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High Frequency of Salivirus in Pediatric Patients with Acute Gastroenteritis

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ABSTRACT

Introduction: Salivirus (SalV), a possible causative agent of gastroenteriti 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 reactio 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 transcriptio 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. Tl highest SalV detection rate was in Feb. (28.2%), and the highest viral load (6.2×1 copies/g) belonged to a 1-month-old patient. **Conclusion:** Salivirus occurs amoı 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 was first detected in 2009 in diarrheic children fecal samples in the United States and Australia, later in non-polio acute flaccid paralysis (NPAFP) and untreated sewage samples in Spain [7-9]. The nucleotide and amino acid sequence analysis demonstrated that early Klassevirus in literature was SalV [7, 8, 10, 11]. On the other hand, based on VP3, 2C, and 3D nucleotide sequence analysis, SalVs are divided into two genotypes, A1 and A2 [10, 12, 13]. SalV, a new member of Picornaviruses, is a naked positive-sense single-stranded RNA virus [7, 11, 14] with a genome component similar to other picornaviruses that include 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 [10, 15]. The length of SalV genomic RNA is 8021 nucleotides with an open reading frame encoding 2369 amino acids polyprotein [10, 15]. The worldwide distribution of SalV in different geographical studies has shown various detection rates (0.1%–8.8%) in patients with gastroenteritis [10, 16-18]. However, detection rates in environmental samples, i.e., river and sewage waters, ranged from moderate to high prevalence [19-22]. The SalV occurrence in environmental and stool samples from 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].

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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 58 (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 80ºC for 10 min, and then placed on ice for 10 min. Then, a second reaction buffer (containing10X RTase reaction buffer, 0.1 M DTT, HyperScript RTase, and RNase inhibitor) was added to the previous mixture and incubated at 42ºC for 60 min. Finally, the RT reaction mixture was incubated at 85ºC 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'-CTCTGCTTGGTGCCAACCTC-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 (10^1 to 10^7 copies/reaction) and negative and positive controls from our previous work [21] were also included. Amplification in a thermocycler (Rotorgen, 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 1.0×10^1 to 1.0×10^7 copies/reaction (Fig. 1). The CT values were directly proportional to the $\log^{10}$ 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 1.0×10^1 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 1.5×10^3 to 6.2×10^7 copies/g. The maximum viral load (6.2×10^7 copies/g) was in March, and the minimum (1.5×10^3) was in November and April (Table 1).

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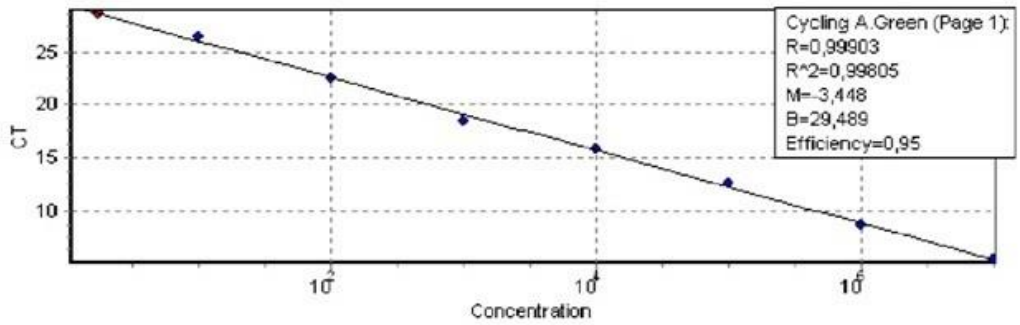

Fig. 1. Standard curve for SalV detection by RT-qPCR using serial 10-fold dilutions of genomic SalV– plasmid (10^1 to 10^7) per reaction [21].

Table 1. Data for SalV RT-qPCR positive patients

Sampling Date	SalV positive specimens	†Max. viral load	†Min. viral load	Age groups
Sep. 2018	3	6.3×10^6	2.8×10^4	2 mo-4 yrs
Oct. 2018	4	1.1×10^6	2.4×10^3	1 mo-6 yrs
Nov. 2018	3	4.2×10^6	1.5×10^3	10 mo-6 yrs
Dec. 2018	6	2.3×10^7	6.5×10^5	8 mo-12 yrs
Jan. 2019	7	1.4×10^6	9.1×10^4	1 mo-10 yrs
Feb. 2019	11	5.5×10^7	2.1×10^5	2 mo-12 yrs
Mar. 2019	5	6.2×10^7	4.4×10^3	1 mo-12 yrs
Apr. 2019	1	1.5×10^3	NA	12 yrs
May 2019	1	3.9×10^6	NA	2 mo
Total	41			

†(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 (6.2×10^7 copies/g) was detected in an 11-year-old in March 2019, and the minimum (1.5×10^3 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

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

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.

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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.

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