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MOLECULAR DETECTION OF HUMAN SAPOVIRUS FROM HEALTHY AND HOSPITALIZED CHILDREN WITH ACUTE GASTROENTERITIS IN BASRAH

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ABSTRACT

Gastroenteritis is a major cause of childhood morbidity and mortality worldwide. Sapovirus is one of the leading cause for hospitalization due gastroenteritis. The objective of this study is to determine the frequency and importance of sapovirus in stool specimens of children aged less than 5 years presented with acute gastroenteritis (symptomatic infections) and healthy children without gastroenteritis (asymptomatic). A total of 400 stool samples (200 from diarrheal cases and 200 from normal children) were collected from infants and young children less than 5 years of age during the period from March 2011 to February 2012, and all relevant information were obtained on special questionnaire form. Viral nucleic acid was extracted from stool specimens using a spin column technique according to the instruction given by QIAamp-MinElute virus spin kit for purification of virus RNA(Qiagen, Germany). Sapovirus was detected by Reverse-Transcriptase-Polymerase Reaction (RT-PCR) using the specific primers: SR80-F and JV33-R of human sapovirus. Sapovirus was detected in 21.5% of diarrheal cases and among 3% of asymptomatic control group. The highest incidence of sapovirus was found in 12-17 and 18-23 months age groups and frequently observed during autumn and winter seasons. In addition to diarrhea; vomiting and dehydration were associated with sapovirus infections among hospital admitted children. The study highlights the significant burden of sapovirus gastroenteritis among children in Basrah and identifies a pathogen target for further prevention efforts in this population.

INTRODUCTION

Sapoviruses are associated with gastroenteritis [1]. Although with low incidence and prevalence, human sapoviruses have been associated with gastroenteritis outbreaks (0.01-9%) and sporadic cases (0.4- 6.6%) [2,3]. Outbreaks have occurred in day-care centers, healthcare facilities and schools, but given the low prevalence and incidence, no information is available about the impact on each setting [4,5]. Sapovirus infections are less common and known to cause disease primarily in young children, usually less than 5 years of age [6,7]. Although sapovirus associated diarrhea is generally mild, severe cases can occur[8]. Recently sapoviruses

have been reported as an occasional cause of outbreaks in hospitals and other health care facilities [9,10].

Sero-epidemiologic studies in adult people showed the worldwide spread of members of genus Sapovirus [11]. The acquisition of antibodies to Sapovirusbegins early in life and antibody prevalence rates for adults in Japan, Canada, United States, China, Singapore, Indonesia, and New Guinea ranged from 70% to 100%..[12]. The antibody prevalence studies showed that virtually all children are infected with Sapoviruses by the time they are 5 years of age, indicating that Sapovirus infection is widespread, although the illness most likely is sporadic with a high rate of asymptomatic infections.

Sapoviruses can be detected by ELISA, EM and/or RTPCR in feces of infected humans [13,14]. Usually when the techniques are compared, RTPCR has the highest sensitivity followed by IEM and ELISA. Despite these differences, all three methods will be useful for epidemiological investigations in gastroenteritis outbreaks, but for individual cases, at least two of these methods should be combined [15].

Because the ELISA technique allows testing of numerous samples in a short time, without any specialized equipment, ELISAs have been developed and used for large-scale epidemiologic studies of human sapoviruses[14]. Moreover, a major weakness of ELISAs is the high specificity that each antiserum has shown for the homologous strain used to produce it, but not for heterologous strains [16]. This is a disadvantage for screening samples in epidemiologic studies because the circulating strains are often highly diverse.

The purposes of this study were: a) to investigate the incidence of sapovirus associated gastroenteritis in infants and young children in Basrah during 2011-2012, b) to detect the season and age related distribution of sapovirus infections as well as the clinical symptoms associated with this virus infection.

MATERIALS AND METHODS

Study population

A total of 400 fecal specimens were collected from infants and children under five years of age in the city of Basrah, south of Iraq during one year (March 2011- February 2012): 200 stool specimens were collected from hospitalized children (including 105 male and 95 female) suffering from acute gastroenteritis admitted to Basrah Hospital for Obstetric and Child, this hospital is a major referral hospital in Basrah city. The remaining 200 stool specimens were collected from non diarrhea healthy children (including 94 male and 106

female) at Al Furdos kindergarten and nursery, Al Zohor kindergarten and nursery, and Baraem Al Amal kindergarten and nursery were studied as an asymptomatic group.

Acute gastroenteritis was defined as the occurrence of diarrhea (at least three soft or liquid stools in 24 h) with or without vomitinglasted no longer than 14 days [17,18]. Excluded children were those with chronic digestive disease including celiac disease based on their medical history. The asymptomatic population was healthy children with no history of diarrhea on the day of stool collection and in the 2 weeks preceding sample collection

Informed verbal consent was obtained from parents/ caregiver of participating children before collecting the samples. A standard structured questionnaire was used to obtain the information regarding age, sex, season, clinical manifestations (diarrhea, fever, abdominal pain and dehydration) and type of feeding (breast, bottle or mixed feeding) for each case.

Spring season was from March to May, summer was from June to August, autumn from September to November, and winter from December to February. According to WHO's recommendation, all children were classified in groups (0–2, 3–5, 6–8, 9–11, 12–17, 18–23, 24–35, and 36–48 months) [19].

Stool samples collection

After vigorously mixing, 20% (w/v, for the solid sample and v/v, for the liquid sample) stool suspension of each specimen was prepared in NaCl 10%, and then stool suspension was clarified by centrifugation at 8000 rpm for 20 min at 4 C. The resulting supernatants were collected and stored at -20 °C until use for nucleic acid extraction. One stool specimen was collected from each child.

Viral Nucleic acid (the genomes of both RNA and DNA viruses) was extracted from 200 µl of supernatant of the stool sample using a spin-column technique, according to the instructions given in the insert of the QIAampMinElute virus spin kit .Viral nucleic acid was then recovered in 100 µl of elution buffer. All the extracted viral nucleic acid examined by Nano drop instrument in order to determine the concentration of viral RNA. This is important step to determine the amount of extracted viral genome will be used in the PCR or RT-PCR and to neglect the negative sample.

Oligonucleotides primers for PCR amplification of sapovirus

Virus	Primer a	Sequence b	Nucleotide position	Amplicon size (Reference)
Sapovirus	SR80-F	5-TGGGATTCTACACAAAACCC-3	4366–4385	320 (Vinje et al. 2000)
	JV33-R	5-GTGTANATGCARTCATCACC-3	4685–4666	

Reverse transcription polymerase chain reaction (RT-PCR)

We used published methods [20] for the detection of sapovirus RNA by one step RT-PCR using Accu Power Rocket Script RT-PCR PreMix kit (Bioneer, South Korea). This kit consist of 96 PCR tubes for 96 tests each tube contains reverse transcriptase, DNA Polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, dried into a pellet. These components for reaction size were20µl.Horizontal agarose gels were used for analysis of DNA after RT-PCR, or PCR amplification. The concentration of agarose used was 2%; gels are prepared as percentage weight/volume solutions. That is, the weight of agarose in grams per 100 ml running buffer. Thus, a 2% gel is 2 g agarose in 100 ml buffer. 8µl of the final PCR product were loaded on to 2% agarose gel containing ethidium bromide (0.2µg/ml). DNA molecular weights were determined by comparison with a 100 bp DNA ladder. Samples showing a specific amplicon were considered as positive. Statistical Package for Social Science (SPSS) version 15 software was used to analyze the data. Chi- square (X2) test was used to assess the significance of differences between groups and variables. P value less than 0.05 was considered to be statistically significant.

RESULTS

The prevalence of sapovirus among children hospitalized due to acute gastroenteritis was 21.5% (43/200). However, sapovirus was detected in 3% (6/200) of specimens collected from asymptomatic children(Table-1).

Table-1 : prevalence of sapovirus among diarrheal cases and healthy children

Specimens from	No.positive/ total	(%)
Cases	43/200	21.5
Healthy control	6/200	3

Age and sex distribution of sapovirus infection

The highest incidence of Sapovirus was in the 12–17 and 18-23 months age groups which were 37.9% and 36.7% respectively (Table -2). Sapovirus infection was not detected in the infants aged less than 6 months. Sapovirus infections were identified in 19% of male and 24.2% of female gastroenteritis patients examined, this difference was not significant

(P>0.05).In asymptomatic children Sapovirus detected in 6 (3%) children their age range from 12-23 months with no significant differences between age groups (P>0.05).

Table -2: Age and sex distribution of Sapovirus-positive in diarrheal and control group of children under 5 years old

Variable	Diarrheal group		Control group	
Age (month)	**No. of +ve cases / No. of tested cases**	**% of +ve cases**	**No. of +ve cases / No. of tested cases**	**% of +ve cases**
0-2	0/21	0%	0/16	0%
3-5	0/22	0%	0/12	0%
6-8	5/26	19.2%	0/11	0%
9-11	8/28	28.6%	0/20	0%
12-17	11/29	37.9%	3/15	12.5%
18-23	11/30	36.7%	3/16	11.1%
24-35	5/23	21.7%	0/15	0%
36-48	3/21	14.3%	0/17	0%
Sex				
Male	20/105	19%	2/104	2.1%
Female	23/95	24.2%	4/96	3.8%
Total	43/192	21.5%	6/200	3.0%

Diarrheal group: age groups $X^2 = 18.8$ df= 2 P>0.05 sex $X^2 = 0.62$ df= 1 P>0.05
Asymptomatic group: sex $X^2 = 0.48$ df= 1 P>0.05

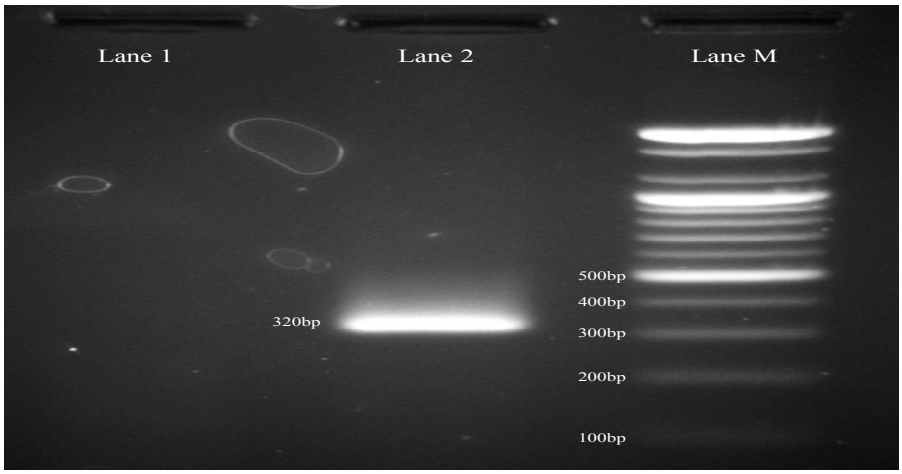

Figure -1: Agarose gel electrophoresis demonstrating Sapovirus. Lane 1, a representative of tested sample that negative for Sapovirus. Lane 2, a representative of tested sample that positive for Sapovirus. Lane M, 100 bp DNA Ladder marker. (2% agarose, 100v for 30min)

Seasonal distribution of sapovirus infection

The incidence in the autumn and winter was higher than that in the summer and spring (p=0.05). The rates of Sapovirus infection were 36% in autumn, 30% in winter, 14% in summer, and 6% in spring. Sapovirus were detected throughout the year except in the months of April and May. November was the month with the highest incidence of Sapovirus infection. In asymptomatic group 6 Sapovirus-positive cases were equally divided into autumn and winter (Table -3).

Table -3: Distribution of Sapovirus in diarrheal and control group of children under 5 years old in different season

Season	Diarrheal group		Asymptomatic group	
	No. of +ve cases / No. of tested cases	% of +ve cases	No. of +ve cases / No. of tested cases	% of +ve cases
Spring	3/50	6%	0/50	0%
Summer	7/50	14%	0/50	0%
Autumn	18/50	36%	3/50	6%
Winter	15/50	30%	3/50	6%
Total	43/200	21.5%	6/200	3%

Diarrheal group: $X^2 = 20.93$ df= 3 P=0.05

Diarrhea was observed in all children and analysis of enteric virus positivity in relation to other gastroenteritis symptoms such as vomiting, fever, dehydration and abdominal pain in association with the diarrhea.Table-4 shows that Children infected with Sapovirus were more likely to have vomiting (79.1%) followed by fever (58.1%), dehydration (58.1%) and abdominal pain (23.3%)

Table-4: Distribution of clinical symptoms associated with sapovirus Infected children below 5 years of age

Clinical symptoms	No. positive	(%)
Vomiting	34	(79.1)
Fever	25	(58.1)
Dehydration	25	(58.1)
Abdominal pain	10	(23.3)
Total	43	(21.5)

The frequency of sapovirus associated with gastroenteritis among infants less than 24 months of age who were breast fed, bottle fed and mixed fed at the time of presentation of infection was found to be the highest with statistical significance in patients who were bottle fed then breast fed and mixed fed (Table-5).

Table-5 : The effect of feeding type on the frequency of sapovirus infections

Type of feeding	No. positive	(%)
Breast feeding	13	(37)
Bottle feeding	16	(46)
Mixed feeding	6	(17)
Total	35/128	

Discussion

Gastroenteritis is one the commonest disease in children especially in developing countries [22]. More than 20 types of viruses cause this illness [23].

The previous studies on viral etiology of AGE in Iraq, reported rotavirus and enteric adenovirus were most frequently associated with AGE in Iraqi children[24-27], these studies used conventional detection methods (eg, latex agglutination and ELISA) had low sensitivity and specificity. Epidemiological studies of sapovirus infection have been published worldwide; there is no data of the illness caused by this virus in Iraq. This is the first study from Basrah (south of Iraq) that used molecular detection methods to investigate the role of sapovirus in acute pediatric gastroenteritis.

Our study indicated high prevalence for sapovirus (22%) (Table -1) when compared with previously published papers on sapovirus epidemiology worldwide, in which sapovirus prevalence was shown to range from 0.3 to 9.3%[28,29] .In this study, we used RT-PCR for the detection of sapovirus. It is very sensitive and effective method for molecular epidemiological research. Some researchers used ELISA for detecting sapovirus. However, ELISA has lower sensitivity as compared to PCR [3].

The prevalence of sapoviral gastroenteritis in different countries has been determined, the RT-PCR method also reported sapovirus percentage higher than the worldwide range and may reached to our result: 11.9% in Iran (Romani et al. 2012), 15% in Thailand [30], 19% in Denmark[31] and 19.2 % in Japan [32].Our findings also confirmed Sapovirus as one of the enter opathogens responsible for viral gastroenteritis among infants and children in Iraq. We advise awareness and improved personal hygiene for the elimination of infections. In order to investigate asymptomatic infection with diarrheal virus, fecal specimens were also collected from children without signs of acute gastroenteritis. Viral infection was identified among asymptomatic children. We used the more sensitive RT-PCR assay to identify a large number of persons with asymptomatic sapovirus infection, who tend to have lower viral loads [33].

In our study, sapoviruswas detected in 5% of control specimens.. Asymptomatic infection of sapovirus also detected by other studies; Two studies were conducted in China [34]

and Japan[3]where sapovirus was detected in 3% and 2.6% of fecal specimens collected from asymptomatic children respectively.

Our findings are the first to clearly indicate that diarrheal viral pathogens cause not only clinical manifestations of acute gastroenteritis but also asymptomatic infection in children in Basrah. A significant finding of this study was the incidence of sapovirus in asymptomatic children, with 5% of surveillance samples in the community positive for sapovirus, which has not been reported previously from Iraq. Detection of sapovirus in asymptomatic children signifies that these viruses can circulate in the general population in the absence of disease in young children. However, asymptomatic infections constitute a significant reservoir for infection in the community, and may act as a source of both endemic and epidemic disease.

Previous studies have demonstrated that smaller quantities of rotavirus are shed during asymptomatic rotavirus infections than during symptomatic rotavirus infections[33]; similarly sapovirus among asymptomatically infected persons may be less infectious, but further work is needed to determine their role in the transmission and persistence of these viruses in human populations.

Age-related prevalence of antibody against sapovirus has demonstrated that infections commonly occur in children less than 5 years old [17]. In our study, the prevalence of Sapovirus infection in all age groups except from children less than 6 months of age there were no positive samples (Table -2) while reports from other countries showed that sapoviral gastroenteritis is very common in children below the age of 1 year[35], especially 0–6-month old . However, another study showed that sapoviral gastroenteritis is very common in children below the age of 3 year [36].We assume that the children in our population had maternal immunity to Sapovirus may persist for first 6 months of life, that is mean the mothers have immunity from asymptomatic infection which is confirmed on 5% (6/122) of tested samples from asymptomatic were positive. Statistical differences were not observed in genders as it's similar to other studies[36]due to this virus.

In this study, sapovirus was identified throughout the year except in April and May and was highest in November as autumn. According to other report, sapovirus has been found mainly in the cold season [28], whereas several other studies did not find a clear seasonal pattern for sapovirus infections[1]

Seasonal variation is likely to be based on biological, environmental and behavioral factors that regulate transmission, virulence and persistence of the virions in host populations[37].

Diarrhea, fever, vomiting, and abdominal pain were associated with sapovirus infected children, these data agrees with those from other studies [38,39].

Evaluation of the feeding status of infants aged <24 months of age with acute diarrhea revealed that enteric viruses-positive cases were significantly higher among those being bottle fed at the time of acute gastroenteritis . This result suggested that breastfeeding may be a protective factor against sapovirus infection by conferring passive immunity from the mothers to her infant. It has been reported that human breast milk contains several factors that may play a role in preventing infection and of these is a glycoprotein, lactoferrin, which is able to bind to and prevent virus replication, beside the natural immune defense mechanisms [40].

On the other hand, the lower frequency of enteric virus infection among those of both breastfed and bottle-fed may be explained with the synergy of these two types of nutrition in reducing the amount of enteric virus disease. The infection with diarrhea increased in infants who used bottle feeding may be because of the contamination of the bottle nipples. This result was similar to those previously described in other studies [41,42]. However, the protective effect of breastfeeding on gastroenteritis caused by sapovirus has been variable.

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