High Frequency of Salivirus in Pediatric Patients with Acute Gastroenteritis

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ABSTRACT

Introduction: Salivirus (SalV), a possible causative agent of gastroenteritis belongs to the genus Salivirus in Picornaviridae Family. The fecal-oral path seen to be the entry route for SalV in susceptible peoples such as children that consun polluted water. We investigated the SalV occurrence in children's stool with acu gastroenteritis using a reverse transcription-quantitative polymerase chain reactive assay (RT-qPCR). Methods: From Sep. 2018 to May 2019, 160 stool samples we collected from children with acute gastroenteritis admitted to Imam Ali Gener Hospital, Alborz University of Medical Sciences, Iran. After viral RNA extractio the 5'UTR region of the SalV genome was amplified by reverse transcription quantitative polymerase chain reaction (RT-qPCR), and the viral load was define Results: Of the 160 stool samples, 41 (25.6%) were positive for SalV RNA. The highest SalV detection rate was in Feb. (28.2%), and the highest viral load (6.2×10^7 copies/g) belonged to a 1-month-old patient. Conclusion: Salivirus occurs amor Iranian children with acute gastroenteritis. Our results suggest that Salivirus mig contribute to acute gastroenteritis.

INTRODUCTION

Although most common viruses, including Rotaviruses, Noroviruses, Astroviruses, and enteric Adenoviruses, are linked to diarrheic disease, new emerging viruses in the Picornaviridae family, i.e., Salivirus (SalV), Aichi virus (AiV), Saffold virus (SAFV), and human Cosavirus (HCoSV) have also received attention as the possible etiologic agent of acute gastroenteritis [1-6]. Salivirus occurs among patients with acute gastroenteritis suggests that people acquire infection from the fecal-oral route. The SalV pathogenesis is still controversial and remains to be clarified [15]. However, in symptomatic SalV infection, the clinical signs include fever, diarrhea, and vomiting [14, 15, 23]. As a gold standard, in environmental and clinical samples, SalV is identified by real-time PCR (RT-PCR) and real-time quantitative PCR (RT-qPCR) that targets either 5'UTR, or VP0/ VP3, 2C, and 3D regions of the virus genomes [7-9, 21-24].

http://jommid.pasteur.ac.ir

The present study investigated SalV infection by RT-qPCR in stool samples from children with gastroenteritis in Alborz Province, Iran. Our study is the first report of SalV occurrence among children with acute gastroenteritis in Iran.

MATERIALS AND METHODS

Clinical samples. From September 2018 to May 2019, 160 fecal specimens were collected from patients admitted to the Imam Ali General Hospital, Alborz University of Medical Sciences, Iran. Samples were obtained from patients ≤ 12 years of age who presented with acute gastroenteritis. According to World Health Organization (WHO), we defined diarrhea as a decrease in the consistency of stools (loose or liquid) and/or as having more stools than is usual for a person (typically, ≥3 in 24 h), with or without fever or vomiting. Two investigators filled out questionnaires for patients, including data such as age, sex, and presenting symptoms, e.g., fever, diarrhea, and duration of illness before admission. The patients were in the > 1, 1-5, and 6-12 years old groups, with the frequency of 38 (36.2%), 54 (33.7%), and 48 (30%), respectively. Initially, the samples were examined for pathogenic bacteria (Escherichia coli, Salmonella spp, Campylobacter spp, and Shigella spp) and parasites (Giardia lamblia and Entamoeba histolytica). The negative specimens for pathogenic bacteria and parasites were stored at -80°C until used.

The study was reviewed and approved by the ethical committee for human experimentation in the Faculty of Medicine, Karaj University, Iran. (Ethics approval ID: IR.IAU.K.REC.1398.043)

In order to prepare stool specimens, 10% phosphate-buffered saline (pH 7.2) homogenate of stool samples was centrifuged at 8000 g for 10 min, and the resulting supernatant was used for the reverse transcription step.

Viral RNA extraction and reverse transcription. According to the manufacturer's protocol, the virus genomic RNA was extracted from 300 μL of 10% fecal sample suspensions using the TRIZOL reagent (Invitrogen). The extracted RNA was used directly in the reverse transcription reaction or stored at -70°C until used. A control, previously shown to be positive for SalV RNA, was included in all assays. Briefly, 10 μL of extracted RNA or positive SalV RNA was added in reverse transcription (RT) mixtures containing random primers and dNTPs, incubated at 80°C for 10 min, and then placed on ice for 10 min. Then, a 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 was incubated at 85°C for 5 min to inactivate the enzyme.

qPCR. The partial 5′UTR region of SalVs is highly conserved, exhibiting 97%-99.2% identity in nucleotides and 99%-100% in amino acids. Thus, the previously published primers, F (5’-CTCTGCTTGGTGCAAACCTC-3’) and R (5’-CTGGTCTGGGACAGCGGAAC-3’) [14], were used to amplify a 134-bp fragment in the 5′UTR region of the SalV genome. A Sybr Green qPCR reaction using forward and reverse primers for detecting the SalV genome in the samples was deployed. A plasmid DNA containing the 134-bp 5′UTR sequence of the SalV genome (10⁵ to 10⁷ copies/reaction) and negative and positive controls from our previous work [21] were also included. Amplification in a thermocycler (Rotorgene, Germany) began with primary denaturation at 95°C for 10 minutes, followed by 40 amplification cycles consisting of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 20 sec. Amplification data were analyzed using the Rotor-Gene® software, defining the Ct value for all samples; negative samples had no measurable Ct value. The standard curves were illustrated based on the average cycle threshold (CT) values of reactions against the plasmid copies per reaction volume. The primers could amplify plasmid DNA in reactions containing 1.0×10¹ to 1.0×10⁷ copies/reaction (Fig. 1). 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 -3.4 (Fig. 1). The lower quantification limit was determined to be around 1.0×10¹ copies per reaction. Based on melting curve analysis for assessing the real-time PCR specificity, each sample produced a single sharp peak, and all of them overlapped and showed the same melting temperature.

RESULTS

Detection of SalV RNA in stool samples. Out of 160 fecal samples examined, 41 (25.6%) were positive for Salivirus (SalV) RNA, and of positive samples, 22 belonged to males and 19 to females. The patient’s age ranged from 1 month to 12 years, with a median of 4.7 years. The maximum SalV detection rate (28.2%) was in Feb., and the minimum was in April and May (2.5 %) (Table 1).

Viral load. According to the standard curve, the concentration of the SalV genomic RNA in stool samples ranged from 1.5×10¹ to 6.2×10⁷ copies/g. The maximum viral load (6.2×10⁷ copies/g) was in March, and the minimum (1.5×10¹) was in November and April (Table 1).
Salivirus in pediatric patients

Fig. 1. Standard curve for SalV detection by RT-qPCR using serial 10-fold dilutions of genomic SalV plasmid (10⁴ to 10⁷) per reaction [21].

Table 1. Data for SalV RT-qPCR positive patients

<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>SalV positive specimens</th>
<th>†Max. viral load</th>
<th>†Min. viral load</th>
<th>Age groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep. 2018</td>
<td>3</td>
<td>6.3x10⁶</td>
<td>2.8x10⁴</td>
<td>2 mo-4 yrs</td>
</tr>
<tr>
<td>Oct. 2018</td>
<td>4</td>
<td>1.1x10⁷</td>
<td>2.4x10³</td>
<td>1 mo-6 yrs</td>
</tr>
<tr>
<td>Nov. 2018</td>
<td>3</td>
<td>4.2x10⁶</td>
<td>1.5x10³</td>
<td>10 mo-6 yrs</td>
</tr>
<tr>
<td>Dec. 2018</td>
<td>6</td>
<td>2.3x10⁷</td>
<td>6.5x10⁵</td>
<td>8 mo-12 yrs</td>
</tr>
<tr>
<td>Jan. 2019</td>
<td>7</td>
<td>1.4x10⁷</td>
<td>9.1x10⁴</td>
<td>10 mo-12 yrs</td>
</tr>
<tr>
<td>Feb. 2019</td>
<td>11</td>
<td>5.5x10⁷</td>
<td>2.1x10⁴</td>
<td>2 mo-12 yrs</td>
</tr>
<tr>
<td>Mar. 2019</td>
<td>5</td>
<td>6.2x10⁷</td>
<td>4.4x10³</td>
<td>1 mo-12 yrs</td>
</tr>
<tr>
<td>Apr. 2019</td>
<td>1</td>
<td>1.5x10⁷</td>
<td>NA</td>
<td>10 yrs</td>
</tr>
<tr>
<td>May 2019</td>
<td>1</td>
<td>3.9x10⁶</td>
<td>NA</td>
<td>2 mo</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†(copies/g stool)

Clinical Characteristics. Among the SalV positive pediatric patients with acute gastroenteritis, the main clinical features were diarrhea (86.8%) and fever (70.1%), followed by vomiting (42.6%). Based on gender, fever and vomiting in females were higher than in males. In contrast, diarrhea was more frequent in males than females (Table 2). The maximum viral load (6.2x10⁷ copies/g) was detected in an 11-year-old in March 2019, and the minimum (1.5x10³ copies/g) in ten-month and 12-year old patients in November and April. (Table 1).

Table 2. Clinical characteristics of patients based on gender

<table>
<thead>
<tr>
<th>Sex</th>
<th>Positive for Salivirus</th>
<th>Median age (years)</th>
<th>Fever (%)</th>
<th>Diarrhea (%)</th>
<th>Vomiting (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>22</td>
<td>4.3</td>
<td>15 (68.1%)</td>
<td>18 (81.8%)</td>
<td>10 (45.4%)</td>
</tr>
<tr>
<td>Females</td>
<td>19</td>
<td>5.1</td>
<td>16 (84.2%)</td>
<td>14/19 (73.6%)</td>
<td>11 (57.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>4.7</td>
<td>31</td>
<td>32</td>
<td>21</td>
</tr>
</tbody>
</table>

DISCUSSION

Salivirus, as a recently diagnosed virus, is a new member of the Picornaviridae family concomitantly detected by three research groups in 2007 from diarrheic and non-polio flaccid paralysis patients and untreated sewage water samples [7-9]. Many studies worldwide have documented the SalV in various age groups, especially in children under five years old, in polluted river waters, and treated and untreated wastewaters [10, 21, 22]. Nucleotide sequence analysis of the VP1 region of the Picornaviridae family is a valuable tool for their classification into genera. On the other hand, sequence regions of 2C, 3D, and VP3 have shown to be sufficient for genotype analysis of SalV, and primers that target 5'UTR, VP0/VP3, 2C, and 3D regions of viral genomic RNA can be used to detect SalV in either stool or environmental samples [7-9, 23, 24].

The present study examined stool samples from children ≤ 12 years of age with acute gastroenteritis presented to Imam Ali General Hospital, Alborz, Iran.
We detected the SalV RNA by the primers complementary to the 5'UTR region of the virus. Previously, we had successfully used these primers to identify SalV in sewage and river waters [21]. The prevalence of SalV in different countries among patients range from 0.1% to 8.8% [10, 14]. In our study, the SalV infection rate among children with acute gastroenteritis was 24.3%, much higher than similar studies worldwide. Although the SalV infection rate in children ≤ 12 years old was higher than in other studies, the conclusion of whether this virus is the causative agent of gastroenteritis in these children remains unresolved since we had no control groups.

Also, we checked for other known major pathogenic viruses that contribute to gastroenteritis. Of the 41 SalV positive stool samples examined in our study, 12 were also positive for AiV in our previous work [25], unlike a similar study that detected no coinfection with SalV [26]. The SalV viral load in positive stool samples ranged from 1.5×10^3 to 6.2×10^7 copies/g. Although the minimum viral load (1.5×10^3) in our study was much lower than a previous study which reported 7.1×10^6 copies/g stool [14], the maximum viral load of our study was almost one log lower. However, a real-time PCR for 1.5-5 days newborns in Hungary showed two logs higher than our study [15]. Our results showed high viral load in patients in different age groups ranging from 1 month to 12 years, and no relationship was between virus load and age.

In contrast to the report by Yu et al. (2015), which detected no SalV in the cold seasons [16], our results showed the highest SalV detection rate in February. Based on our results, we cannot refuse the possible effect of double infections in inducing gastroenteritis in children. However, regarding the lack of mono-infection, the possible effect of double coinfections in gastroenteritis severity remains to be investigated.

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CONFlict of INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

REFERENCES


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