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Detection of rotavirus and norovirus in the elderly population of Caxias do Sul, Rio Grande do Sul, Brazil, from 2010 to 2012

Detecção de rotavírus e norovírus na população idosa na cidade de Caxias do Sul, Rio Grande do Sul, Brasil, de 2010 a 2012

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ABSTRACT

Aims: Rotavirus (RV) and norovirus (NoV) are the most common causes of diarrhea in children aged less than five years throughout the world. However, RV and NoV have frequently been overlooked as pathogens in elderly individuals. This study examined the frequency of RV and NoV among elderly patients with complaints of diarrhea in the city of Caxias do Sul, southern Brazil.

Methods: Between 2010 and 2012, stool samples from patients aged 60 years or older with acute gastroenteritis treated at a private healthcare center were analyzed, seeking to identify RV and NoV. RV detection was performed by latex agglutination (LA) methods and polyacrylamide gel electrophoresis (PAGE). One third of the samples were further tested for NoV by reverse transcription polymerase chain reaction (RT-PCR).

Results: A total of 145 stool samples from patients aged 60 to 105 years were analyzed. RV was detected in 6/145 (4.14%) and 5/125 (4.00%) of the samples by LA and PAGE, respectively. NoV was detected in 6/51 (11.76%). Mixed infection (RV and NoV) was detected in a single sample: 1/51 (1.96%).

Conclusions: This study adds further evidence that viral agents are involved in the etiology of gastroenteritis in the elderly, contributing significantly to the understanding of RV and NoV infections in the mature population.

KEY WORDS: rotavirus; norovirus; aged; gastroenteritis; diarrhea.

RESUMO

Objetivos: Rotavírus (RV) e norovírus (NoV) são as causas mais comuns de diarreia em crianças menores de cinco anos em todo o mundo; entretanto, como patógenos em idosos, esses vírus têm sido pouco investigados. Este estudo examinou a frequência de RV e NoV entre idosos com queixas de diarreia na cidade de Caxias do Sul, no sul do Brasil.

Métodos: Entre 2010 e 2012 foram analisadas amostras de fezes de pacientes de um Centro de Atenção à Saúde privado, com idade de 60 anos ou mais, com gastroenterite aguda, buscando-se identificar RV e NoV. A detecção de RV foi realizada pelos métodos Aglutinação em Látex (LA) e Eletroforese em Gel de Poliacrilamida (PAGE). Uma terceira parte das amostras foi ainda testada quanto a NoV por Transcrição Reversa e Reação em Cadeia da Polimerase (RT-PCR).

Resultados: Ao todo foram analisadas 145 amostras de fezes de pacientes com idade entre 60 e 105 anos. RV foi detectado em 6/145 (4,14%) e 5/125 (4,00%) das amostras por LA e PAGE, respectivamente. NoV foi detectado em 6/51 (11,76%). Infecção mista (RV e NoV) foi detectada em uma única amostra: 1/51 (1,96%).

Conclusões: Este estudo acrescenta mais evidências de que agentes virais estão envolvidos na etiologia da gastroenterite em idosos, e contribui para a compreensão das infecções por RV e NoV na população idosa.

DESCRITORES: rotavírus; norovírus; idoso; gastroenterite; diarreia.

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INTRODUCTION

Elderly people present diverse clinical manifestations that are characteristic of their age group. Diarrhea, which can be caused by multiple factors, including infectious agents, is one of the most common symptoms in the elderly [1]. Viruses are the most frequent cause of acute diarrhea at all ages [2,3]. Dehydration secondary to diarrhea can trigger degenerative diseases, such as myocardial ischemia, and renal, cerebrovascular and pancreatic failure [4]. Moreover, immunosenescence further increases the mortality rates associated with infectious diseases during old age [5].

The frequencies of viruses implicated in gastroenteritis in the elderly are not well known in Brazil and few research groups have investigated such condition. Most studies are limited to events in childhood or outbreaks in nursing homes [6-8]. In the world population, viral diarrhea accounts for over half of the outbreaks recorded at healthcare centers and involves mainly two viruses: rotavirus (RV) and norovirus (NoV) [9]. RV is the most important etiologic agent of diarrhea, especially in children under five years of age. The disease has been attributed to two million hospitalizations, 11 million episodes of diarrhea and 600,000 deaths in children and young people worldwide [10].

RV belongs to the family *Reoviridae*, is a non-enveloped virus with 11 segments of double-stranded RNA (dsRNA) and is classified into eight groups (A-H) [11,12]. Group A is recognized as the single most important cause of severe acute enteritis in infants in developed and developing countries. Groups B and C, which are antigenically and genetically distinct, also infect humans; however, they are not epidemiologically important [13,14].

NoV is a small non-enveloped single-stranded RNA virus of the family *Caliciviridae*, responsible for many outbreaks at all age groups. Transmission of NoV and RV occurs through the fecal-oral route or by contamination of water and food. The genus NoV is divided into five genogroups (GI to GV), so far only GI, GII and GIV have been detected in humans [3].

Viral diarrhea caused by RV and NoV does not generate specific symptoms that could be diagnosed based on patient's report, since other viral infections may also produce similar clinical outcomes [15]. Laboratory diagnosis is based primarily on the presence of the viruses in the stools of infected patients. The immunological technique is used routinely for agglutination of latex particles, but it is only effective in detecting group A RV, which is the most prevalent in humans [16]. Enzyme-linked immunosorbent assay (ELISA) kits are rapid and efficient for detection; however, these kits are suitable for assessing only the group A RV by latex agglutination (LA) and incur high costs associated with equipment and with personnel training [17,18]. The polyacrylamide gel electrophoresis (PAGE) technique is applied to detect non-group A RV and to differentiate between long and short group A RV electropherotype profiles, also allowing for the detection of mixed infections [19]. This method allows evaluating the diversity of surrounding electropherotypes in a particular region [20]. The diagnosis of NoV is not usually offered in health services given its cost-benefit ratio; it involves robust molecular analyses such as conventional reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR, which are also time-consuming. Both detection techniques are highly sensitive, enabling the direct study of different types of the same virus, most of the times not identified by conventional methods [19,20]. More recently, a third-generation ELISA kit has also been used successfully in the diagnosis of NoV. This system offers a quick test to improve the surveillance of gastroenteritis outbreaks, but it has limited use in clinical laboratories because of its costs [3,21].

The aim of this study was to monitor RV and NoV infections among elderly patients with acute diarrhea in the city of Caxias do Sul, Rio Grande do Sul, Brazil, in order to help guide public health actions that may improve the quality of life of this particular age group.

METHODS

Study design and population

A prospective study of fecal samples collected between 2010 and 2012 from patients with diarrhea aged 60 years or older was performed. The patients were not hospitalized and were attended to at a private healthcare center in the city of Caxias do Sul, in southern Brazil. Samples of non-diarrheal stools from patients who sought the laboratory for routine examination were used as control. Control samples were taken from the same age group, at Alfa, a privately-owned laboratory located in Caxias do Sul. Alfa is a reference laboratory for clinical analysis and complementary services in healthcare in southern Brazil and treats patients from all socioeconomic levels.

The detection methods used in the present study were selected based on the infrastructure and economic resources available at the research laboratory of the Universidade de Caxias do Sul. The study was approved

by the Ethics Committee of the Universidade de Caxias do Sul under protocol no. 0075.0.397.000-10.

Rotavirus detection

The samples were tested using a commercial LA kit (Richmund Immuno systems Diagnostics GmbH, São Paulo, Brazil) following the manufacturer's instructions. Briefly, 20% of the fecal suspensions were centrifuged (6000 rpm) for about 10 minutes. An aliquot of 25µL was transferred from the supernatant to two wells on the test slide, 20µL of the test reagent was added in the first circle (test circle), and 20µL of the control reagent suspension was added in the second circle (control circle). Then, LA was further monitored.

The samples were also tested by polyacrylamide gel electrophoresis (PAGE) according to standard procedures [19]. The RNA was extracted by suspending the samples in 40µl of 10% sodium dodecyl sulfate (SDS), 40µl of 1M sodium acetate and 400µl of phenol-chloroform, followed by centrifugation (8000 rpm) for 15 minutes [19]. After centrifugation, the supernatant was removed and 1ml of ethanol and 20µl NaCl were added. Samples were frozen for 17 hours. Once thawed and centrifuged, 20µl of the dissociation solution was added, followed by a water bath at 56°C for 15 minutes. The gel was then prepared with 3.5% acrylamide/0.2% Bis-acrylamide, 6 mM Tris/HCl pH 6.8, 0.015 mM ammonium persulfate and 0.2% tetramethylethylenediamine (TEMED). After complete polymerization of the gel, the plates were attached to the electrophoresis tank with running buffer (0.01 M Tris, 0.083 M glycine, pH 8.3). Electrophoresis was run at 20 mA for approximately 8 hours. The gel was fixed with a solution of 10% ethanol and 0.5% acetic acid for 30 minutes, and silver nitrate solution 0.01 M. Positive samples presented visible bands. The SA11 simian RV RNA was used as a pattern for the migration profile analysis [19].

Norovirus Detection

RNA extraction was obtained from fecal suspensions stored in 20% phosphate buffer with 1ml of TRIzol® Reagent and 200 ml of chloroform. Samples were shaken, incubated at 4°C and centrifuged (9500 rpm) for 10 minutes. The aqueous upper phase was mixed with the same volume of isopropyl alcohol, subsequently incubated at 4°C and centrifuged (10,000 rpm). Finally, the precipitate was washed with 75% cold ethanol and allowed to dry on filter paper. The RNA was resuspended in 20µl of Milli-Q water with diethylpyrocarbonate (DEPC).

The extracted nucleic acids were subjected to reverse transcription and subsequently amplified with primers MON 431, MON 432, MON 433 and MON 434 [21]. These primers amplify the 213-bp fragment of the gene that encodes the enzyme viral RNA polymerase, from the B region of ORF1. The *SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen®)* commercial kit was used. The following reagents were added to 20µl of the reaction mix: 6.96µl of Milli-Q water, 9.72µl of 2x Reaction Mix, 0.11 µl of each primer, 0.3µl of *SuperScript II RT/Platinum Taq Mix* and 2.5µl of RNA extracted from the sample. The negative control consisted of the reaction of the same reagents without the RNA sample. The positive control was a nucleic acid extract of NoV. Amplification was performed in a Tonederm® thermocycler using the following protocol: 15 minutes at 42°C for reverse transcriptase action, 3 min at 94°C for enzyme inhibition, followed by a series of 40 cycles for denaturation, annealing, and extension of the target segment, respectively, with 30 seconds at 94°C, 90 seconds at 50°C and 30 seconds at 60°C, after 7 minutes at 72°C for the final extension and storage at 4°C.

The RT-PCR products were analyzed on a 2% agarose gel with 0.35% ethidium bromide solution (0.1 mg/ml) in 1X TBE buffer (Trisma, boric acid and EDTA) at a constant voltage of 80 volts. A molecular weight marker of 100-bp DNA ladder was used. The bands were visualized under an ultraviolet transilluminator.

RESULTS

A total of 145 fecal samples from patients aged 60 to 105 years were analyzed. Ten samples of non-diarrheal stools were used as control.

Rotavirus Detection

LA detected RV in 6 (4.14%) of 145 specimens collected from elderly patients. RV infection was found predominantly in the 60-70 year-old group (7.14%; 5/70) (**Table 1**). The 10 samples used as control tested negative for RV infection in the LA assay.

A total of 125 fecal specimens were evaluated by PAGE, and 4.00% (5/125) were positive for RV infection. Twenty specimens yielded indeterminate results in the PAGE assay, and were considered to be negative. All five samples detected showed the same long group A RV electrophoretic pattern. RV infection was also predominant in the 60-70 year-old group (60.00%; 3/5). Three samples (2.4%; 3/125) were positive in the LA and PAGE assays (**Table 1**).

Table 1. Determination of rotavirus by Latex Agglutination and Polyacrylamide Gel Electrophoresis and of norovirus by Reverse Transcription Polymerase Chain Reaction among elderly patients with complaints of diarrhea in the city of Caxias do Sul, southern Brazil, 2010-2012.

Age (years)	Determination of Rotavirus by LA				Determination of Rotavirus by PAGE				Determination of Norovirus by RT-PCR			
	Negative	Positive	Total	(%)	Negative	Positive	Total	(%)	Negative	Positive	Total	(%)
60 to 70	65	5	70	(48.2)	60	3	63	(50.4)	25	1	26	(51.0)
71 to 80	40	0	40	(27.6)	33	1	34	(27.2)	12	2	14	(27.4)
Over 80	34	1	35	(24.1)	27	1	28	(22.4)	8	3	11	(21.6)
Total	139	6	145	(100)	120	5	125	(100)	45	6	51	(100)

LA: Latex agglutination; PAGE: Polyacrylamide gel electrophoresis; RT-PCR: Reverse transcription polymerase chain reaction.

Norovirus Detection

Due to scarce resources, a limited number of samples (~1/3 of the specimens) were selected for NoV screening in the present study. A total of 51 fecal specimens were analyzed by RT-PCR and NoV infection was detected in 11.80% (6/51). Mixed infection (NoV-RV) was detected in 1.96% (1/51) (**Table 1**).

DISCUSSION

This study was designed in order to investigate the frequency of RV and NoV infections in elderly patients with acute gastroenteritis in Caxias do Sul, Brazil. Outbreaks of viral gastroenteritis have a high incidence worldwide, leading to hospitalization and mortality of elderly people in nursing homes. Literature reviews show 69 outbreaks in the United States, Canada, Australia, Europe, and Asia recorded in the past 10 years, infecting 2,423 people and resulting in 89 hospitalizations and 17 deaths from infection [2].

RV spreads mainly through the fecal-oral route, with transmission to adults and elderly probably because of their being cared for by sick children or because of the ingestion of contaminated food or water [22]. Cultural aspects contribute to this dissemination, since a fraction of grandchildren look after their grandparents, especially in cases of illness. The proximity of children to the elderly may facilitate the transmission of the disease. In addition, the population over 60 years of age can be considered to be immunocompromised, depending on factors such as quality of life and genetic predisposition, thus facilitating the transmission [5].

The frequency of RV infection in the elderly detected in this work (4.00-4.14%) was higher than that observed in a study carried out in England (2.5%) [23]. Lewis et al. [7] described an outbreak of viral gastroenteritis, which occurred in seven psychogeriatric wards of a psychiatric hospital in England. This RV involved seniors whose age averaged 78.7 years [7], comparable to the data observed in the present study (7.14% in the 60-70-year-old group). In present study, three samples (2.4%) were positive for RV in the LA and PAGE assays. The combination of methods to confirm RV is essential; it seems that none of those methods is reliable when applied separately.

RV infection occurs mainly in children aged 0-5 years [9]. The overall percentage of RV detected among children in Brazil has been much higher (~30%) than that found in adults and elderly [9]. Two new RV vaccines have shown efficacy against severe disease. Between 2006 and 2010, a total of 27 countries introduced vaccination in children, including Brazil, and later, rates of severe RV-related diseases decreased in vaccinated and unvaccinated patients [24]. In children from Caxias do Sul, the RV vaccine coverage reached 90.3% and increased further until 2013. The research conducted by Anderson et al. [25] showed that the vaccination of children against RV is correlated with an almost 50% reduction in RV among adults [25]. Other reports reviewed the number of registered cases of nonspecific gastroenteritis caused by RV [26]. In this context, the RV vaccine was able to reduce cases of this disease in the United States and indirectly protected the population aged 5 to 44 years [1]. Curiously enough, people under 5 and over 44 years of age were not protected by the vaccine [1]. In Brazil, it was shown that the immunization of children did not reduce diarrhea cases in individuals older than 18 years [26]. The adult population is susceptible to the same RV genotypes as children, and infections tend to appear at the same time of the year. The authors suggest that adults act as reservoirs for viruses and are in part responsible for outbreaks among infants [26].

The frequency of NoV infection in the elderly in this work (11.8%) was similar to that found in Spain (11.1%) in 2011 [27]. In England, distinct prevalence rates have been reported for NoV infection among elderly inpatients, ranging from 0.3% [28]to 14.9% [23]. Previous studies conducted in different Brazilian states showed that the prevalence of NoV among hospitalized children ranges from 9% to 36% [29,30].

In 2002, a severe NoV gastroenteritis outbreak was recorded in a geriatric hospital in France, where over 38% of elderly patients and 26% of nurses were infected. Healthcare workers are commonly associated with the spread of NoV infection in hospitals and in long-term care facilities [3,8]. In Brazil, there was an outbreak of NoV infection in a long-term care facility in 2010, with an incidence of 41.3% and 16.25% among patients and employees, respectively [8]. Given its high frequency, gastroenteritis in the elderly can be viewed with contempt and can lead to more serious consequences, as reported in Gothenburg, Sweden, during 2008 and 2009, when death cases increased among NoV-infected elderly outpatients compared to inpatients [6].

In the present study, mixed infection (NoV-RV) was detected in 1.96%. Viral coinfection has also been reported in nursing homes, where 12% of RV-positive samples were also positive for adenovirus, astrovirus, and NoV [7]. Multiple cases of infection are also frequently associated with nosocomial infections [2]. Besides the diversity of viruses circulating in the community, higher levels of mixed infections should be expected; however, these levels seem to be lower in the Brazilian population [31].

The fact that the identification of gastroenteritis caused by RV and NoV relies on distinct methodologies (i.e., detection of morphologically distinct viral particles by electron microscopy, of virus genome by RT-PCR or of viral antigens by ELISA) is noteworthy. The results obtained with these techniques are often poorly correlated due to distinct targets and inherent problems. The present study is no exception in this respect. The LA test is widely used in clinical laboratories; it is fast, inexpensive, and does not require high technical skills [19]. This method is very effective for detection of group A RV, the most prevalent in humans [32]. Nevertheless, the LA technique is unable to verify the presence of other RV groups or of possible mixed infections [33]. RV is attributed almost exclusively to childhood and there is a scarce literature available to date. On the other hand, PAGE could be used in epidemiologic studies for detecting different RV groups, allowing for the detection of mixed infections [34]. However, this method is dependent on viral load, and fecal samples with a small amount of RV particles may yield false negative results.

RT-PCR allows identification of the virus even in a sample with a small number of viral particles [35]. This method is also suitable for detecting the virus several days after the resolution of clinical symptoms [36]. This molecular approach is applied more often in epidemiologic studies and not usually accessible by clinical laboratories, given its high costs and the paucity of clinical investigative interest. Morillo et al. [21] showed that NoV detection could also be performed using a third-generation enzyme immunoassay (EIA) kit. However, due to its sensitivity, RT-PCR is still required as a routine NoV detection method in sporadic cases [21]. The results of the present study reinforce the need for implementation of different diagnostic tests for enteric viruses at hospital clinical laboratories [37].

In conclusion, this study adds further evidence that viral agents are involved in the etiology of gastroenteritis in elderly patients in Caxias do Sul, Brazil, and contributes significantly to the understanding of RV and NoV infections in the mature population. Prevention is still the best strategy to generate health benefits at all ages, especially for the elderly. A more appropriate treatment for these groups of patients should be provided, improving both the quality of life and life expectancy.

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