Gastroenterology and Hepatology From Bed to Bench.

ORIGINAL ARTICLE

High viral load detection of human Cosavirus in Iranian pediatric patients with acute gastroenteritis

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ABSTRACT

Aim: The present study implemented an RT-qPCR assay for the detection and quantification of human cosavirus in stool specimens from pediatric patients involved in acute gastroenteritis.

Background: Human cosavirus is a newly recognized virus that seems to be partly related to acute gastroenteritis in pediatric patients. However, the relationship between human cosavirus and diseases in humans is unclear

Methods: From January 2018 to December 2019, a total of 160 stool samples were collected from pediatric patients presenting with acute gastroenteritis in a hospital in Karaj, Iran. After viral RNA extraction, RT-qPCR was performed to amplify the 5'UTR region of the human cosavirus genome and viral load was analyzed.

Results The human cosavirus genomic RNA was detected in 4/160 (2.5%) stool samples tested. The maximum viral load was determined to be 4.6×10^6 copies/ml in one sample obtained from a 4-year-old patient.

Conclusion: The human cosavirus as a new member of the Picornaviridae family was illustrated in fecal samples from pediatric patients with acute gastroenteritis in Iran. This is the first documentation of human cosavirus circulation in Iranian children.

Keywords: Cosavirus, Acute gastroenteritis, Pediatric Patients, Stool.

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Introduction

Picornaviridae is a growing family with newly discovered viruses, including Aichi viruses, saliviruses, saffold viruses, and human cosaviruses, among which the latter was first detected in fecal specimens of

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nonpolio acute flaccid paralysis and healthy children in Pakistan in 2008 (1). Human cosaviruses were subsequently detected in different samples obtained from HIV-infected patients with gastroenteritis (3), children with gastroenteritis (4), and pediatric patients with nonpolio flaccid paralysis (2) and from environmental samples such as sewage waters (2-6). Although the similar rates of human cosavirus detection in humans with nonpolio flaccid paralysis and in patients with gastroenteritis has led to the pathogenicity of human cosavirus being unknown, a study implemented in Japan suggested human cosavirus as a causative agent of gastroenteritis in pediatric patients because of the lack of known pathogenic viruses in fecal samples of involved patients (7). From the detection of human cosavirus in various samples consisting of sewage and feces samples across the world, the geographical distribution of the virus has been determined to be large (8). Some characteristics of human cosaviruses including the high presence of co-infections, low viral load detection, and identification of different strains of human cosaviruses along with various symptoms have suggested that human cosaviruses may not be related to diseases in humans (9). Taxonomically, human cosavirus belongs in the Cosavirus genus with five species (A, B, D, E, and F) and more than 30 genotypes (4). The high distributed genotypes are A and D (9-11). Although cell culture has been used to amplify and identify human cosaviruses, the detection of them in environmental and clinical samples has relied on RT-PCR and RT-quantitative PCR (RT-qPCR) as the gold standard in which 5′UTR, VP1, and 3D regions of their genomes have been targeted (12, 13). In the present study, stool samples from children affected with gastroenteritis, who were admitted to a hospital in Karaj, Iran, were tested. Human cosavirus was detected by RT-qPCR using primers targeting the 5'UTR region of viral genome. To the best of our knowledge, this is the first report of human cosavirus occurrence among children with acute gastroenteritis in Iran. Documenting the pathogenesis of human cosavirus among children can determine the importance of the virus in gastroenteritis with unknown agents and in other diseases in the future.

Methods

Specimens

The current retrospective study was conducted during the 12-month period from January 2018 to December 2019 to analyze human cosavirus occurrence from a total of 160 fecal specimens collected from walk-in clinics. All fecal samples were obtained from a hospital in the city of Karaj (Emam Ali Hospital) from pediatric patients under 12 years of age who presented with acute gastroenteritis. According to the World Health Organization (WHO), diarrhea was defined as a decrease in the consistency of stools (loose or liquid) and/or as having more stools than normal for that person (typically, ≥3 in 24 h), with or without fever or vomiting. A checklist was completed by two investigators for each patient comprising information including age, sex, and presenting symptoms such as fever, diarrhea, and duration of illness before admission. The study was reviewed and approved by the Ethical Committee for Human Experimentation of the Faculty of Medicine, Islamic Azad University, Karaj Branch (Code: IR.IAU.K.REC.1398.044). The parents of these children signed an informed consent for participation form. Following parasitology and bacteriological tests, the negative specimens were saved at -80 °C. The samples were examined for pathogenic bacteria such as

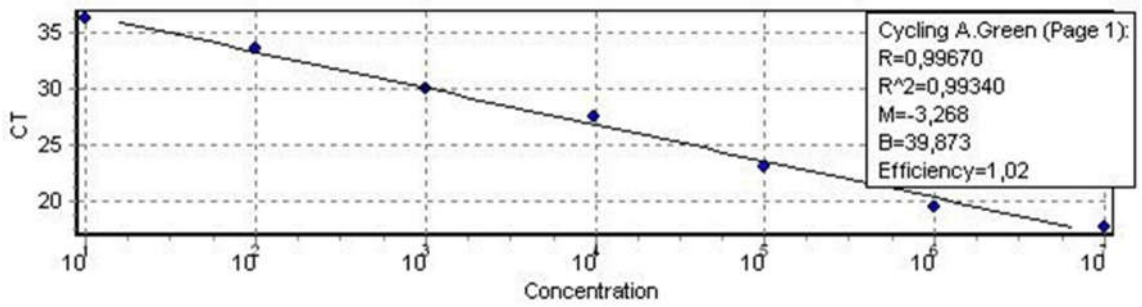

Figure 1. Standard curve for human Cosavirus detection by Rotorgen Real-time PCR in a series of 10-fold dilutions of genomic human Cosavirus– Plasmid (10^1 to 10^7) per PCR reaction

Escherichia coli, *Salmonella ssp, Campylobacter,* and *Shigella spp*. To prepare 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 at -80 °C until RNA extraction.

Viral RNA extraction and reverse transcription

Viral genomic RNA was extracted from 300 µL of 10% fecal sample suspension using the TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. The extracted RNA was used directly in the reverse transcription reaction or stored at −70 °C until use. Briefly, 10 µL of extracted RNA was added in RT mixtures (containing random primer and dNTPs), incubated at 80 ºC for 10 minutes, and 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 42 ºC for 60 minutes. Finally, the RT reaction mixture was incubated at 85 ºC for 5 minutes to inactivate the enzyme.

Implementation of the qPCR Assay

A Syber Green qPCR reaction using forward (CTCCCGTTCCTTCTTGGAC) and reverse (CACTGTGTGGGTCCTTTCG) primers was performed to detect human cosavirus genotypes for each sample and for synthesized plasmid DNA containing human cosavirus sequence in length of 215-bp (10^1 to 10^7 copies/reaction) (14). The negative control reaction was also included. PCR amplification was done with Rotorgen under the following program: primary denaturation at 95 °C for 10 minutes, followed by 40 amplification cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 25 seconds, and extension at 72 °C for 30 seconds. Amplification data was analyzed with Rotor-Gene Q software. All samples were characterized by a corresponding Ct value. Negative samples gave no Ct value. The standard curves were illustrated based on 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 of 1.0×10^1 to 1.0×10^7 copies/reaction (Figure 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 (Figure 1). The lower quantification limit was determined to be around 1.0×10^1 copies per reaction. Based on melting curve analysis for assessment of specificity of real-time PCR, each sample was able to produce a single sharp peak, and all of them overlapped and showed the same melting temperature.

Results

Human cosavirus viral genomic RNA detection in stool samples

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 rates of 58 (36.2%), 54 (33.7%), and 48 (30%), respectively. Out of 160 fecal samples tested, 4 (2.5%) were positive for human cosavirus by RT-qPCR and gel electrophoresis demonstrated bonds of positive RT-qPCR (Figure 2).

Of the 4 stool samples diagnosed with HCosV, 3 were related to males and 1 sample was from a female; the median age of these four patients was 4.6 years (range of 1 month to 12 years). The maximum detection rate of HCosV was in February with a frequency of 75% (3 out of 4) and the minimum HCosV detection rate was related to April with 25% (1 out of 4) (Table 1).

Human cosavirus viral load determination

According to the standard curve, the concentration of viral genomic RNA in stool samples ranged from 1.2×10^3 to 4.6×10^6 copies/ml. The maximum of HCosV viral load (4.6×10^6 copies/ml) was related to February, obtained from a 4-year-old male, and the minimum HCosV viral load (1.5×10^3 copies/ml) was associated with February and obtained from an 11yearold male (Table 1). Although the maximum viral load was seen in a 4-year-old case (4.6×10^6 copies/ml), low viral loads

Tables 1. Clinical characteristics of patients based on gender and viral load determination

Sex and month	Viral load (copies/ml)	Age	Fever	Diarrhea	Vomiting
Male, February	4.6×10^6	4 years	Positive	Positive	Positive
Male, April	1.7×10^3	3 months	Positive	Positive	Negative
Male, February	1.2×10^3	11 years	Negative	Positive	Negative
Female, February	6.8×10^5	3 years	Positive	Positive	Negative

were seen for patients of both 11 years (1.2×10^3 copies/ml) and 3 months (1.7×10^3 copies/ml) of age (Table 1).

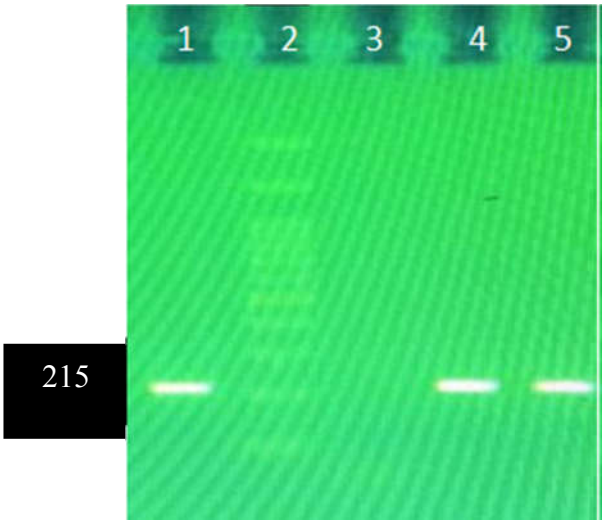

Figure 2. Gel electrophoresis demonstration, Line 1 is related to positive control, Line 2 is DNA marker, line 3 shows negative control, and Line 4 and 5 are related to positive samples

Clinical Characteristics

Among pediatric patients admitted with acute gastroenteritis, the main symptoms for positive HCosV children were diarrhea and fever in percentages of 100% and 75%, respectively. Vomiting as another sign was also seen with less frequency (25%). On the basis of viral load, the maximum viral load (4.6×10^6 copies/ml) was related to a patient who was positive for all three symptoms. The minimum viral loads were seen in two patients who had at least one negative sign when they were admitted.

Discussion

Human cosaviruses have been considered as a new member of the *Picornaviridae* family, which can possibly be related to non-polio acute flaccid paralysis (AFP) and acute gastroenteritis (1, 7). In general, many studies around the world have documented the prevalence of human cosavirus in a variety of age groups, especially among pediatric and immunocompromised patients, as well as in environmental polluted samples such as untreated wastewater (5, 7, 15-17). However, human cosavirus has also been detected in stool samples from healthy children (13). Here, 160 stool samples obtained from pediatric patients with acute gastroenteritis who were under 12 years of age and presented to a hospital in Karaj, Iran were tested. The specimens were collected from January 2018 to December 2019. A set of primers targeting 5'UTR region of human cosaviruses was used, which was previously used to identify these viruses by RT-qPCR assay (14). According to previous studies, the prevalence of human cosaviruses in different samples was determined to range from 0% to 42.8% in stool samples of different cases (14, 15). In the current study, the occurrence rate of human cosavirus was 2.5% in pediatric patients with acute gastroenteritis, which was slightly higher than previous studies in Japan, the UK, Thailand, Tunisia, and Italy with rates of HCoSV detection ranging from 0.1% to 1% (4, 5, 7, 18). On the other hand, the positive rate of human cosavirus was lower in the current study than in studies conducted in China (3.2%) and Brazil (3.6%) (13, 19). Even though the minimum viral load (1.2×10^3 copies/ml) in this research was comparable with previous studies performed in China and Brazil for children infected with human cosavirus, the maximum viral load (4.6×10^6 copies/g) was considerably higher in this study than in those reports (9, 13). In contrast, research suggesting that a low viral load could not be related to human cosavirus in affected patients and detection of a viral load around 100-fold higher may be evidence on the importance of symptomatic human cosavirus infection in pediatric patients with acute gastroenteritis (3). This study reports seasonality for the detection of human cosavirus, as high detection rates were seen in the cold season. Considering the fecal-oral route for infection with human cosavirus, it may be suggested that the presence of a high viral concentration in polluted water sources during the cold season can likely promote the risk of virus transmission to humans, such as occurs in other members of the *Picornaviridae* family.

After the recent detection of Aichi virus, salivirus, and saffold virus as new members of the *Picornaviridae* family (20-22), the first occurrence of human cosavirus in fecal samples from pediatric patients with acute gastroenteritis in Iran is illustrated in the current study. The use of Syber green method instead of the Taqman procedure can confer the advantage of low cost for the detection of viral agents in gastroenteritis cases.

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Conflict of interests

The authors declare that they have no conflict of interest.

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