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ORIGINAL PAPER

Occurrence of Salivirus in Sewage and River Water Samples in Karaj, Iran

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Abstract

Salivirus is a newly discovered virus which seems to be related to acute gastroenteritis in children. Salivirus may infect susceptible children by fecal–oral route after exposure to contaminated water. The present study aims to evaluate the occurrence and quantity of Salivirus in treated and untreated sewage water and river water samples collected in the city of Karaj, Iran by reverse transcription-quantitative PCR assay. A total of 50 samples were collected from environmental waters containing 22 treated and untreated sewage water in volume of 1 l and 28 river water samples in volume of 5 l were included in this study. After viral RNA extraction, the Real-time PCR was performed to amplify the 5′UTR sequence of Salivirus genome and viral load was assessed. Out of the 50 samples tested, the Salivirus genomic RNA was identified in 5/12 (41.6%) of treated and 3/10 (30%) of untreated sewage samples and in 8/28 (28.5%) of river water samples. The maximum viral load was 4.8×10^6 copies/l in treated sewage water sample in September and the lower viral load was 4×10^5 copies/l related to treated sewage water taken in December. This is the first report of Salivirus occurrence in the environmental waters in Iran. The viral prevalence of Salivirus in each of the three sets of tested samples was within low to moderate in range.

Keywords Salivirus · Sewage water · River water · Real-time PCR

Introduction

Acute gastroenteritis is considered to be induced with infection of humans by pathogens such as rotaviruses and noroviruses as main etiologic agents of diarrheic diseases (Jin et al. 2009; Nasab et al. 2016). However, approximately 40% of diarrheic cases with unknown etiology remain to be identified (Denno et al. 2007; Itta et al. 2016). Among newly discovered viruses, Salivirus (SalV) is an emerging virus which was detected concomitantly first in 2009 from stool samples of diarrheic children in United State and Australia, specimens collected from children with non-polio acute flaccid paralysis (AFP), and untreated sewage samples in Spain (Holtz et al. 2009; Greninger et al. 2009; Li et al. 2009).

Although Salivirus (named Klassevirus only in early literature) was illustrated to be closely related to Aichi virus of the *Kobuvirus* genus, the nucleotide and amino acid sequence analysis showed that Salivirus or Klassevirus belong to different genus named *Salivirus* in *Picornaviridae* family (Reuter et al. 2017; Greninger et al. 2009; Holtz et al. 2009; Tapparel et al. 2013). In addition, Salivirus isolates on the basis nucleotide similarity have been included in two different genotypes containing Salivirus A1 and Salivirus A2 (Reuter et al. 2017; Ng et al. 2012). Salivirus genotypes including A1 and A2 have been determined based on comparison of VP3, 2C, and 3D nucleotide sequences in the phylogenetic analysis (Reuter et al. 2017; Lasure and Gopalkrishna 2016).

Salivirus as a new member of picornaviruses is non-enveloped virus with one segment of single-stranded RNA genome in positive sense (Aldabbagh et al. 2015; Holtz et al. 2009; Tapparel et al. 2013). The viral genome organization in length of 8021 nucleotides (nt) is the same as picornaviruses including 5′UTR-L protein- viral structural VP0, VP3 and VP1 proteins-non-structural 2A, 2B, 2C, 3A, 3B, 3C and 3D proteins-3′UTR that end with a 3′ poly (A) tail (Reuter et al. 2017; Boros et al. 2016).

The epidemiological studies in several different geographical areas in the world propose that Salivirus has a

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worldwide distribution and it can be the possible etiologic agent of gastroenteritis in humans (Yu et al. 2015; Reuter et al. 2017). However, there is a low detection rate of Salivirus with a prevalence of 0.1–8.8% in patients with gastroenteritis (Yip et al. 2014; Kumthip et al. 2017). Although Salivirus infections are reported in different age groups, the overall most frequent infections have been detected in under 5-year-old children (Richman et al. 2009; Reuter et al. 2017). In contrast, a moderate and high prevalence of Salivirus has been reported in environmental samples containing raw and treated sewage water and river waters from 15–93% in United States and Japan and 16–33% in Japan and Brazil, respectively (Haramoto and Otagiri 2013; Kitajima et al. 2014). Regarding highly prevalence identification of Salivirus in environmental samples, it seems that the possibly transmission route of Salivirus infections occurs by the fecal–oral route.

On the basis of previous studies, the pathogenesis of Salivirus and the relationship between Salivirus infections and gastroenteritis remain to be clarified (Boros et al. 2016). However, when Salivirus infection occurs symptomatically, it is associated with a clinical sign such as fever, diarrhea, and vomiting (Boros et al. 2016; Aldabbagh et al. 2015; Han et al. 2010).

Molecular methods including RT-PCR and RT-quantitative PCR (RT-qPCR) have been introduced as a gold standard to detect Salivirus isolates with targeting 5′UTR, VP0/VP3, 2C, and 3D regions of their genomes (Li et al. 2009; Greninger et al. 2009; Holtz et al. 2009; Han et al. 2010; Shan et al. 2010). In the present study, two different sample collection from sewage water (treated and untreated sewage water) collected from Mahdasht sewage treatment plant and river water collected from Karaj River in Karaj, Iran were evaluated to detect and to quantify viral genomic RNA for Saliviruses by RT-qPCR using primers targeted to 5′UTR region.

Materials and Methods

Sample Collection and Processing

During cold season between September 2016 and May 2017, river water as well as treated and untreated samples were collected every week in the city of Karaj, Iran. A total of 28 sewage water samples and 22 river water samples in a volume of 1 l for sewage water and 5 l for river water samples were collected in sterile plastic bottles and shipped on ice. The samples were left to settle for 3 h and 100 ml of clarified waters were stored in − 20 °C for 2 years. After thawing the water samples, they were concentrated by procedure which was previously described by Hovi (Hovi et al. 2001). Briefly, a 100 ml of samples was centrifuged at 1500×*g* for 20 min to produce pellet and supernatant. The supernatant was concentrated with the dextran–polyethylene glycol (PEG6000) and incubated for overnight at 4 °C. A volume of 4 ml was mixed with the pellet and after adding 1 ml chloroform and shaking, the suspension was clarified by centrifugation at 1500×*g* for 10 min. Finally, the concentrated water samples were used for viral RNA extraction.

Viral RNA Extraction and Reverse Transcription

Viral genomic RNA was extracted from 100 µl of the river and sewage water concentrated samples using the TRIZOL (Invitrogen) reagent according to the manufacturer's instruction. The viral RNA was eluted in a final volume of 50 µl. The concentration and quality of the extracted RNA were assessed by Nanovue spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNA was used directly in the reverse transcription reaction (GeneAll) or stored at − 70 °C until use. Briefly, 10 µl of extracted RNA was added in RT mixtures containing random primer and dNTPs, and incubated at 80 °C for 10 min and after then placed on ice for 10 min. Then, second reaction buffer (containing 10X RTase reaction buffer, 0.1 M DTT, HyperScript RTase, and RNase inhibitor) was added to the previous mixture and incubated at 42 °C for 60 min. Finally, the RT reaction mixture incubates at 85 °C for 5 min to inactivate the enzyme. Nucleotide sequences of the partial 5´UTR region of Salivirus are highly conserved, thus the selected previously used primers containing forward (5′-CTCTGCTTG GTGCCAACCTC-3′) and reverse primers (5′-CTGGTC TGGGACAGCGGAAC-3′) can amplify a 134-bp fragment located at 5´UTR region of the Salivirus (Aldabbagh et al. 2015). The primer set which was previously used for Salivirus detection by probe-based procedure, here was applied to Syber green method.

Implementation of the qPCR Assay

The sequences of Salivirus genome located at 5´UTR region in length of 134 nt were synthesized and cloned into pGH plasmid (Shanghai Generay Biotech Co.). A qPCR reaction using forward and reverse primers to detect of Saliviruses for each sample was performed in a 20 µl reaction volume containing 5 µl of synthesized cDNA or 1 µl plasmid DNA (10^1 to 10^7 copies/reaction) as a template, 4 µl Syber master mix (Rotor-Gene SYBER green PCR), and 10 mM of each primer. The negative control reaction was also included. PCR amplification was done with Rotor-Gene under the following program: primary denaturation at 95 °C for 10 min, followed by 40 amplification cycles consisting denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for

20 s. Amplification data were analyzed with Rotor-Gene Q software. All samples were characterized by a corresponding Ct value. Negative samples gave no Ct value.

Diagnostic Sensitivity and Specificity

The viral copy number was quantified through 10-fold serial dilution of plasmid DNA reactions. The slope (S) of the linear regression curve to access efficiency and melting curve to determine PCR reaction quality were analyzed and the results were interpreted (Fig. 1). In order to confirm the results, three positives of Real-time PCR products were imaged by gel electrophoresis and purified. Then, sequencing was performed with the same primers used for amplification by using an ABI Prism Big Dye Terminator cycle sequencing. The obtained nucleotide sequences were compared to Salivirus sequence that is accessible in the GenBank database (Accession Number GQ184145).

Results

Development of RT-Quantitative PCR

The standard curves were illustrated on the basis of the average cycle threshold (CT) values of reactions against the amount of the plasmid copies per reaction volume.

The primer set was able to amplify plasmid DNA dilutions consisting 1.0×10^{1} to 1.0×10^{7} copies/reaction. (Fig. 2). The CT values were directly proportional to the log10 of the viral genome copies/reaction with correlation coefficients (r) of 0.99, and the slope of the standard curve was −3.44 (Fig. 2). The lower quantification limit was determined to be around 1.0×10^{1} copies per reaction. These results indicated that using the primers to amplify 5′UTR region of Saliviruses by Syber green method can be used for detection of the presence of low viral genomic RNA in environmental specimens including river water and sewage water samples.

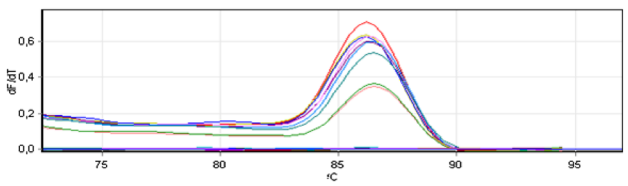

Fig. 1 Melting curves (x = temperature, y = changes in density of fluorescence signal) of the samples. Each sample produced a single sharp peak, and all of them overlapped and showed the same melting temperature

Detection of SalV Genomic RNA in River Water

To assess the applicability of the RT-qPCR assay for detection of Salivirus in river water samples, we tested a total of 28 samples. Out of 28 samples for detection of SalV genome, the developed RT-qPCR showed positive signals for 8 samples (28.5%). According to the standard curve, the concentration of viral genomic RNA was seen from range 8×10^{5} to 4×10^{6} copies/l of river water sample. The maximum of SalV viral load (4×10^{6} copies/l) was related to September. (Table 1).

Detection of SalV Genomic RNA in Treated and Untreated Sewage Water

This assay was also implemented in order to determine the presence of SalV in sewage water samples including treated and untreated samples. Out of 22 collected samples consisting 12 treated and 10 untreated sewage water for detection of SalV RNA genome, the RT-qPCR was positive for 5 samples of treated sewage samples (41.6%) and positive for 3 samples of untreated sewage water samples (30%). The viral RNA load in untreated and treated sewage samples were shown to be within range from 1.2×10^{6} to 2.8×10^{6}

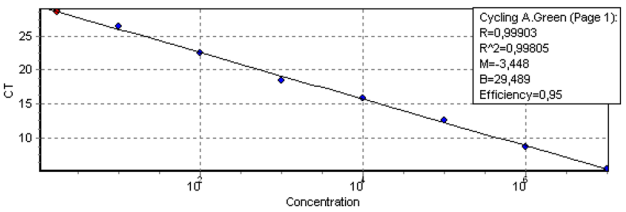

Fig. 2 Sensitivity of Salivirus detection by Rotor-Gene Real-time PCR in a series of 10-fold dilutions of genomic SalV– Plasmid (10^{1} to 10^{7}) per PCR reaction

Table 1 The viral load in positive untreated and treated sewage water and river water samples

Date of sampling	Untreated sewage water (copy/l)	Treated sewage water (copy/l)	River water (copy/l)
September	ND	4.8×10^{6}	4×10^{6}
October	1.2×10^{6}	ND	ND
November	ND	2.4×10^{6}	1.2×10^{6}
December	2.8×10^{6}	4×10^{5}	8×10^{5}
January	ND	ND	8×10^{5}
February	1.2×10^{6}	8×10^{5}	8×10^{5}
March	ND	1.6×10^{6}	1.6×10^{6}
April	ND	ND	2.8×10^{6}
May	ND	ND	2.8×10^{6}

ND Not determined

copies/l and 4×10^5 to 4.8×10^6 copies/l, respectively. The maximum of SalV viral load in treated and untreated sewage water were seen in September and December, respectively (Table 1).

Specificity of qPCR

To assess nucleotide sequence of the positive qPCR reactions, three positive products of qPCR containing treated, untreated sewage water, and river water samples were sequenced. The results of sequencing showed the highest nucleotide sequence homology to 5′UTR sequence of Salivirus obtained from genebank with accession number of GQ184145.

Discussion

After the initial detection of Salivirus in fecal samples in the U.S and Australia, in untreated sewage specimens in Spain (Greninger et al. 2009; Holtz et al. 2009), and from non-polio acute flaccid paralysis (AFP) in Nigeria (Li et al. 2009), many studies have reported Salivirus prevalence among different age groups especially among children under the age of five as well as in environmental polluted samples including river water, treated, and untreated wastewater (Greninger et al. 2009; Holtz et al. 2009; Li et al. 2009). The VP1 region has been generally used to classify members of *Picornaviridae* family into genera because of its nucleotide sequence variability features. However, 2C, 3D, and VP3 regions have been shown to be sufficient for genotype analysis of Salivirus. On the other hand, using the primer set targeting 5′UTR, VP0/VP3, 2C, and 3D regions of genomic RNA can be used to identify Salivirus isolates in either stool specimens and environmental samples (Li et al. 2009; Greninger et al. 2009; Holtz et al. 2009; Han et al. 2010; Shan et al. 2010). In this study, we implemented RT-qPCR methods for the detection and the quantification of Salivirus in river water as well as treated and untreated sewage water samples. A previously reported set of primer targeting conserved 5′UTR region of Salivirus (Aldabbagh et al. 2015) was used to the RT-qPCR assay. This is the first report of Salivirus occurrence in Karaj, Iran.

To date, several studies have conducted the RT-qPCR assay to detect and to measure SalV RNA in clinical and environmental samples including wastewater and river water (Greninger et al. 2009; Han et al. 2010; Holtz et al. 2009; Li et al. 2009; Shan et al. 2010). Here, we used Syber Green method instead of Probe-based method which was previously described by Aldabbagh et al. Although our results showed the primer set is applicable to detect SalV genome in environmental samples, it seems that the RT-qPCR can also be capable to detect Salivirus viral genomic RNA in clinical samples with low to high copy of viral genome. According to the previous reports, which have demonstrated the higher rates of prevalence of SalV during monsoon season (Lasure and Gopalkrishna 2016), the 50 samples for this study were collected from September 2016 to May 2017. The results of SalV detection rate in our study indicated that virus dissemination in environmental waters occurs from September to May. However, the maximum of SalV viral load in both river water and treated sewage water was seen in September and in untreated sewage water was related to December. Similar to previous report by Kitajima, no SalV detection was seen by RT-qPCR in our results in April in both treated and untreated sewage water samples. On the other hand, in contrast to Kitajima report, we detected a SalV in treated sewage water sample in March by RT-qPCR. In general, the maximum viral load in treated and untreated sewage water samples were higher than those of Kitajima report, but in comparison with Haramoto report, the viral load in our study was relatively similar in both treated and untreated sewage water samples. Many studies from around the world have documented Salivirus prevalence in different types of water samples with low to high concentration ranging between 15 and 93% (Reuter et al. 2017). In this study, SalV was detected in 28.5, 30, and 41.6% of river water, treated, and untreated sewage water samples, respectively. Our results were similar to the previous studies conducted in South Korea, United States and France which have reported low to moderate prevalence of SalV in environmental samples. However, in contrast to Japanese study with high detection of Salivirus (93%) (Haramoto and Otagiri 2013), the maximum SalV detection rate in our study was 41.6% in treated sewage samples. The Salivirus RNA detection in both treated (41.6%) and untreated sewage water (30%) could explain the treatment processes of sewage water are not efficient to the removal of SalV particles because of possible virus aggregation forms or crystalline formation groups, as such previously described in other picornaviruses (Ibrahim et al. 2017). The aggregation of viruses has documented that have an effect to increase viral survival in environment water samples. On the other hand, regarding the maximum range of viral load isolation of SalV genomic RNA in both treated and untreated sewage water and also in river water samples after 2 years of storage at -20 °C similar to previous work, which were shown the SalV genomic RNA could be amplified from 4 °C storage samples after 2 years of collection, it may be concluded that SalV particles have stable features similar to other enteric viruses (Boros et al. 2016). However, detection of SalV genomic RNA in environmental samples cannot certainly be related to virus infectivity.

The presence of Saliviruses in environment waters reported by several environmental surveillance studies suggests a potential fecal–oral route of transmission to humans (Reuter et al. 2017). Consequently, the first report of SalV

occurrence in Karaj, Iran from environmental samples as a viral etiologic agent inducing enteric illness could be partly cause of unknown acute gastroenteritis cases which no explanation for them have determined. Regarding previously published documents, demonstrating extended distribution of Salivirus, here we explain the occurrence of Salivirus in Alborz province may result in occurrence of the Salivirus on the other locations in Iran. In general, whether the SalV infections can result in diarrhea in Iranian affected peoples remains to be determined.

The occurrence of SalV in our collected environment samples suggests that the virus is released from the feces of infected individuals in the community resulting in the presence of virus in the samples.

Our results showed that there is a relatively higher detection rate of Salivirus in treated sewage water in comparison with untreated sewage water. However, we cannot justify whether the sewage treatment for elimination of Salivirus could be efficacious since the positive results in both collected samples were related to different days.

The levels of viral contamination in treated of the wastewater treatment plant must be considered by the regulatory and local authorities for improving the efficiency of the treatment processes for the removal of pathogens.

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