition.

Results

Sapovirus RNA standard

Construction of the SaV RNA standard was successful. The standard curve created from the RNA standard has a R^2 value of 0.991 and the PCR efficiency was 1.98. The detection limit was approximately 100 copies per reaction of the RNA standard. The detection range of the standard curve was 10^7 to 10^2 copies/reaction (Fig 2).

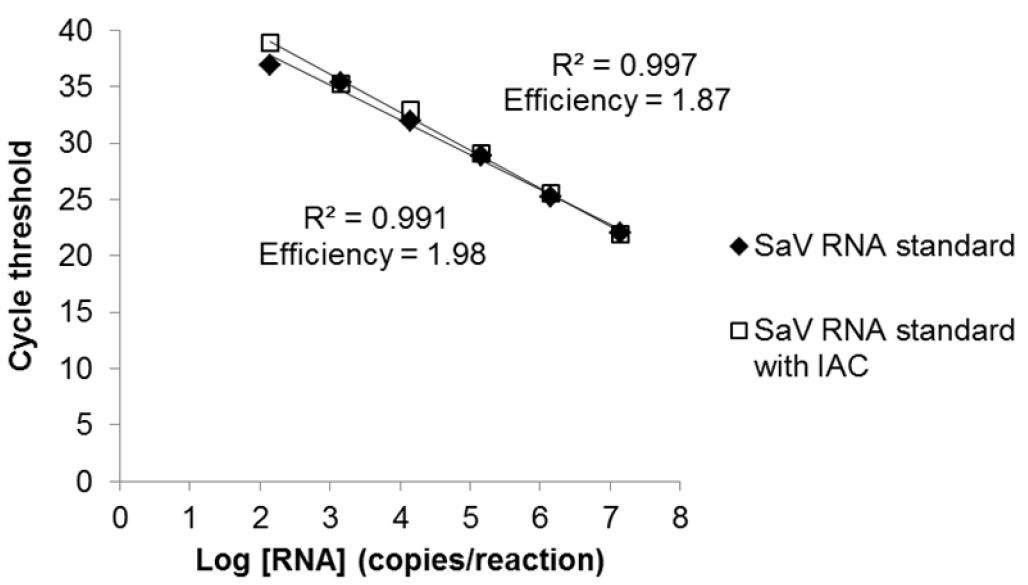

Fig 2 Sapovirus standard curve created using the SaV RNA standard (◆) and with the inclusion of the IAC in each SaV RNA standard dilution, at its optimum working concentration (□). PCR efficiencies were calculated automatically by the LightCycler 2.0 software (Roche Diagnostics) using the slope of the straight portion of a polynomial regression line in the standard curve

Internal amplification control

The IAC DNA consisted of 161 bp *T.parva* DNA flanked by the SaV primers CU-SV-FI and SaV1245R to yield a final product size of 201 bp (Fig 3). The 201 bp IAC is 97 bp longer than the 104 bp SaV target amplicon. This allows competition between SaV and the IAC to favour SaV. The IAC is amplified with SaV-specific primers but can only be detected using the VIC-labelled ThPP probe. This enables differentiation between SaV and IAC in a single reaction during real-time PCR as the SaV amplicon is detected using the FAM-labelled CU-SV-probe. Following optimisation, the working concentration of the IAC to be added to each reaction was determined to be 1000 copies (8.08×10^{-9} ng/µl). This was the lowest concentration that consistently gave a positive signal during the real-time RT-PCR assay. The addition of the IAC did not adversely affect the SaV standard curve. The limit of detection remained the same, the R^2 value increased slightly from 0.991 to 0.997 and the PCR efficiency was still above the recommended minimum of 1.8 (Hoffman 2011) (Fig 3).

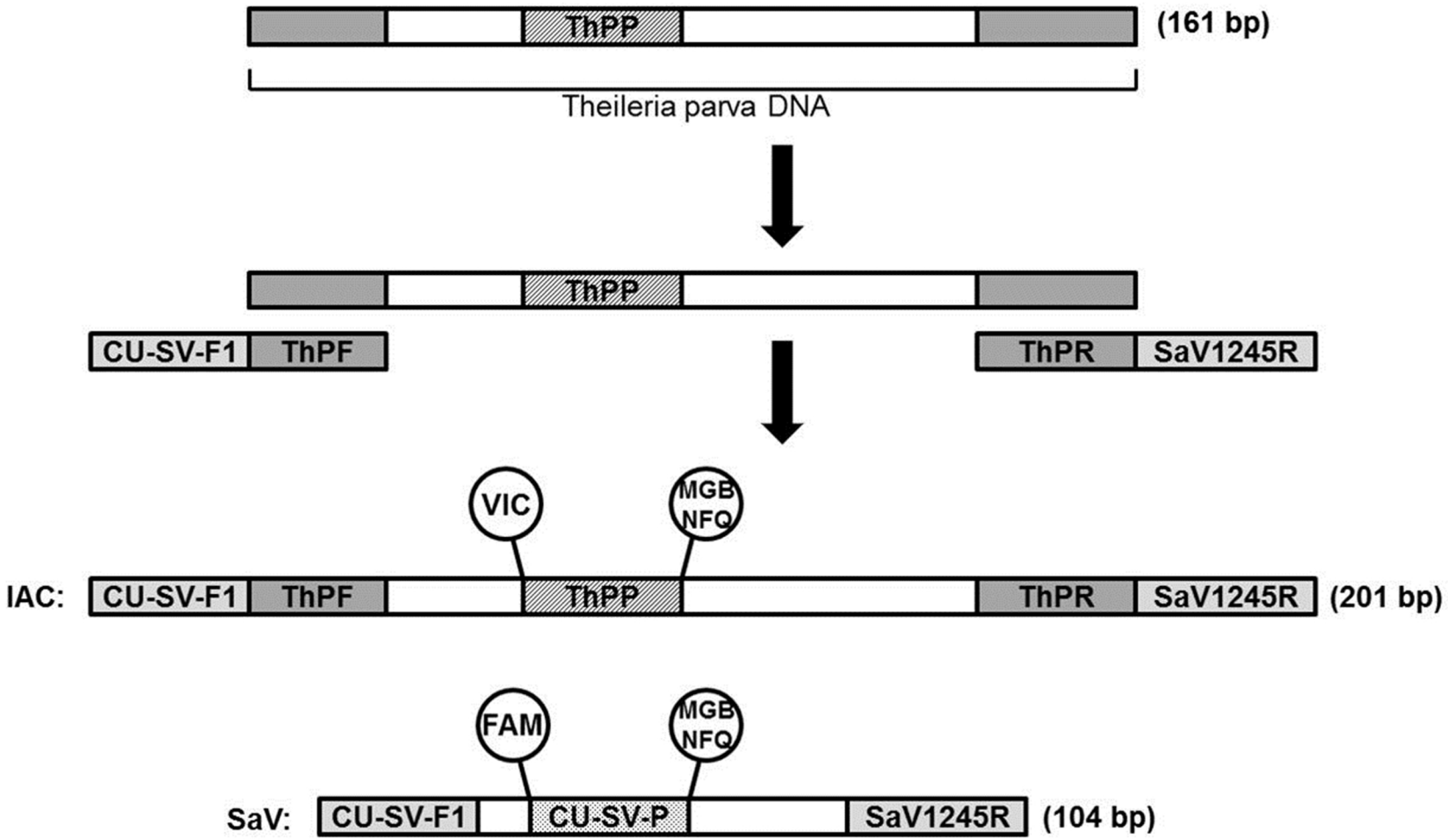

Fig 3 A schematic outline of the competitive IAC design with *T.parva* as the non-relevant nucleic acid, flanked by SaV-specific primers using the hybrid primer approach. The *T.parva*-specific primer regions and probe are indicated by "ThPF/R" and "ThPP" respectively. The SaV forward primer "CU-SV-F1" and reverse primer "SaV1245R" were used to amplify SaV and the IAC. A schematic representation of the SaV RNA standard ("SaV") has also been included for comparison and includes the SaV-specific probe "CU-SV-P" region to identify SaV. Note the larger size of the IAC (201 bp) and the different fluorophores in the TaqMan probes used to distinguish between IAC (VIC) and SaV (FAM)

Environmental samples

Following the initial automated extraction of nucleic acid from the samples and real-time RT-qPCR detection, 32/51 (63%) samples tested positive for SaV. Four samples were considered true negatives as they tested negative for SaV but the IAC and mengovirus both tested positive. Of the remaining 15 samples, 3 were negative for SaV and mengovirus, but the IAC was successfully amplified and 12 samples showed inhibition, indicated by an undetected IAC. RNA from the 12 inhibited samples was diluted ten-fold in nuclease-free water and the real-time RT-PCR was repeated. Following the ten-fold dilution, a further four samples were found to be positive for SaV, one remained inhibited and seven were negative for SaV and mengovirus, but positive for the IAC. The ten samples that were negative for SaV and mengovirus and the one sample that was inhibited after the ten-fold dilution were re-extracted manually. Of these, one was positive for SaV and ten were negative, even after 1:10 RNA dilutions to overcome inhibition. All ten SaV-negative samples tested positive for mengovirus. In total, 37/51 (72.5%) wastewater samples were positive for SaV.

Sapoviruses were detected in each of five provinces of SA from which samples were collected. The majority of samples were collected from MP, where 16/20 (80%) were positive for SaV. In the NW, 12/16 (75%) samples tested positive and in GP, 6/9 (67%) samples were positive for SaV. KwaZulu-Natal and WC each had 1/2 (50%) sample positive for SaV and both samples from unknown provincial origin were also positive.

Based on the RNA standard used to quantify SaV present in these samples, SaV concentrations ranged from 4.24×10^3 copies/ml (May 2011) to 1.31×10^6 copies/ml (October 2011). In order to observe a potential seasonal distribution, the average concentration from SaV-positive samples from all provinces was calculated for each month in which samples were received (Fig 4). No samples were collected in March or April 2011. The average concentration of SaV per month ranged from 4.24×10^3 copies/ml (May 2011) to 6.04×10^5 copies/ml (June 2011). The month with the second lowest SaV concentration was December 2010, at 2.29×10^4 copies/ml. This was followed by two months, January and February 2011, where no SaVs were detected in four and two

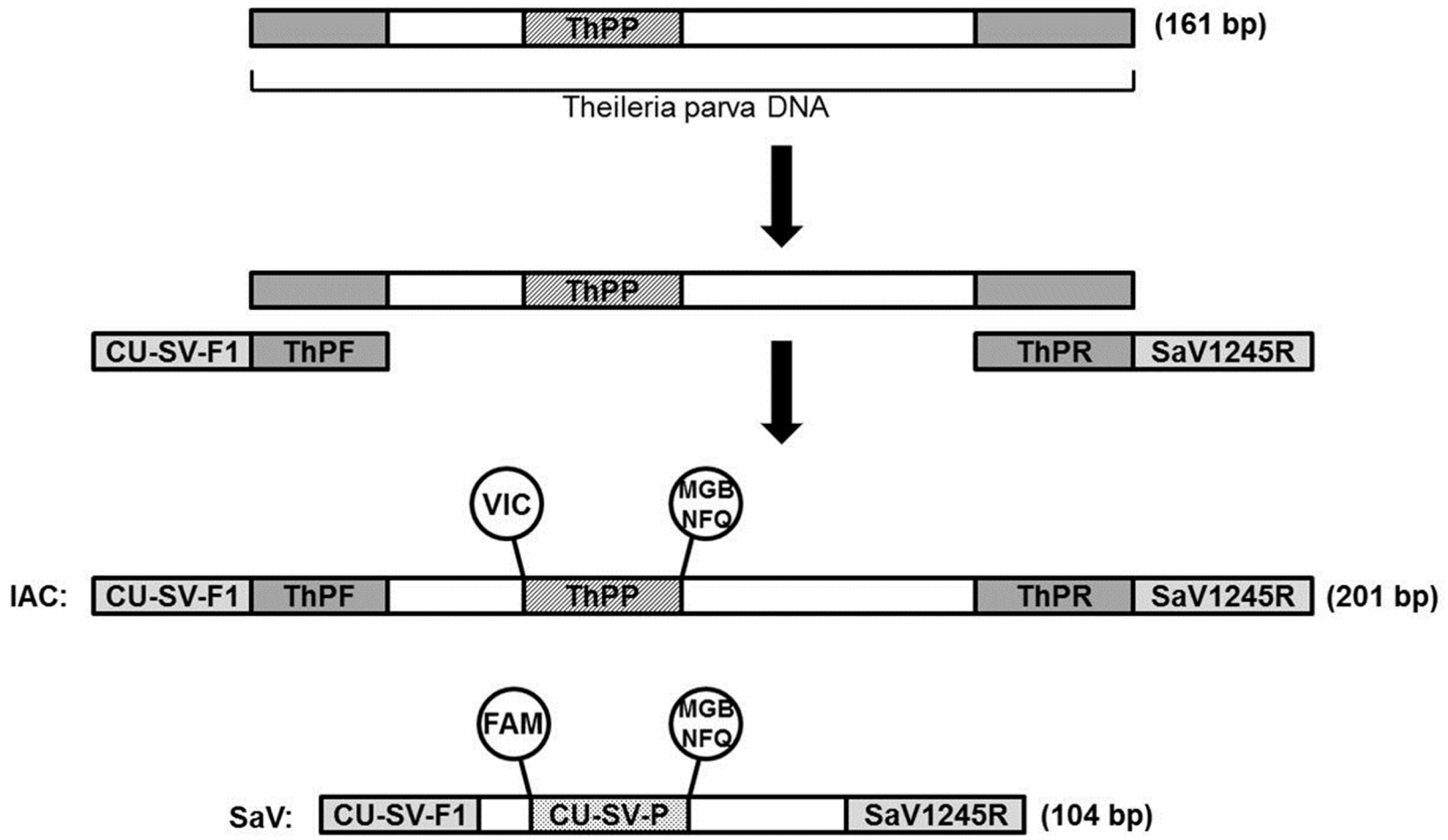

Fig 4 Monthly distribution of wastewater samples collected from five provinces in South Africa and the number of SaV-positive samples (indicated by the black portion of each bar) in each month's collection. The average SaV concentration per month is indicated as log [RNA] in copies/ml on the secondary y-axis. March and April 2011 have been excluded from the graph as no samples were collected in either month

samples, respectively. Although no samples were received in March and April 2011, the lowest SaV concentration for the study was recorded in the following month (May 2011).

Discussion

A competitive IAC was successfully developed and implemented in the monitoring of inhibition during the detection and quantification of SaVs in wastewater samples. Quantification is essential in determining the level of SaV contamination in the environment and useful in identifying potential seasonal distributions. Without an IAC, the level of contamination could be underestimated. The IAC identified four samples which were negative for SaV due to PCR inhibition, but following a ten-fold dilution of the RNA were positive for SaV. Dilution of RNA to overcome RT-PCR inhibition has been successfully applied in previous studies on enteric viruses (Ratcliff et al. 2002; Radin and D'Souza 2011). Had the IAC not been included, these samples would have been considered negative for SaV and this would have resulted in only 65% (33/51) of samples being positive for SaV rather than the true representation of 72.5% (37/51).

The average monthly concentration of SaVs circulating in the environment in SA ranged from 4.24×10^3 to 6.04×10^5 copies/ml, with the highest average concentration occurring in June 2011. No SaVs were detected in January and February 2011, two months which were preceded by the second lowest SaV concentration recorded in December 2010 and followed by the lowest SaV concentration in May 2011. This decline in SaV concentrations could indicate a period of low SaV circulation, however since no samples were received in March and April 2011 this cannot be confirmed. The other months were consistently higher ($> 3 \times 10^4$ copies/ml) with average SaV concentrations ranging from 3.05×10^4 copies/ml in November 2011 to 6.04×10^5 copies/ml in June 2011. The SaV concentrations in SA are significantly higher than those found in wastewater in Japan (Haramoto et al. 2008; Kitajima et al. 2011), but similar to that determined in Spain (Sano et al. 2011). The months in which SaV peaked (August 2010, June and July 2011) differ from what was found in Japan and Spain where SaV concentrations were highest in the winter months of December to February.

However, this corresponds to the same season in the southern hemisphere. In SA, no SaVs were detected in the summer months of January and February 2011. Likewise, in the northern hemisphere SaV concentrations were at their lowest or not detected in the summer months of July and August (Haramoto et al. 2008; Sano et al. 2011).

Sapoviruses were detected in the majority of samples from all three provinces where more than two samples were collected (MP, NW and GP). Sapoviruses were also detected in one sample from each of the other two provinces (KZN and WC). This indicates that SaVs are circulating in all five provinces from which samples were received, suggesting a country-wide distribution of the virus. The high detection rate in wastewater indicates that SaVs are circulating in many different communities in SA. However, the clinical impact that SaVs have on the population is unknown and further studies are needed to establish this impact.

This study has shown that the inclusion of an IAC in the detection of SaVs in environmental samples is essential to avoid underestimation of the virus due to inhibition of the RT-qPCR assay. Over 72% of the samples were positive for SaV, indicating that the virus is widespread in wastewater in SA. Quantification of SaVs showed high concentrations of the virus circulating in the wastewater, averaging 10^5 copies/ml. This information provides valuable data on the presence and quantities of SaV circulating in SA and is the first data of its kind in the southern hemisphere. Further analysis of SaVs in the environment and in a clinical setting in SA are required to better understand and compare the distribution of this virus in the two settings.

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