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Cent. Eur. J. Med. • 5(5) • 2010 • 640-645
DOI: 10.2478/s11536-009-0130-6

Central European Journal of **Medicine**

Molecular characterization of rotaviruses in mid-western Turkey, 2006-2007

Research Article

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Received 19 May 2009; Accepted 6 October 2009

Abstract: Vaccines against rotaviruses are now available in numerous countries, including Turkey. As the vaccines may show various efficiencies against different type specificities and routine vaccination in infants might result in selection and immune escape of wild-type rotavirus strains, strain surveillance has been initiated before and during the vaccine introduction. We aimed to provide corresponding information on local strain prevalence in Anatolia, mid-western Turkey during the introduction of rotavirus vaccines. Stool samples positive for group A rotavirus by commercial enzyme immunoassay were subjected to reverse transcription-polymerase chain reaction based genotyping of the outer capsid antigens, VP7 and VP4, determining G and P type specificities respectively. Among 36 fully and 5 partially typeable strains we detected genotype G1, G2, and G9 VP7 specificities and genotype P[4], P[6] and P[8] VP4 specificities in 5 individual and 4 mixed combinations. The most common strain was G2P[4] (n=17), followed by G9P[8] (n=9). Other strains were G1P[8] (n=2), G2P[8] (n=2), G1+2P[8] (n=2), G9P[4] (n=1), G2+9P[8] (n=1), G4+9P[6] (n=1), and G2P[4+8] (n=1). Partially typed strains included 2 G1P[NT] and 3 G2P[NT] strains. Our data may help determine a baseline of the rotavirus genotype prevalence in Turkey and see if changes in the incidence of individual strains will be observed after routine use of vaccine.

Keywords: *Rotavirus • Genotyping • VP7 • VP4 • Reassortment • Vaccine*

1. Introduction

Group A rotaviruses are the single most important cause of severe acute diarrhea in infants and young children worldwide. An estimated annual 611,000 deaths, 2 million hospitalizations and 25 million medical visits worldwide represent the major economic and societal burden due to rotavirus infections [1]. Recently, two vaccines, the monovalent Rotarix (GlaxoSmithKline) and the polyvalent RotaTeq (Merck) have been licensed or are already available for use in more than 100 countries to decrease this burden [2]. Group A rotaviruses are genetically and antigenically heterogeneous members of the genus *Rotavirus*, family

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Reoviridae. They are classified into G and P types based on antigenic and genetic characteristics of the outer capsid proteins, VP7 and VP4 respectively [3]. Thus far, at least 11 G types and 12 P types have been reported in humans. Of these, only 5 G types (G1 to G4, and G9) and 3 P types (P[4], P[6] and P[8]) mainly in six combinations (G1P[8], G2P[4], G3P[8], G4P[8], G9P[6] and G9P[8]) have been identified in >90% of rotavirus-associated hospitalizations [4,5].

Large-scale clinical trials demonstrated a good efficacy and safety profile for both vaccines; however, it is still unclear if any of the two vaccines has differential protective levels for any given serotype [6-8]. These findings raise the question of whether or not these vaccines will be effective in parts of the world where strains other than those represented by these vaccines are in circulation. Thus, numerous countries have launched pre- and post-licensure rotavirus strain surveillance to provide baseline information on the strain prevalence and to monitor the effectiveness of these vaccines against this heterogeneous virus family.

Consistent with these goals we setup a pilot study during the introduction of these vaccines in Anatolia, Turkey in 2006.

2. Material and Methods

2.1. Patients and specimens

Fecal samples were collected from 675 children ≤6 years of age presenting with acute gastroenteritis (AGE) to outpatient clinics and emergency departments of university hospitals in 3 counties in Anatolia, Afyonkarahisar (n=365), Kirikkale/Ankara (n=250) and Bolu (n=60), between November 2006 and June 2007. Ethics were obtained by permission and subjects whose parents gave written, informed consent were eligible for inclusion in the study. AGE was defined as an episode of at least 3 loose stools, 3 watery stools, or forceful vomiting associated with gastroenteritis within a 24-hour period during the 7 days before the medical visit. Common indications for hospitalizations were: duration of diarrhea (2-4 days), number of diarrheal stools (>4-5 / 24 h), frequency of vomiting (2-4 / 24 h), fever (≥38.1°C), and dehydration (1-5%). Health care resource utilization data (medication, laboratory tests, and health care visits/ contacts) were collected via questionnaires.

Children who had participated in a trial of a rotavirus vaccine or who had nosocomial AGE were excluded. All samples were stored at 4°C before EIA testing was carried out. Subsequently the samples were put in a freezer (-80°C) and were kept there until shipment to Hungary.

2.2. Laboratory diagnosis of rotavirus infection

Samples were screened for rotavirus by enzyme immunoassay (EIA; Serazym Rotavirus, Virotech; Germany) according to the manufacturer's recommendations. Briefly, stool suspensions were diluted at 1 in 11 and then 50 μl were added to the wells that already contained 2 drops of the conjugate. The plate was incubated for 60 minutes at room temperature. After incubation the wells were emptied and then washed 5 times. Subsequently, 2 drops of the TMB/Substrate solution was added and incubated for 10 minutes at room temperature. The enzymatic reaction was stopped by adding 2 drops of the stop solution. The absorbance was read at 450 nm using a 620 nm reference filter. The assay's analytical sensitivity (94.4%) and specificity (99.2%) and the detection limit (10^6 rotavirus particles per gram of stool) were determined by the manufacturer.

2.3. RNA extraction and genotyping

Rotavirus-positive stool samples were subjected to RT-PCR genotyping by the Hungarian collaborating laboratory. The genomic RNA was extracted using the QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol or using the TRIzol reagent (Invitrogen) as described elsewhere[9]. Purified dsRNA was added to a mixture of random hexamers, heat denatured and then immediately cooled. After complementary DNA was synthesized, the amplification was performed using type-specific primers for genotypes G1 to G4, and G9 VP7 and for genotypes P[4], P[6], P[8], and P[9] VP4 [10-12]. Those samples that gave no specific products in this single-round genotyping PCR were then subjected to semi-nested PCR using the VP7 and VP4 gene specific consensus primers (VP7, 9con1 and RVG9; VP4, con3 and con2) [10,11,13], respectively, in the first-round PCR. In the second-round, the above-mentioned genotype-specific primer mixtures were utilized. Alternatively, an oligonucleotide primer mixture containing G1 to G4 specific typing primers described by Gouvea et al. [13] was used in the second-round PCR. cDNAs that gave no amplicons in any of these reactions were subjected to VP6 gene-specific PCR using consensus primers VP6F and VP6R in the reverse transcription and the same primers were utilized in the amplification step [14].

Table 1. Detection of rotavirus infections in various settings, Anatolia, 2006-2007.

	Outpatient setting		Emergency department		Hospitalization		Total
	RV +	RV -	RV +	RV -	RV +	RV -	
Afyon	10	245	7	62	3	38	365
Kirikkale/Ankara	16	157	9	46	2	20	250
Bolu	3	38	3	7	1	8	60
Total	29	440	19	115	6	66	675

Table 2. Strain prevalence of Turkish rotavirus strains identified in Anatolia, 2006-2007.

County	Common strains			Unusual strains		Mixed*	Partially typeable**	Non-amplifiable***	Total
	G1P[8]	G2P[4]	G9P[8]	G2P[8]	G9P[4]				
Afyonkarahisar	-	10	5	-	1	-	2	2	20
Kirikkale / Ankara	2	6	2	-	-	3	3	11	27
Bolu	-	1	2	2	-	2	-	-	7
Total	2	17	9	2	1	5	5	13	54

** Includes the following mixed types: 2 G1+2P[8], 1 G2P[4+8], 1 G9+4P[6], and 1 G2+9P[8].*
*** Includes the following P non-typeable strains: 2 G1PNT and 2 G2PNT.*
**** Includes samples positive by ELISA, but negative by VP6-specific PCR. Please see the text for further information.*

3. Results

Out of 675 diarrheic stool samples, 54 were positive for rotavirus by ELISA; 20 (5.5%) were in Afyonkarahisar, 27 (10.8%) in Kirikkale and 7 (11.6%) in Bolu. Among the 54 identified cases only six patients (11.1%) were hospitalized, 19 (35.2%) were treated in emergency departments and 29 (53.7%) sought primary care physicians (Table 1). The proportions of rotavirus-positive cases were 9.1% (6/66) among hospitalized children, 16.5% (19/115) in the emergency departments, and 6.6% (29/440) in the primary care setting. Rotavirus infections were identified between November 2006 and April 2007, with a higher number of cases during the winter months (36/54, 66.7%).

The mean and median ages of children with rotavirus gastroenteritis were 1.8 and 1.3 years respectively (range 0.2-6 years). The ratio of genders showed a preponderance of girls (57.4% vs. 42.6%). Most of the patients (33/54, 61.2%) came from families of >5 members, very low (<340 USD/month/family) to middle (up to 1,540 USD/month/family) income (50/54, 90.7%) and low education level (52/54, 96.3%). Family members of some patients (12/54, 22.2%) had simultaneous diarrhea with the presenting patient's episode. According to the case definition all children presented with diarrhea. In addition, 35 (64.8%) patients had vomiting, 33 (61.1%) had fever and 29 (53.7%) had abdominal pain.

All 54 samples were subjected to genotyping; 36 samples could be typed for both surface antigens, while 5 were partially typed by RT-PCR. Thirteen samples remained completely non-typeable and none of these samples gave amplicon with a broadly reactive primer pair specific for the VP6 gene [14]. To further investigate if these samples were non-typeable due to the possibly low amount of template we obtained with column based RNA extraction, they were re-amplified after utilization of an alternative RNA extraction method (TRIzol). In addition, to investigate if this unsuccessful amplification was due to the presence of substances interfering with the amplification, the samples were diluted at 1 in 100 in nuclease-free water before repeated amplifications. None of these efforts gave positive results so we considered these samples to be non-amplifiable.

Among the 36 completely typed and 5 partially typeable samples, we identified genotype G1, G2, and G9 VP7 specificities and genotype P[4], P[6], and P[8] VP4 specificities (Table 2). These 3 G types and 3 P types were identified in 5 individual combinations, representing 3 globally common strains (i.e., G1P[8], n=2; G2P[4], n=17; G9P[8], n=9) as well as 2 rare combinations of these common specificities (such as G2P[8], n=2 and G9P[4], n=1). Partially typed strains included 2 G1P[NT] and 3 G2P[NT] strains, while multiple strains were found in 5 samples (including 4 samples with multiple G types and 1 sample with multiple P types; 2 G1+2P[8], 1 G2+9P[8], 1 G4+9P[6], and 1 G2P[4+8]).

4. Discussion

Previous studies reported that 5.5% to 39.8% of Turkish children treated in hospital with acute gastroenteritis are hospitalized due to rotavirus infection [15-21]. This finding together with the estimated 3,000 fatal cases annually [1] indicates the significant economic and societal burden of rotavirus disease in Turkey. In the present study the incidence of rotavirus infections (~8%) in Anatolia, mid-western part of Turkey, fell in the range of previous estimates. This lower detection rate might be explained with a combination of several variables. First, children who had only vomiting, abdominal pain, fever, or a combination thereof without diarrhea have been frequently seen in a previous study [22]. Thus, according to the case definition, rotavirus-positive cases without diarrhea may have been overlooked in our present study. Second, in this study a significant proportion of children were outpatients and rotaviruses are frequently detected at a lower incidence in this setting [23,27]. Third, the overall incidence of rotavirus infections is known to vary year by year [28], raising the possibility that the 2006-2007 rotavirus season was less severe in the study area. Fourth, we cannot exclude that we have failed to recognize some factors that might have affected the sample quality during collection, shipment, storage, preparation or the process of antigen detection EIA.

Three recent papers presented rotavirus strain prevalence data from Turkey [16,18,20]. Kurugöl et al. [16] reported the predominance of G1 rotaviruses followed by G4, G3, and G2 strains during a study in 2000-2001, while Cataloluk et al. [18] demonstrated the dominating prevalence of G4 strains followed by G1 to G3, and G9 strains between 2000 and 2002, providing the first evidence for the circulation of the globally emerging G9 rotaviruses in Turkey. A more recent study by Bozdayi et al. [20] demonstrated the predominance of G1 strains followed by G9 strains during a 2004-2005 study. All these data demonstrate a fluctuating temporal prevalence of different rotavirus types at different areas of Turkey, and show that G2 strains represented an epidemiologically minor serotype. In contrast with these surveys we identified G2 strains prevailing followed by G9 and G1 strains. In addition, we found a relatively high rate of mixed infections in the sample set. *In vivo* mixed infections with strains of different type specificities are important to generate novel reassortant strains [3]. Two rarely identified reassortants of common type specificities, G2P[8] and G9P[4], were detected at a combined incidence of 7.3%. Unfortunately, due to the lack of resources it was not possible to determine if these unusual strains were generated by reassortment among co-circulating common strains identified in the study area or if they have been in circulation for some years since their first detection in Turkey [18,20]. Nonetheless, the higher detection rate of these strains contrasts with data presented in a recent review on global rotavirus strain prevalence, where only 49 G2P[8] (0.3%) and 7 G9P[4] (0.04%) strains were identified among 16,474 rotaviruses [5]. Altogether, our present study is the first from Turkey that documents the rotavirus strain distribution during the introduction of rotavirus vaccines. All these data may help determine a baseline of the overall rotavirus genotype prevalence and see if changes in the incidence of individual strains in line with the increase of vaccine use in our country will be observed.

At present, rotavirus vaccines can be purchased on the private market in Turkey; the full vaccination regimen costs ~200 USD for the Rotarix and ~240 USD for the RotaTeq. More recently it has been announced that the World Health Organization (WHO) recommends that rotavirus vaccination be included in all national immunization programs [29]. The WHO's recommendation on global use of rotavirus vaccines opens a new chapter in the rotavirus vaccine saga, raising hope that rotavirus vaccines will be practically available for all infants in Turkey and other countries of low to upper middle income economics where vaccines are available now only on the private market at relatively high costs. However, because corresponding health policy decisions are made on solid data of reliable disease burden estimates and cost-benefit calculations, appropriate analyses are urgently needed in these countries.

Acknowledgements

We would like to thank Dr Ahmet Gayretli for his contributions to improve the paper. The helpful remarks of the anonymous Reviewer of our paper are also acknowledged. The study was supported in part by the Hungarian Scientific Research Fund (OTKA, T049020).

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