Original Article

First Molecular Detection of Saffold Virus in Children with Acute Gastroenteritis in Iran

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Abstract

Background: Saffold virus as a new member of cardiovirus genus in *picornaviridae* family has been suggested to be related to diarrheic cases and human airway diseases. However, relationship between Saffold virus and human diseases is unclear. In order to establish an investigation for the occurrence of Saffold virus among pediatric patients involved to acute gastroenteritis, we implemented a RT-PCR assay for detection and quantification of Saffold virus in stool specimens.

Materials and Methods: In this study, a total of 160 stool samples from September 2018 to May 2019 were collected from presenting pediatric patients with acute gastroenteritis in a Karaj hospital, Iran. After viral RNA extraction, the RT-PCR was performed to amplify the 5'UTR region of Saffold virus genome.

Results: Out of the 160 samples tested, the Saffold virus genomic RNA was detected in 26/160 (16.2%) of stool samples. The high Saffold virus detection rate was related to February (6/26 or 23%). The co-infection of Saffold virus with Aichivirus and Salivirus as other new emerging viruses was also assessed, among which high double or triple mixed-infections were determined.

Conclusion: This is the first documentation of Saffold virus detection in stool samples that demonstrates Saffold virus has been circulating among Iranian pediatric patients. Our results indicated that Saffold virus in association with Aichivirus and Salivirus may be possibly considered as causative agent of acute gastroenteritis.

Keywords: Saffold virus, Pediatric patients, RT-PCR, Acute gastroenteritis

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Introduction

Acute gastroenteritis is considered to be induced with infection of humans by various determined pathogens such as viruses including rotaviruses and noroviruses as main etiologic agents of diarrheic diseases[1,2]. However, almost up to 40% of diarrheic cases with unknown etiology remain to be identified[3-

[5]. Among newly discovered enteric viruses, the human cardiovirus (named Saffold virus) is an new emerging virus which was firstly detected in 2007 from supernatant of human diploid fibroblast cell culture inoculated with 1981 archived stool sample of eight-month child in United State[6]. After first isolation of Saffold virus (SAFV), SAFV has detected by several research groups from stool of children with gastroenteritis symptoms, nasopharyngeal samples from affected child with airway diseases, and the cerebrospinal fluid (CSF) samples of aseptic meningitis[7-16]. However, Saffold viruses have also identified from stool samples of children suffering non-polio acute flaccid paralysis (AFP)[7,17,18]. On the other hand, the viral genome of Saffold virus has diagnosed in sewage water samples only in three studies in American different cities, Japan and Iran[19,20]. After discovery of Saffold virus, the nucleotide and amino acid sequence analysis showed that Saffold virus is related to species *Theilovirus* in the genus *Cardiovirus* of the family *Picornaviridae*[6]. *Theilovirus* species includes Theiler-like rat virus (TRV), Theiler's murine encephalomyelitis virus (TMEV), Vilyuisk human encephalomyelitis virus (VHEV), and Saffold virus as a new member[6,7]. In addition, Saffold virus isolates on the basis of nucleotide similarity of VP1 region and phylogenetic analysis have been included in 11 different genotypes for which geographical distribution and intertype recombination have been determined[18,21,22]. Epidemiological studies in several different geographical areas on the world propose that Saffold viruses have a worldwide distribution and they can be the possible etiologic cause of gastroenteritis, respiratory and neurological diseases in humans[7]. Saffold virus surveillance in epidemiological studies has shown a low rate of detection of SAFV in a gastrointestinal cohort study with 1.2% positive range in stool samples and in other study for determination of the SAFV genotype 2 detection rate in patients with acute respiratory diseases was 3.5% positive[12,23]. However, a serological study to determine the virus neutralizing antibody illustrated that SAFV genotype 3 infections for children under age of 24 months and in older children or in adults were >75% and >90% positive, respectively[24].

Co-infections of Saffold virus with other enteric viruses in analysis of patients with gastroenteritis have reported[15,16,25]. In addition, the pathogenesis of Saffold virus and the relationship between Saffold virus infections and gastroenteritis, respiratory and neurological diseases remains to be exactly determined. Saffold viruses similar to picornaviruses are non-enveloped viruses with one segment of single-stranded RNA genome in positive sense[26]. The viral genome organization in length of 8050 nucleotides is the same as picornaviruses including 5´UTR- L protein- viral structural VP0, VP3 and VP1 proteins- nonstructural 2A, 2B, 2C, 3A, 3B, 3C and 3D proteins- 3´UTR that end with a 3´ poly (A) tail[6,18,22]. Molecular methods including RT-PCR and RT-quantitative PCR (RT-qPCR) have been introduced as a gold standard to detect SAFV isolates with targeting 5'UTR region of their genomes[6,8,11,14-16,18]. However, virus cell cultures have been used for Saffold virus isolation, virus adaptation and virus cytopathogenesis[27,28].

Here, we tested stool samples from affected children with gastroenteritis, which were admitted to Karaj hospital in Iran for detection and quantification of Saffold virus by RT-PCR using primers targeted to 5'UTR region of viral genome. This is the first report of Saffold virus occurrence among involved children with acute gastroenteritis in Iran.

Methods

Specimens

In order to conduct a retrospective study during 9-month period from September 2018 to May 2019 for assessment of Saffold virus occurrence, a total of 160 fecal specimens were collected from walk-in clinics. All fecal samples were obtained from a hospital in the city of Karaj from pediatric patients under 12 years of age that presented with acute gastroenteritis. The study was reviewed and approved by the ethical committee (with ethical number of IR.IAU.K.REC.1398.044) for human experimentation in Faculty of Medicine, Karaj University. According to age, the pediatric patients were divided, based on age (year), into under 1, 1-5 and 6-12 with frequency of 58 (36.2%), 54 (33.7%), and 48 (30%), respectively. The samples were examined to be negative for pathogenic bacteria such as *Escherichia coli, Salmonella spp, Campylobacter*,

and *Shigella ssp*. The study was reviewed and approved by the ethical committee for human experimentation in Faculty of Medicine, Karaj University. In order to preparation of stool specimens, 10% phosphate-buffered saline (pH 7.2) homogenate of stool samples were centrifuged in 8000 ×g for 10 minutes. Supernatants were stored in -80 °C until RNA extraction step.

Viral RNA extraction and reverse transcription

Viral genomic RNA of Saffold virus was extracted from 300 µL of 10% fecal sample suspensions using the TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. The viral RNA was eluted in a final volume of 50 µL. The concentration and quality of the extracted RNA were examined by Nanovue spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNA was used directly in the reverse transcription reaction or stored at −70°C until use. The known positive Saffold virus RNA as a control which was previously extracted from sewage and documented for Saffold virus RNA presence was also included[29]. Briefly, 10 µL of extracted RNA or positive Saffold virus RNA was added in RT mixtures (containing random primer and dNTPs) and incubated at 80ºC for 10 minutes and after then placed on ice for 10 minutes. Then, 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 minutes. Finally, the RT reaction mixture was incubated at 85ºC for 5 minutes to inactivate the enzyme. Nucleotide sequences of the partial 5'UTR region of Saffold virus are highly conserved (99.2% to 97%), with amino acid identities of 99 to 100%, thus the selected previously used primers containing forward (5'-CTAWCATGCCTCCCCGATT-3') and reverse primers (5'- GYTTAGACCGGGGGAACC -3')[29,30] can amplify a 111-bp fragment located at the 5'UTR region of the Saffold virus genotypes.

Implementation of the PCR Assay

A PCR reaction using forward and reverse primers to detect all of Saffold virus genotypes for each sample was performed in a 20 µL reaction volume containing 2 µL of synthesized cDNA as a template, 10 µL master-mix (BioFact), and 10 mM of each primer. The negative and positive control reactions also were included. PCR amplification was done with ABI under the following program: primary denaturation at 95°C for 10 minutes, followed by 40 amplification cycles consisting denaturation at 95°C for 30 seconds, annealing at 60°C for 25 seconds, and extension at 72°C for 25 seconds. Amplification products were analyzed with gel electrophoresis.

Results

Saffold virus viral genomic RNA detection in stool samples

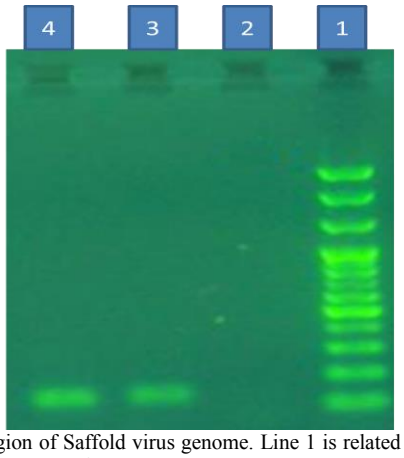

Figure 1. Gel electrophoresis for amplified 5'UTR region of Saffold virus genome. Line 1 is related to DNA marker, lines 2 is negative control, and lines 3 and 4 are related to positive stool samples in length of 111bp.

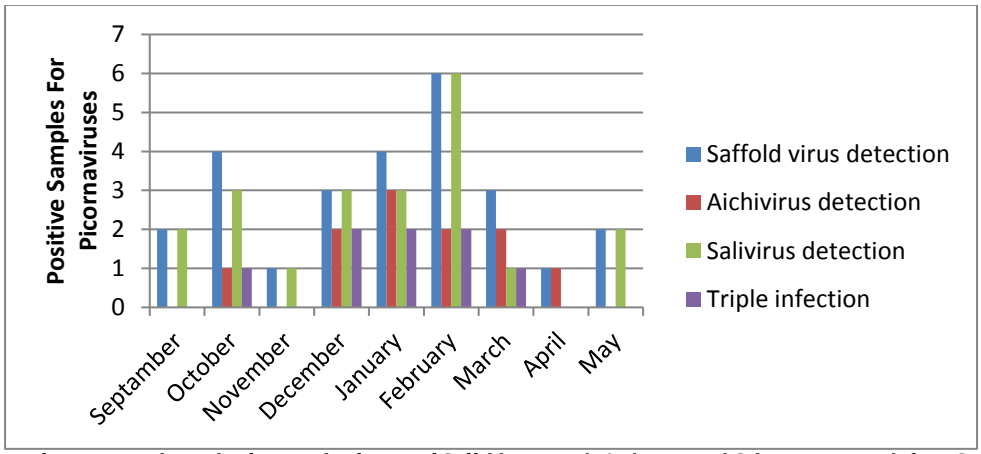

Figure 2. Number of positive stool samples for mixed-infection of Saffold virus with Aichivirus and Salivirus per month from September 2018 to May 2019.

Table 1: Characteristics of patients, and co-infections.

Date of sampling	Positive for Saffold virus	Co-infection with Aichivirus	Co-infection with Salivirus	Triple infection
September/ 2018	2	0/2 (0%)	2/2 (100%)	0/2 (0%)
October/ 2018	4	1/4 (25%)	3/4 (75%)	1/4 (25%)
November/ 2018	1	0/1 (0%)	1/1 (100%)	0/1 (0%)
December/ 2018	3	2/3 (66.6%)	3/3 (100%)	2/3 (75%)
January/ 2019	4	3/4 (75%)	3/4 (75%)	2/4 (50%)
February/ 2019	6	2/6 (33.3)	6/6 (100%)	2/6 (33.3%)
March/ 2019	3	2/4 (50%)	1/4 (25%)	1/4 (25%)
April / 2019	1	1/1 (100%)	0/1 (0%)	0/1 (0%)
May/ 2019	2	0/2 (0%)	2/2 (100%)	0/1 (0%)

The RT-PCR assay was implemented for detection of Saffold virus in a total of 160 diarrheic stool samples collected from Karaj hospital, Iran. Out of 160 fecal samples tested, 26 (16.2%) were positive for SAFV. The positive samples showed an 111bp amplification product in gel electrophoresis (Figure 1). According to the characteristics of the 26 patients with SAFV tested positive in stool samples, 10 samples were related to male and 16 samples were female. The median age of them was 3.8 years (range of 1 month to 12 years). The maximum detection rate of SAFV was in February with frequency of 23% (6 out of 26) and minimum of SAFV detection was related to November and April with 3.8% (1 out of 26) (Table 1).

Although co-infection of the positive SAFV cases with other pathogenic viruses such as Rotaviruses, Noroviruses, human Adenoviruses, and Enteroviruses were not assessed, mixed-infection of SAFV with other newly discovered viruses from *Picornaviridae* family including Aichivirus and Salivirus was examined, among which 11 samples were also positive for Aichivirus and 20 samples were also positive for Salivirus. However, co-infections could be as triple infection instead of double infection with concomitant infection of Aichivirus and Salivirus in one patient. The triple co-infected cases were associated with 8 patients. High frequent triple infections were related to

December (75%), January (50%), and February (33.3%) (Figure 2).

Clinical Characteristics

Among pediatric patients which admitted with acute gastroenteritis, the main symptoms were diarrhea and fever in rang of 84.8% and 63.1%, respectively. Vomiting as another sign was also seen in less frequency (48.8%). Diarrhea and fever symptoms were seen with high severity for around half of pediatric patient (49.7%) which had mixed-infection.

Discussion

After initial identification of Saffold virus by Jones and et al, in California through inoculation of archived stool samples of a child with fever of unknown origin in line of fetal human diploid fibroblast, many studies have identified SAFV prevalence among different age groups especially among children as well as in environmental polluted samples including sewage water[6,7,15,17,20,29]. The viral VP1 region has been generally used to divide *Picornaviridae* family into genera because of its nucleotide sequence variability features, but the same region of Saffold viruses have been shown to be sufficient for SAFV genotype analysis[6,21]. On the other hand, the using primer set which has targeted 5'UTR region of Saffold virus genomic RNA can be applied to detect Saffold virus isolates in either of stool, respiratory, cerebrospinal fluid, and environmental specimens[16,18,19,29,30]. In this study, we used a set of primers targeting 5'UTR region of Saffold virus, which was previously used to detect Saffold virus by RT-PCR and RT-qPCR assays[30]. In general, the previous study conducted by our research group showed using the primers could be beneficial to determine viral detection and quantification of Saffold virus in sewage and river water samples[29].

Here, we tested 160 stool samples obtained from pediatric patients with acute gastroenteritis who were under 12 years and were presented to Karaj hospital in Iran. The specimens were collected from September 2018 to May 2019. The detection rate of Saffold virus in our report was 16.2% for pediatric patients who presented with acute gastroenteritis. This was the highest report of detection rate of Saffold virus compared with other implemented studies worldwide. Generally, regarding the role of geographical regions in detection rate of Saffold virus in various area, we could conclude that high occurrence of Saffold virus in Iran is reasonable. Although in this study detection rate of Saffold virus infection in children under 12 years of age (median 3.8 years) was slightly higher than that of other studies, concluding whether Saffold virus might causative agent of gastroenteritis in children is not clear since we have not included control groups (healthy children). On the other hand, we did not analyze other known pathogenic viruses (Rotaviruses or Noroviruses) as major agent of gastroenteritis in these stool samples for assessment of double or more co-infection of Saffold virus with other viral pathogens. However, we also tested the positive samples for detection of other new emerging viruses which belong to *Picornaviridae* family such as Aichivirus and Salivirus (data are not shown). From the 26 positive stool samples for Saffold virus, 11 samples were also positive for Aichivirus, and 20 samples were positive for detection of Salivirus. Generally, triple infections of Saffold virus with Aichivirus and Salivirus in patients were also seen. In contrast to Nielson and Chuchaona studies[30,31], which had no double infection of Saffold virus with Aichivirus, our results showed mixed-infection of these two viruses in high frequency (42.3%). Based on the results, we cannot exclude the effect possibility of mixed-infections in these viruses in the induction of diarrheal diseases in children. Meanwhile, whether double or triple infections of Saffold virus with Aichivirus and Salivirus instead of mono-infection can exacerbate the severity of gastroenteritis remains to be clarified.

In accordance with findings in other studies indicating high seasonality distribution of Saffold virus infections occur in the autumn and winter, we had a high number of positive samples in October, January, and February. However, in this study Saffold virus detection rate was low in November, which was previously shown to be a month with highly detection of Saffold virus[30].

Conclusion

For the first time our results indicate that Saffold virus is circulating in pediatric patient under 12 years in

Iran.

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