Original Article

First Molecular Detection of Aichivirus in Pediatric Patients with Acute Gastroenteritis in Iran

Mohammad Taghinejad[1], Mostafa Ghaderi[1*], Seyed Dawood Mousavi- Nasab[2]

[1] Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran
[2] Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, Tehran, Iran

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Abstract

Background: Aichivirus as a new member of *Picornaviridae* family was detected and isolated in Japan. Aichivirus species, which belongs to genus *Kobuvirus*, include of three genotypes A, B and C. Based on previous reports to detect aichiviruses in stool samples as well as environmental samples such as river waters and sewage waters, it has been demonstrated that Aichivirus infect humans by fecal-oral routs. In order to establish an examination for the prevalence of Aichivirus among pediatric patients involved to acute gastroenteritis, we conducted a RT-qPCR assay for detection and quantification of Aichivirus in collected stool samples.

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 Karaj hospital, Iran. After viral RNA extraction, the RT-PCR was performed to amplify the 3CD junction region of Aichivirus.

Results: Out of the 160 samples tested, the Aichivirus genomic RNA was detected in 13/160 (8.1%) of stool samples. The maximum viral prevalence rate was related to December (30.7%). The co-infection of Aichivirus with Salivirus and Saffold virus also assessed, among which high double or triple mixed-infections were determined.

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

Keywords: Aichivirus, Pediatric Patients, Acute Gastroenteritis

***Corresponding Author:** Mostafa Ghaderi, Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran. Tel: (+98) 912 2507455, Fox: (+98) 26 34418156. E.mail: ghaderi_viro@yahoo.com

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Introduction

Most commonly diarrheic viruses including Rotaviruses, Noroviruses, Astroviruses, and enteric Adenoviruses have been related to acute gastroenteritis[1]. However, there are many unknown etiological agents of causative viral diarrheal cases, which remain to be demonstrated[2]. Conducting of several studies for detection of new emerging enteric viruses have recently applied to detect new discovered viruses from *Picornaviridae* family among which human Aichivirus was firstly identified by Yamashita et al from a patient as a possibly agent for nonbacterial oyster-associated gastroenteritis in Japan in 1989[4]. Up

to now, Aichiviruses (AiV) have been isolated from patients with acute gastroenteritis after consumption of polluted see-foods and from contaminated water sources consisting river, ground, and sewage waters[5]. Through nucleotide sequence analysis of Aichivirus genome, Aichivirus was included in *Picornaviridae* family as a separate genus named *Kobuvirus*[6]. Aichivirus particles are determined as a small-round naked virus with one positive-sense segment of viral genomic RNA[5,6]. The length of Aichivirus genomic RNA is 8251 nucleotides with an open reading frame encoding 2432 amino acids polyprotein. In general, Aichivirus genomic RNA encodes viral leader protein, viral structural proteins (VP0, VP3, and VP1) and viral nonstructural proteins (2A, 2B, 2C, 3A, 3B. 3C, and 3D)[6,7]. Three genotypes consisting genotype A, B, and C by analysis of nucleotide sequence of human Aichivirus isolates were determined, which geographical distribution of Aichivirus genotypes is slightly different. Genotype A has been identified in Japan and Europe, but genotype B has been found in Brazil and other countries in Asia other than Japan and genotype C has been circulating in Africa[5,8]. After detection of human Aichivirus, bovine Kubovirus species and porcine kubovirus species as other members of Kobuvirus species have also characterized which infect animals[5,8]. Since mixed-infection of Aichivirus with potential pathogenic enteric viruses and bacteria has been shown, the clinical importance of Aichivirus infections as a causative agent of acute gastroenteritis in sporadic and epidemic cases is rather unclear[3,10]. On the other hand, detection of Aichivirus without presence of other pathogens in conducted studies in Japan, Brazil, Hungry, and China have justified that Aichivirus can be induce acute gastroenteritis[10,15-17]. Based on high detection rate of antibody against Aichivirus (80-99%) and low incidence rate of Aichivirus infections (0.4-6.5%)[8,15,18-23], it suggests that Aichivirus infections occur asymptomatically. However, in symptomatic cases the clinical signs are defined as fever, diarrhea, abdominal pain, nausea, and vomiting[4,24].

Since Aichivirus culture is time-consuming, molecular detection Aichivirus based on reverse transcription-polymerase chain reaction (RT-PCR) and RT-qPCR have been determined as gold standard for detection of Aichiviruses[5,22,25-27]. In this study, we examined stool specimens of pediatric patients with acute gastroenteritis presenting to Karaj hospital in Iran for detection of Aichivirus by RT-PCR assay. This is first report of Aichivirus 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 March 2019 for assessment of Aichivirus 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. According to age, the pediatric patients were divided into under 1 year of age, 1-5 years of age, and 6-12 years of age 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 (IR.IAU.K.REC.1398.045). 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. The supernatants were stored in -80°C until RNA extraction step.

Viral RNA extraction and reverse transcription: Viral genomic RNA of Aichivirus 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 Aichivirus RNA as a control, which was previously documented for Aichivirus RNA presence, was also included. Briefly, 10 µL of extracted RNA or positive Aichivirus 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 (containing 10X 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 3CD region of AiV are highly conserved (99.2% to 97%), with amino acid identities of 99 to 100%, thus the selected previously used primers containing forward (5'-GACTTCCCCGGAGTCGTCGTCT-3') and reverse primers (5'-GCRGAGAATCCRCTCGTRCC-3')[28] can amplify a 158-bp fragment located between the 3CD junction region of the Aichivirus genotypes.

Implementation of the PCR Assay: A PCR reaction using forward and reverse primers to detect all of AiV 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 25 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 20 seconds. Amplification products were analyzed with gel electrophoresis.

Results

Aichivirus viral genomic RNA detection in stool samples: The RT-PCR assay was implemented for detection of AiV in a total of 160 diarrheic stool samples collected from Karaj hospital, Iran. Out of 160 fecal samples tested, 13 (8.1%) were positive for AiV. The positive samples showed a 158bp amplification product in gel electrophoresis (Figure 1). Regarding the characteristics of the 13 patients with AiV diagnosed in stool samples, among which four samples were related to male and nine samples were female, the median age of them was 2.6 years (range of 3 months to 12 years). The maximum detection rate of AiV was in December with frequency of 30.7% (4 out of 13) and minimum of AiV detection was related to October 7.6% (1 out of 13) and April 7.6% (1 out of 13). However, AiV Viral genomic RNA was not detected in September, November, and May (Table 1). Although co-infection of the positive AiV cases with other pathogenic viruses such as rotaviruses, noroviruses, human adenoviruses, and enteroviruses were not assessed, mixed-infection of AiV with other newly discovered viruses from picornaviridae family including Salivirus and Saffold virus was examined, among which 12 samples were also positive for Salivirus and 11 samples were also positive for Saffold virus (Figure 2). However, co-infections could be as triple infection instead of double infection with

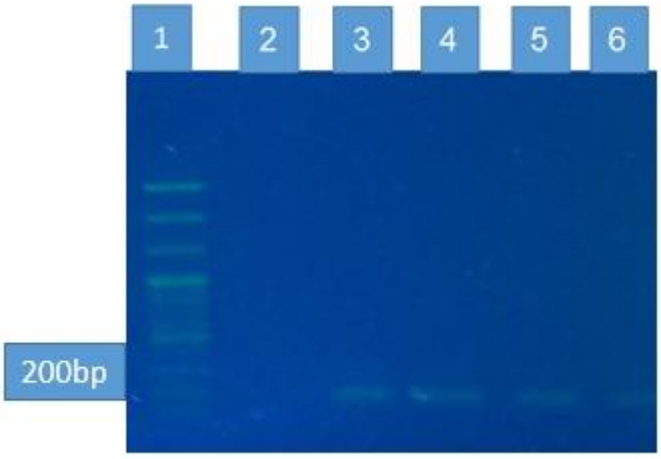

Figure 1. Gel electrophoresis for amplified the 3CD junction region of Aichivirus genome. Line 1 is related to DNA marker, lines 2 and 3 are negative and positive control, respectively, and lines 4 to 6 are related to three positive stool samples in length of 158bp.

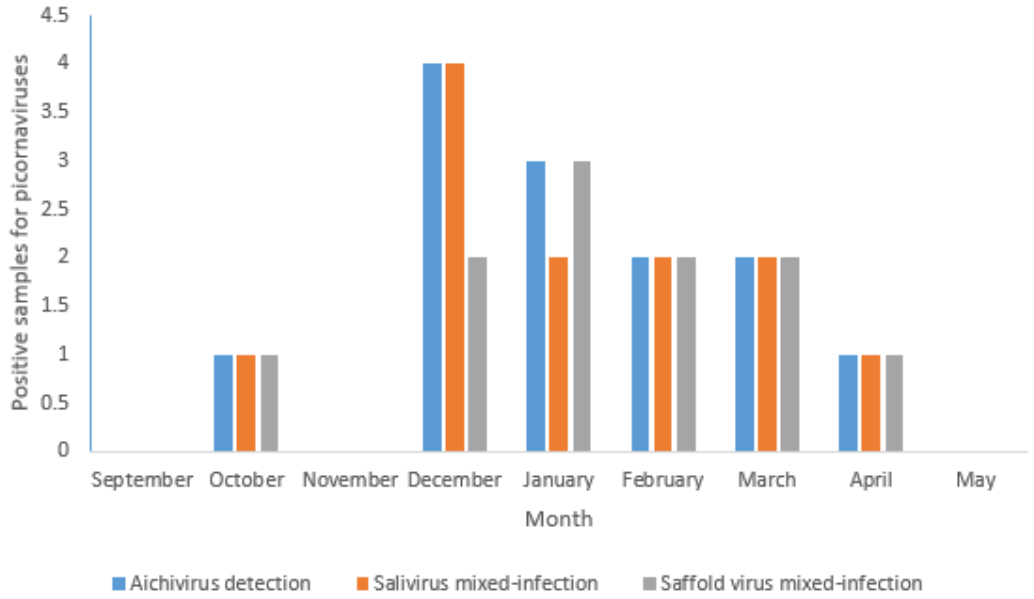

Figure 2. Number of positive stool samples for mixed-infection of Aichivirus with Salivirus and Saffold virus per month from September 2018 to May 2019.

Table 1: Characteristics of patients and assessment of co-infections in stool samples by RT-PCR assay.

Sample number	Date of sampling	Sex	Age	Co-infected viruses
1	October/ 2018	Male	4 Months	Salivirus/Saffold virus
2	December/ 2018	Male	4 Years	Salivirus
3	December/ 2018	Female	2 Years	Salivirus
4	December/ 2018	Female	1 Year	Salivirus/Saffold virus
5	December/ 2019	Male	1 Year	Salivirus/Saffold virus
6	January/ 2019	Male	2 Years	Salivirus/Saffold virus
7	January/ 2019	Male	1 Month	Saffold virus
8	January/ 2019	Female	2 Years	Salivirus/Saffold virus
9	February/ 2019	Female	2 Years	Salivirus/Saffold virus
10	February/ 2019	Male	4 Years	Salivirus/Saffold virus
11	March/ 2019	Male	10 Months	Salivirus/Saffold virus
12	March/ 2019	Male	1 Month	Salivirus/Saffold virus
13	April / 2019	Male	12 Years	Salivirus/Saffold virus

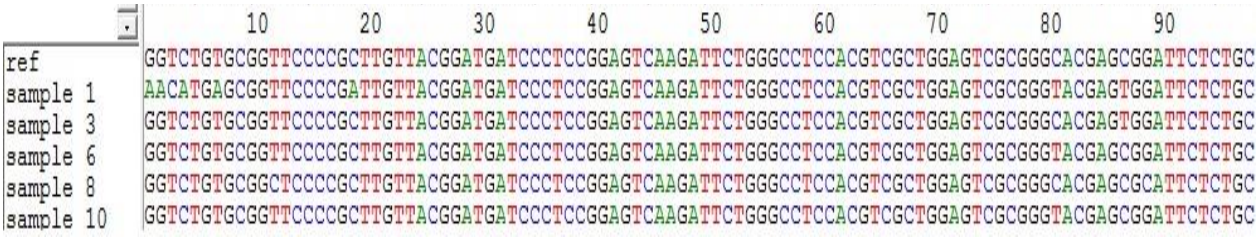

Figure 3. Sequencing results for five sequenced positive stool samples. Sample numbers of selected positive RT-PCR products were compared with sequence of Aichivirus with accession number AB040749 gained from NCBI.

concomitant infection of Salivirus and Saffold virus in one patient. The triple co-infected cases were associated with 10 patients (Table 1).

Confirmation of Aichivirus detection: In order to confirm RT-PCR results, sequencing for five positive samples were done. The results of sequencing showed the highest nucleotide sequence homology to 3CD sequence of Aichivirus obtained from genebank with accession number of AB040749 (Figure 3).

Discussion

Human Aichivirus including three genotypes A, B, and C are introduced as new emerging viruses

infecting children and adults during epidemic periods and in form of sporadic cases[3,8,26]. After the first detection of Aichivirus in stool sample during an outbreak in 1989 in Japan, many studies have been identified the different Aichivirus genotypes around the world[4,8,10-13,18,21,27]. However, Aichiviruses have also been detected from environmental samples containing treated and untreated sewage waters, river waters, and ground waters[27,29,30]. Assessment of structural VP1 region and nonstructural 3CD junction region of Aichiviruses have been shown to have advantage for Aichivirus detection and Aichivirus genotyping analysis[6,31,32]. In the present study, we used a set of primers targeting 3CD junction region of Aichivirus, which was previously used to identify Aichivirus by RT-PCR and RT-qPCR assays[27,28]. In general, previous study, which was conducted by our research group, demonstrated using the primers could be useful to determine viral detection and quantification of Aichivirus in sewage and river water samples[27]. Here, we tested 160 stool samples obtained from pediatric patients with acute gastroenteritis, which were under 12 years of age and were presented to Karaj hospital in Iran. The specimens were related to a collection of stools from September 2018 to March 2019. From the literature search, in contrast of frequent detection rate of antibody against Aichivirus (80-99%), the prevalence of Aichivirus in both sporadic and outbreak cases were estimated to be in range of 0.8-6.5%, which have appraised in different countries[26,31]. The prevalence rate of Aichivirus detection in our reports was 8.1% for children presenting with acute gastroenteritis. This is maximum of reported detection rate of Aichivirus at compared with other implemented studies in worldwide. Although in this study the prevalence of Aichivirus infection in pediatric patients less than 12 years of age was slightly higher than other studies, conclusion for whether Aichivirus is causative agent of gastroenteritis in children should be demonstrated since we have not used control groups (healthy children). With together, we did not test other known pathogenic viruses, which account for major agent of gastroenteritis in our examined stool samples for assessment of double or more co-infection of Aichivirus with another viral pathogens. On the other hand, we tested the positive samples for detection of new emerging viruses including Salivirus and Saffold virus which belong to picornaviridae family (data are not shown). Out of the 13 positive stool samples for Aichivirus, 12 samples were also infected with Salivirus, and 11 samples were positive for Saffold virus. Generally, mixed-infection in form of triple co-infection of Aichivirus with Salivirus and Saffold virus in one patient were seen. In contrast to Nielson and Chuchaona studies[12], which had no double infection of Aichivirus with Saffold virus, our results showed mixed-infection of these viruses in high frequency (84.6%). Based on the results, we cannot exclude the possibility of effect of mixed-infections of these viruses in the induction of gastroenteritis in children. Meanwhile, whether double or triple co-infections of Aichivirus with Salivirus and Saffold virus instead of mono-infection can exacerbate the severity of gastroenteritis remains to be defined. Similar to other studies, which have been, indicated the high seasonality distribution of Aichivirus infections in December and January, we also detected high number of positive samples in cold months[12,32].

Conclusion

Finally, our results for the first time indicate that Aichivirus may be agent of gastroenteritis, which is circulating in pediatric patients under 12 years of age in Iran.

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