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Research paper

Detection and molecular characterization of enteric viruses in children with acute gastroenteritis in Northern Italy

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ABSTRACT

Enteric viral infections are a major concern for public health, and viral acute gastroenteritis is the principal cause of pediatric morbidity and mortality worldwide, mostly in developing countries. The purpose of this study was to determine the prevalence of different enteric viruses detected in a pediatric population with acute gastroenteritis symptoms, and to characterize the strains detected.

Stools were collected from children, aged from 2 months to 15 years old, admitted to one of the main hospitals of Northern Italy, between November 2015 and October 2016. Stools were tested for nine enteric viruses (adenovirus, rotavirus A, norovirus, astrovirus, sapovirus, enterovirus, parechovirus, bocavirus and aichivirus) by molecular methods. Furthermore, rotavirus, norovirus and adenovirus were deeply characterized by nucleotide sequencing and phylogenetic analysis.

A total of 151 out of 510 (29.6%) stools analyzed resulted positive for at least one of the enteric virus investigated. The most common virus detected was rotavirus A (53/151, 35.1%), followed by norovirus (39/151, 25.8%), adenovirus (35/151, 23.1%), sapovirus (9/151, 6%), enterovirus (5/151, 3.3%), astrovirus (5/151, 3.3%), parechovirus (4/151, 2.6%) and bocavirus (1/151, 0.6%). Aichi virus was not detected in any sample. Co-infections were detected in 12 out of 510 faecal samples (2.3%).

These data improved the knowledge of the enteric viruses circulating in children in Northern Italy. In fact, besides rotavirus, adenovirus and norovirus, several viruses circulated across the whole year in the pediatric population object of this study. The introduction of specific viral diagnosis in our clinical setting will improve patient care by reducing unnecessary use of antibiotics addressing the right etiologic diagnosis.

1. Introduction

Acute diarrhea is a major concern for public health, causing about 0.8 million deaths annually (Liu et al., 2012), affecting mainly children under 5 years of age. Although most of the deaths occurs in developing countries, diarrheal disease is the principal cause of pediatric morbidity and mortality worldwide (WHO, 2011; Wilhelmi et al., 2003). Enteric viruses are recognized as the most significant etiological agent of acute gastroenteritis (AGE), accounting for approximately 70% of episodes (Chow et al., 2010). Four viral families are commonly associated with AGE: group A rotaviruses (RVA), noroviruses (NoV), adenoviruses (AdV), and astroviruses (AstV).

Rotaviruses belong to the *Reoviridae* family. Based on nucleotide differences in gene 9 (VP7) and 4 (VP4), RVA are currently classified in 35 G- and 50 P-genotypes (Matthijnssens et al., 2011).

RVAs are the leading cause of AGE in young children worldwide, and are estimated to cause up to 250.000 deaths every year among children aged 0–5 years, mostly in developing countries of Sub-Saharan Africa and South-East Asia (Tate et al., 2016).

Although several G/P genotypes combinations have been reported (Iturriza-Gomara et al., 2011; Matthijnssens and Van Ranst, 2012), the five G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] RVA genotypes cause up to 75% of human RVA infections worldwide as well as in Italy (Santos and Hoshino, 2005; Ruggeri et al., 2011).

Noroviruses (NoVs) are enteric pathogens of humans and animals, recognized as the most common agents of AGE in humans worldwide (van Beek et al., 2013). NoVs are small (27–40 nm) non-enveloped viruses with a single-stranded RNA genome of approximately 6.5–7.5 kb, classified in the genus norovirus of the family *Caliciviridae*. NoV can be subdivided into six genogroups GI–GVI (Martella et al.,

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Table 1
Oligonucleotide primers used for detection of enteric viruses by conventional PCR and RT-PCR assays.

Virus	Primer	Target region	Sequence (5′ to 3′)[a]	Size	Reference
Adenovirus	HexA	Hexon	GCC GCA GTG GTC TTA CAT GCA CAT C	300	19
	HexB		CAG CAC GCC GCG GAT GTC AAA GT		
Aichivirus	C94b	3C region	GACTTCCCCGGAGTCGTCGTCT	158	17
	AIMP		GCRGAG AAT CCR CTC GTR CC		
Astrovirus	Mon269	ORF2	CAA CTC AGG AAA CAG GGT GT	449	21
	Mon270		TCA GAT GCA TTG TCA TTG GT		
Bocavirus	Adel1	NS1	AGG GTT TGT CTT TAA CGA TTG CAG ACA AC	518	22
	Adel2		TAT ACA CAG AGT CGT CAG CAC TAT GAG		
Enterovirus	F1	5′NTR[b]	CAA GCA CTT CTG TTT CCC CGG	440	17
	R1		ATT GTC ACC ATA AGC AGC CA		
Norovirus	JV12	RdRp[c]	ATACCACTATGATGCAGATTA	327	20
	JV13		TCATCATCACCATAGAAAGAG		
Parechovirus	Ev22 (+)	5′UTR[d]	CYC ACA CAG CCA TCC TC	270	17
	Ev22 (−)		TRC GGG TAC CTT CTG GG		
Rotavirus A	Beg 9b	VP7	GGC TTT AAA AGA GAG AAT TTC CGT CTG G	1067	8
	End 9b		GGT CAC ATC ATA CAA TTC TAA TCT AAG		
Rotavirus A	Con-3	VP4	TGG CTT CGC CAT TTT ATA GAC A	876	18
	Con-2		ATT TCG GAC CAT TTA TAA CC		
Sapovirus	SLV5317	Capsid	CTC GCC ACC TAC RAW GCB TGG TT	100	17
	SMPR		CMWWCC CCT CCA TYT CAA ACA C		

[a] Bases within nucleotide sequence of primers, R = A or G; W = A or T; Y = C or T.
[b] 5′ non translated region.
[c] RNA dependent RNA polymerase.
[d] 5′ untranslated region.

2009; Patel et al., 2009). Human noroviruses belong to genogroups I, II and IV, further subdivided into at least 33 genotypes (9 GI, 22 GII, and 2 GIV genotypes) (Vinje, 2015). The genotype GII.4 has been responsible of outbreaks worldwide (Vinje, 2015).

Other viruses associated with AGE include adenovirus type 40 and 41 (AdV, family *Adenoviridae*), human astroviruses (AstV, family *Astroviridae*), human sapoviruses (SaV, family *Caliciviridae*), human bocaviruses (BoV, family *Parvoviridae*) and some viruses belonging to the *Picornaviridae* family such as aichiviruses (AiV), parechoviruses and enteroviruses (Sdiri-Loulizi et al., 2008; Ouyang et al., 2012; Lekana-Douki et al., 2015).

Routine diagnostic methods for viral AGE are based on the detection of common enteric viruses (RVA, AdV, NoV and AstV) by immunoassays or by molecular methods, while the presence of co-infections or other enteric viruses is poorly investigated.

The aim of this study was to determine the prevalence of different viral pathogens including, besides the well-recognized enteric viruses (adenovirus, rotavirus and norovirus), also astrovirus, sapovirus, enterovirus, parechovirus, bocavirus and aichivirus in a pediatric population of Northern Italy with AGE symptoms, between November 2015 and October 2016. The rotavirus, norovirus and adenovirus strains detected were characterized at molecular level in order to understand better the epidemiology of these pathogens in Northern Italy.

2. Materials and methods

2.1. Clinical specimens collection

Between November 2015 and October 2016, a total of 510 stools were collected from 510 children, aged from 2 months to 15 years old (mean: 4 years; median age: 2 years), with AGE symptoms admitted to the Spedali Civili's Hospital, Brescia, Italy. The male/female (M/F) sex ratio for children was 1.3 (293 M/217 F). AGE symptoms included fever, abdominal pain, vomiting, bloating and diarrhea. Diarrhea was defined according to the WHO criteria for children as the occurrence of three or more loose liquid or watery stools within a 24-hour period. The specimens were tested by routine assays and then stored in phosphate-buffered saline at 10% suspensions at −80 °C until use.

In particular, samples were tested for the presence of rotavirus and adenovirus by a commercially available immunochromatographic assay (Rapid Strip ROTA-ADENO, Meridian Biosciences, Europe) and for the presence of norovirus by a rapid immunoassay (ImmunoCard-STAT! Norovirus, Meridian Biosciences, Europe). The tests were performed according to manufacturers' instructions. All molecular assays were performed retrospectively on faecal samples collected during the study period.

2.2. Viral nucleic acid extraction

Viral nucleic acid was extracted from 10% clarified stool suspensions prepared in phosphate-buffered saline by using the Nucli-SENS EasyMag platform (bioMérieux, Marcy l'Etoile, France) or by the QIAamp Viral RNA mini kit (Qiagen, Milan, Italy) according to manufacturers' instructions.

2.3. PCR and RT-PCR assays

The presence of rotavirus, norovirus, adenovirus, astrovirus, sapovirus, enterovirus, parechovirus, bocavirus and aichivirus was determined by RT-PCR or PCR using the virus specific oligonucleotide primers (Table 1). For RNA viruses, reverse transcription was performed at 37 °C for 120 min followed by inactivation of reverse transcriptase at 85 °C for 5 min by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Milan, Italy). PCR cycling conditions were performed according to the published protocols (Table 1) (Khamrin et al., 2011; Iturriza-Gómara et al., 2004; Chhabra et al., 2013; Vinjé and Koopmans, 1996; Medici et al., 2012). For the detection of sapovirus, enterovirus, aichivirus and human parechovirus, a multiplex PCR was performed as previously described (Khamrin et al., 2011).

The PCR products were separated by electrophoresis on 2% agarose gel, stained with GelRed Nucleic Acid Stain (D.B.A. Italia s.r.l. Milan, Italy) and visualized under blue-light transilluminator. The presence of enteric viruses was determined by the presence of the expected PCR product size corresponding to each virus as shown in Table 1.

2.4. Genotyping of rotavirus A, adenovirus and norovirus

After the first round RT-PCR, RVA genotyping was carried out by performing a multiplex semi-nested PCR able to detect the VP7genotypes G1, G2, G3, G4, G8, G9, G10 and G12 and the VP4 genotypes P[4], P[6], P[8], P[9], P[10], and P[11], using a mixture of