Incidence of Rotavirus and Adenovirus: detection by molecular and immunological methods in human faeces

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ABSTRACT

Rotaviruses and Adenoviruses are reported worldwide among the main agents of gastroenteritis and, consequently, the development and validation of sensitive and cost-effective methods of detection is necessary. In this study, two approaches for detection of Rotavirus A and Adenovirus in samples of human faeces were compared: the immunological kit VIKIA Rota-Adeno and the nested-polymerase chain reaction (nested-PCR for Adenovirus) and Reverse Transcriptase-PCR (RT-PCR for Rotavirus) molecular methods. From January 2006 to July 2009, 467 samples of faeces from individuals with gastroenteritis symptoms assisted at the Hospital Infant D. Pedro (Aveiro, Portugal) were analysed for Rotavirus and Adenovirus, using the VIKIA kit. From the 467 samples, 59 (12.6%) were positive for Rotavirus and 5 (1.1%) for Adenovirus. Between December 2008 to July 2009, 18 samples were analysed by both immunologic and molecular methods. From the 18 samples, 10 were positive for Rotavirus (55.5%) and 16 for Adenovirus (88.9%) when analysed by RT-PCR and nested-PCR, respectively With VIKIA kit, 11 samples were positive for Rotavirus (61.1%) and only one was positive for Adenovirus (5.5%). Sequencing of PCR products confirmed the presence of Rotavirus in 1 sample and Adenovirus in the 10 samples that were classified as negative with VIKIA Kit. The results of VIKIA kit suggest that from the both viruses studied Rotavirus are the most incident viruses in gastroenteritis, however, molecular analysis results suggest that Adenovirus could be the most responsible for the viral gastroenteritis studied.

Key words: Adenoviruses; Rotavirus A; PCR; VIKIA Rota-Adeno; gastroenteritis.

Introduction

Despite the improvement of sanitary conditions, diarrhea remains the leading cause of disease worldwide and viruses have been responsible for most outbreaks of gastroenteritis [1, 2]. Among enterovirus, Rotavirus A, Astrovirus, Adenovirus, Sapovirus and Norovirus are the main agents of gastroenteritis, especially in young children. Person-to-person contact, ingestion of contaminated water, consumption of contaminated food and exposure to polluted recreational waters are the main transmition routes [3-6].

The incidence of sporadic gastroenteritis and acute infectious diarrhea is variable among different countries. This can be, in part, due to the fact that the confirmation of these cases is rare because most patients do not request for medical assistance [7] and also because the resources to perform viral analysis are restricted to a small number of laboratories in urban areas [8]. Recent studies suggest, however, that in children, the number of deaths due to diarrhea has decreased in the past 20 years and the number of cases requiring hospitalization declined between 2000 and 2004, because of the improvement in sanitary conditions, hygiene habits and microbiological quality of water and food [9].

Rotaviruses A and Adenoviruses are among the most commonly recognized causes of epidemic viral acute gastroenteritis worldwide [10]. The Rotavirus A are considered the major etiological agent of acute diarrhea in infants and young children [11]. There is a tendency to seasonality in temperate areas for Rotavirus infection, with peaks occurring predominantly in winter, but outbreaks can occur throughout the year in tropical areas [12, 13]. Rotavirus A belong to family *Reoviridae* and are non-enveloped double-stranded RNA viruses [14]. They are distributed by seven groups (A through G) [15].

Adenovirus is considered a gastroenteritis emerging virus, responsible for a high number of outbreaks in nurseries, schools and hospitals [16-20]. Human Adenoviruses belong to the

family *Adenoviridae* and are non-enveloped double-stranded DNA viruses. They are classified into seven species A-G, and at least 51 different serotypes (and 5 proposed types, HAdV-52 to HAdV-56) have been described to date [21- 22]. Adenoviruses, namely the species F (serotypes 40 and 41) and species A (serotypes 12, 18, 31), are associated with gastroenteritis in children. Serotype 40 and 41 are the main agents of gastroenteritis and the most frequent serotypes detected in hospitalized children [23-25].

The methods most frequently used to detect Rotavirus and Adenovirus in routine clinical analysis are enzyme immunoassays (EIA) and latex agglutination (LA) tests since these immunological methods are easy to use, fast and highly specific, however, these tests present low sensitivity for many antigen detection [26-29]. More recently, techniques like PCR have been introduced as convenient and powerful methods to detect viruses. These techniques are highly sensitive and specific [26-29] and, additionally, genome amplification by PCR allows further characterization of the viruses by analyses of nucleic acid sequence [30, 31]. Logan et al. [26] showed that the efficiency of detection of Rotavirus A by PCR was 111 % superior to that obtained by LA test, and that the detection of Adenovirus by PCR was 175 % higher relatively to LA detection. It was also been reported that the detection rate of Rotavirus A by molecular methods is up to 48 % higher than that of EIA test [32, 33]. However, Fau et al [34] showed good sensitivity and specificity for the immunological method (using the kit VIKIA Rota-Adeno) when compared to PCR for Rotavirus A.

The main objective of this work was to compare the detection of Rotavirus and Adenovirus by immunological tests (kit VIKIA Rota-Adeno) and by molecular techniques (RT-PCR and nested PCR) in samples of human faeces of patients with gastroenteritis symptoms. Additionally, the seasonal variation and the distribution by age of the viral infection were also studied.

Material and methods

Samples

A total of 467 samples of human faeces were collected from babies, children and adults with gastroenteritis symptoms in Hospital Infante D. Pedro, Aveiro (Portugal) between January 2006 and July 2009. All samples were analysed by an immunochromatographic technique (VIKIA Rota-Adeno; BioMérieux) for double detection of Rotavirus A and Adenovirus. Between December 2008 to July 2009, a total of 18 samples positive for VIKIA Rota-Adeno were analysed in parallel for Rotavirus and Adenovirus by RT-PCR and nested PCR, respectively.

Nucleic acid extraction and purification

Only the samples that produced positive results for VIKIA Rota-Adeno were used to Rotavirus and Adenovirus detection by PCR analysis. A suspension of faecal material was prepared diluting 0.1 g of faeces in 0.9 mL of distilled water that was vortexed and centrifuged at 5000x g for 15 min. The viral nucleic acids were extracted from the supernatant using geneMAG-RNA/DNA kit (Chemicel, Germany). For DNA and RNA purification the GeneClean Kit (MP Biomedicals, LLC), was used according to the instructions of the manufacturer. After purification, the nucleic acids were stored at -80°C until analysis.

Detection of Rotavirus A by RT-PCR

RT-PCR was performed on RNA extracted using the OneStep RT-PCR kit (Qiagen Germany) according to the recommendations of manufacturer. Detection of Rotavirus A was performed using the primers described by Villena et al. [35] that correspond to an highly conserved region of group A Rotavirus: VP6-3 (5´ GCT TTA AAA CGA AGT CTT CAA 3´; positions 2 to 23 of human strain) and VP6-4 (5´ GGT AAA TTA CCA ATT CCT CCA G 3´; positions 187 to 166 of human strain). The reaction mixture consisted of 2 mM Taq Buffer, 3 mM $MgCl_2$, 0.08 mM of each dNTP, 0.1U/µl of Taq polymerase (Fermentas) and 0.48 µM of each primer. The RT-PCR was performed at 42°C for 30 min, 95°C for 15 minutes followed by 40 cycles at 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes [35].

PCR products were analyzed by electrophoresis in a 2 % agarose gel stained with ethidium bromide and visualized under UV light. The PCR was considered positive when specific band product with 186 bp was observed.

Detection of Adenovirus by PCR

A nested PCR approach using the primers described by Allard et al. [36] was followed for the detection of Adenovirus. The primers hex1deg (5´ GCC SCA RTG GKC WTA CAT GCA CAT C 3´; S=C+G; R=A+G; K=T+G; W=A+T) and hex2deg (5´ CAG CAC SCC ICG RAT GTC AAA 3´; I=deoxyinosine) created a 301 bp products. The nested primer pair, nehex3deg (5´ GCC CGY GCM ACI GAI ACS TAC TTC 3´; Y=C+T; M=A+C) and nehex4deg (5´ CCY ACR GCC AGI GTR WAI CGM RCY TTG TA 3´) produced a 171 bp products. The amplifications were carried out in 20 mL reaction mixtures containing 1x Taq Buffer with KCl (Fermentas), 1.5 mM MgCl2 (Fermentas), 0.28 mM dNTP (Fermentas), 0.4

µM of each primer and 1 U/µL of Taq DNA polymerase (Fermentas). For the first PCR, 5 µL of sample were added and for the second (nested) PCR, 2 µL samples were used. The first PCR included an initial denaturation at 95°C for 10 minutes, immediately followed by 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minutes and a final extension at 72°C for 5 minutes. The second amplification was initiated by denaturation at 94°C for 3 minutes, immediately followed by 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute and a final extension at 72°C for 5 minutes [36].

PCR products were analyzed by electrophoresis in a 2 % agarose gel stained with ethidium bromide and visualized under UV light. Positive PCR results were determined by visualization of a band product of 171 bp.

Positive and negative controls

Rotavirus A and Adenovirus 41 suspensions of unknown concentration were used as positive controls. Nucleic acids were extracted and stored at -80°C. Negative controls, in which RNase free water was used instead of sample, were also included

Nucleotide sequence analysis

To confirm PCR results, the PCR products of positive samples for Rotavirus A and Adenovirus were sequenced. PCR products were used as templates in the sequencing reactions which were carried out in an ABI PRISM –BigDye-Terminator v1.1 (Applied Biosystems), using forward primer VP6-3 for Rotavirus identification and the forward primer Nehex3deg for Adenovirus identification.

Results

Detection of Rotavirus A and Adenovirus by molecular and immunological methods

From the 18 samples analyzed for Rotavirus A, 11 (61 %) were positive when analysed by VIKIA Rota-Adeno and 10 (56 %) were positive when analysed by RT-PCR (Table 1). For Adenovirus, VIKIA Rota-Adeno kit produced 1 positive result (5.5 %) and nested PCR produced 16 (88.9 %) positive results (Table 1). In the analysis of Rotavirus, the molecular and immunological methods had an agreement of 83%, while for Adenovirus the agreement of the two methods was only 17 %.

Bands corresponding to fragments of 186 bp for Rotavirus and 171 bp for Adenovirus were observed in the positive controls. No bands were observed in the samples corresponding to the negative controls.

Amplified DNA fragments from samples in which PCR and immunochromatographic methods produced conflicting results were sequenced. Positive results from PCR analysis were due to the presence of the viruses and were true positives. The sequencing of sample 8, that was positive PCR and negative VIKIA Rota-Adeno kit, confirmed that the sample was positive for Rotavirus A. The sequence, presented 97% homology with human Rotavirus A. In the 15 samples of Adenovirus witch the PCR and VIKIA Rota-Adeno kit were not concordant showed the presence of human Adenovirus specie F serotype 41, being the lower percentage of homology 91 %.

In the 18 samples analysed by PCR, 9 (50 %) were simultaneously positive for Rotavirus A and Adenovirus.

Incidence of patients with gastroenteritis symptoms

The number of patients with gastroenteritis symptoms assisted in each year of the study period (2006-2009) increased (Figure 1). It was not observed a clear seasonal pattern of gastroenteritis during the different years of the study (Figure 1).

From the 467 patients with gastrointestinal symptoms, the larger number of cases corresponds to children younger than 12 months and 73 % were observed in the two first age groups (< 5 years) (Figure 2).

Incidence of Rotavirus and Adenovirus gastroenteritis

In the 467 cases of patients with gastroenteritis symptoms, Rotavirus was detected by the VIKIA Rota-Adeno kit in 59 patients (12.6 %). The incidence of viral gastroenteritis caused by Rotavirus was higher in the cold months (December to March) (Figure 3). The incidence of Adenovirus detected by the VIKIA Rota-Adeno kit consisted of only 5 cases (1.1 %), registered in different monts.

The incidence of Rotavirus infections was highest in children younger than 5 years old (56 % of the cases) (Figure 4). Although Adenovirus was only detected by the VIKIA Rota-Adeno in 5 cases during the study period, 4 of these cases were children less than 5 years old (Table 2).

Discussion

Molecular epidemiogical studies gained relevance in the field of clinical virology [31]. The development of molecular methods for the diagnosis of infections has allowed the detection of enteric viruses that previously could not be detected in conventional cell culture systems

because of their inability to grow in cell lines [2]. Moreover, the detection of enteric viruses by molecular methods is now possible in samples that were considered negative by the immunological approach [26, 37].

The results of this study show that, although the VIKIA Rota-Adeno kit is a rapid and simple method of diagnostic for Rotavirus infections, it may not be the most efficient approach to the diagnosis of Adenovirus gastroenteritis. The percentage of Rotavirus detected by PCR and by the VIKIA Rota-Adeno kit was similar (56 and 61 %, respectively), but for Adenovirus the nested PCR approach was much more efficient (detected in 88.9 % of the studied samples) than the VIKIA Rota-Adeno kit (detected in 5.5 % of the studied samples).

VIKIA Rota-Adeno is described by BioMérioux as a rapid three-step chromatographic immunoassay that the mainly targets Rotavirus A and only providing the detection of Adenovirus as supplementary information (BioMérioux, 2003). This may explain the results obtained in this study for Adenovirus when the VIKIA Rota-Adeno kit was used. In fact, all the PCR positive results for Adenovirus and VIKIA Rota-Adeno negative for Adenovirus were identified by sequencing as Adenovirus F, serotype 41. This kit was also tested by comparison with PCR detection in a previous work that showed poor agreement (30 %) between the immunoassay kit and the RT-PCR method [38]. This can be attributed by the false negative result that is produced by the VIKIA Rota-Adeno kit when the number of Adenovirus is too low [39, 40]. Although PCR is more time consuming than immunological tests, nowadays, this method is the most sensitive to detect Adenovirus in clinical samples. However, this comparison is still controversy and results indicating a higher sensitivity of commercial immunologic tests in relation to PCR are also reported in the literature [28, 41].The fact that from the 18 samples analysed by PCR, approximately 60 % were positive for Rotavirus and 90 % were positive for Adenovirus, indicated that Rotavirus A and

Adenovirus are common agents of gastroenteritis. Moreover, the double presence of Rotavirus and Adenovirus in half of the 18 samples analysed, confirm that both viral agents can act in combination as the causative agents of gastroenteritis as proposed by Aminu et al. [42].

Although the results of VIKIA Rota-Adeno kit clearly suggest that Rotavirus are the most incident of both studied viruses in the gastroenteritis study cases, the molecular analysis indicates that Adenovirus can be the most responsible from the both for the studied viral gastroenteritis episodes. The sequencing of PCR amplification products for samples with contradictory results (results negative for VIKIA Rota-Adeno kit and positive for molecular methods) confirmed Adenovirus as agents of viral gastroenteritis (presence of human Rotavirus A in 1 sample and the presence of human Adenovirus 41 in 15 faecal samples analyzed).

The seasonal incidence of viral gastroenteritis transmitted by Rotavirus was similar to that observed in other studies [43, 44]. Although the occurrence of viral gastroenteritis episodes was observed throughout the year, lower incidence was observed between July and September. As observed before, children in pre-school age are most affected by Rotavirus and Adenovirus [45].

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Table 1. Rotaviruses and Adenoviruses detected by VIKIA Rota-Adeno kit and molecular methods (nested-PCR for Adenovirus and Reverse Transcriptase-PCR for Rotavirus) in samples of human faeces of patients assisted at the Hospital Infant D. Pedro in Aveiro (Portugal) from December 2008 to July 2009.

		Sample number																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
RotaV	VIKIA® Rota-Adeno	+	+	+	+	-	+	+	-	+	-	-	-	-	+	+	-	+	+
	RT-PCR	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	+	+
AdV	VIKIA® Rota-Adeno	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	nested-PCR	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+

RotaV – Rotaviruses A, AdV – Adenoviruses

Table 2. Incidence of gastroenteritis caused by of Adenovirus (detected by VIKIA Rota-Adeno kit) in patients assisted at the Hospital Infant D. Pedro in Aveiro (Portugal) during the study period (2006 - 2009).

	Nº of cases	**Month**	**Age group**
2006	1	July	45/55
2007	1	March	<1
2008	1	May	<1
	1	October	<1
	1	December	1/5

Figure legends

Figure 1. Seasonal variation of gastroenteritis incidence in patients assisted at the Hospital Infant D. Pedro in Aveiro (Portugal) during the study period (2006 - 2009).

Figure 2. Gastroenteritis incidence by age group in patients assisted at the Hospital Infant D. Pedro in Aveiro (Portugal) during the period of study (2006 - 2009). N.D. age not determined.

Figure 3. Seasonal incidence of Rotavirus A in patients assisted at the Hospital Infant D. Pedro in Aveiro (Portugal) between 2006 and 2009.

Figure 4. Incidence of Rotavirus A by age group in patients assisted at the Hospital Infant D. Pedro in Aveiro (Portugal) between 2006 and 2009.

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