Detection and characterization of human rotavirus in hospitalized patients in the cities of Ponta Grossa, Londrina and Assai - Pr, Brazil

ABSTRACT

Acute diarrheal disease is still one of the major public health problems worldwide. Rotaviruses (RV) are the most important viral etiologic agents and children under five years of age are the target population. **Objective:** To investigate the rate of RV infection in hospitalized patients due to acute diarrhea in the cities of Ponta Grossa, Londrina and Assai - Paraná. **Methods:** Latex agglutination (LA); immunochromatography (ICG); polyacrylamide gel electrophoresis (PAGE) and negative staining electron microscopy (ME) tests were used to detect the virus. For the genotyping, RT-PCR and RT-PCR-ELISA were used, respectively, for NSP4 and VP4/VP7. **Result:** Out of 124 samples there were 69 positive stool samples for RV, for at least one of the used tests, 67 of them being RV group A (RV-A). Overall, most of the RV positive stool samples came from children under thirteen years of age. However, 12 positive cases occurred in patients aged 13 years or above, including an 81-year old patient. **Conclusion:** The data showed similar electropherotypes and genotypes G, P and NSP4 of the inland wild circulating strains of RV.

Keywords: rotavirus; determination; hospitalized patients; genotyping.

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INTRODUCTION

Acute diarrheal disease is still one of the major public health problems worldwide. Rotaviruses (RV) are the most important etiologic agents and children under five years of age are the preferred target population. Moreover, a variety of infant animals are equally affected by species-specific virus strains. Worldwide, RV cause approximately 112 millions of domestic episodes of diarrhea, 25 millions of clinic visits, 2 millions of hospitalizations and about 611,000 deaths of children under five years of age, annually.[1]

RV are icosahedric virions, non-enveloped, and present a triple concentric layers of proteins.[2,3] The inner layer is formed by virus protein 2 (VP2) that involves the genome, the VP1 (RNA polymerase dependent of RNA) e a VP3 (guanilyltransferase and methylase). The intermediate layer is made up of VP6 associated with VP2 and confers the structure the so-called double-layered particles (DLP). The outer layer is constituted by trimeric structures of VP7 glycoprotein and the dimeric spikes of VP4 forming the triple-layered particles (TLP), the infectious form of the virus. Virus genome is represented by 11 segments of double-stranded RNA that encode, respectively, six structural proteins (VP1-VP4, VP6 and VP7) and six nonstructural proteins (NSP1-NSP6).

Based on antigenic specificity of VP6, RV are classified into seven groups (A-G), prevailing the infections by group A strains (RV-A).[2] Moreover, VP6 epitopes allow the differentiation of group A strains into sub-groups (SG-I, SG-II, SG-I/II, and non-SG-I/II), prevailing SG-II in human infections.[4] These strains are molecularly distinguished into genogroup I (SG-I) and genogroup II (SG-II, SG-I/II, and SG non-I,non II).[5] G and P genotyping is attributed, respectively, to VP7 and VP4.[2] Twenty-three G and 31 P genotypes have been described.[6,7] Due to the importance of NSP4 protein in the virion morphogenesis, replication and in the pathogeny of the infection this protein was also defined molecularly into six genotypes (A-F).[4]

Genotypes G1-G4 and G9 combined with P[8] and P[4] are the most prevalent worldwide (approximately 90%).[9,10] Concerning NSP4 genotyping, B type has been shown to be the most common in the world.[11,12]

A more complex though complete classification of group A RV has been proposed based on the molecular analysis of all eleven genome segments.[8]

Authors
Carlos M Nozawa[1]
Gustavo Franthesco Kerntopf[2]
Érika da Silva Czernisz[3]
Daniele Albuquerque[3]
Priscila Romanin[4]
José Felipe Eliseu Freitas[4]
Norma Santos[5]
Fabrício José Benati[6]
Eduardo Pietruchinski[7]
Rosa Elisa Carvalho Linhares[8]

[1]PhD in Sciences (Virology)/ Universidade Estadual de Londrina, Londrina, Pr.
[2]Master's Degree in Microbiology/Universidade Estadual de Londrina, Londrina, Pr.
[3]Bacheloring in Biological Sciences/ Universidade Estadual de Londrina, Londrina, Pr.
[4]Secondary School – Fellowship of IC Jr/ Universidade Estadual de Londrina, Londrina, Pr.
[5]PhD in Sciences (Microbiology)/ Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ.
[6]Master's Degree in Microbiology/Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ.
[7]Master's Degree in Microbiology/Centro de Ensino Superior de Campos Gerais, Ponta Grossa, Pr.
[8]PhD in Sciences (Microbiology)/ Universidade Estadual de Londrina, Londrina, Pr.

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Correspondence to:
Universidade Estadual de Londrina
Rodovia Celso Garcia Cid, (Pr 445) Km 380
Caixa Postal 6001
CEP: 86.051-990 - Londrina
Paraná - Brasil
Phone: +55 (43) 3371-4000
E-mail: cnoz@uel.br

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By poliacrylamide gel electrophoresis (PAGE) RV are classified into seven electropherotypes (e-type) from A-G, according to the migration pattern of the 11 RNA segments. Moreover, group A strains can be further classified according to the mobility of the segments 10 and 11 into long (L), short (S) and super-short (SS) electropherotypes according to the migration pattern.[13,14]

Presently, two types of RV vaccine are commercially available for human use (Rotarix and RotaTeq) and their safety and efficacy to prevent and/or attenuate severe diarrheal episodes have been proved.[15,16]

Bearing in mind the genetic variability of the virus, either vaccine or host natural immunity to the virus or both may pose a selective pressure that may result in emergence of unusual genotypes. Crossing species barrier is also another possibility for the appearance of mutant strains. In fact, hitherto undescribed genotypes have been found.[17] These selective events may represent a significant antigenic "shift" or "drift", as has been shown for influenza virus with a real impact in the epidemiology of the disease. Therefore, it is important to monitor wild strains of the virus in order to evaluate all these consequences, and to accompany the evolution of the infection.

In this paper we evaluated circulating human RV strains in three locations of the State of Paraná, Brazil.

MATERIAL AND METHODS

Feces

One hundred and twenty four fecal samples were collected from April 2005 to March 2009 from hospitalized patients suffering from acute diarrhea, admitted to private and public hospitals in the cities of Assai *(Hospital Climas)*, Londrina *(Hospital Universitário Regional do Norte do Paraná)* and Ponta Grossa *(Hospital Bom Jesus)* - Paraná. The study protocol was approved by the Experimental Ethics Committee of the *Universidade Estadual de Londrina*, under the nº 01840268000-07.

Negative staining electron microscopy

For transmission electron microscopy (EM) raw stool samples were processed by super direct negative staining with 2% sodium phosphotungstate, pH 6.3, as described elsewhere.[18]

Immunochromatography and latex agglutination tests

Stool samples were also homogenized at 20% (vol/vol) in PBS, pH 7.3, and clarified by centrifugation at 450xg/10 min. Clarified homogenate were submitted to Vikia - Rota Adeno, Biomérieux SA, Fr. and/or Virotect Rota, Omega Diagnostic Ltd., UK., according to the manufacturers recommendations.

Polyacrilamide gel electrophoresis

Clarified fecal homogenates were further submitted to virus RNA extraction, as described before,[19] for PAGE.

RT-PCR and PCR-ELISA

For RT-PCR virus RNA was extracted from stools using the TRIzol method (Invitrogen, Carlsbad, USA) and subjected to reverse transcription followed by PCR.[20] The cDNA of VP7 or VP4 gene was synthesized by using primers labeled with biotin at their 5' ends. For PCR-ELISA detection of labeled PCR products and identification of the genotypes of positive samples, briefly, 96-well microplates (Nunc-Immuno module, Nunc, Roskilde, Denmark) coated with streptavidin (Roche Diagnostic GmbH, Mannheim, Germany) were used. To each well the biotin-labeled PCR product was distributed (one reaction per genotype) followed by the addition of type-specific digoxigenin-labeled probe. Individual G- or P-type specific probe mixtures (three type-specific probes/genotype/mixture) were distributed onto the plates. This was followed by the addition of anti-digoxigenin peroxidase conjugate (Roche Diagnostic GmbH); the substrate (TMB peroxidase substrate system; KPL, Gaithersburg, MD), and absorbance read at 450 nm within 10 min.[21] For NSP4 typing, nested-PCR was used and primers are listed in Table 1, including those for VP7 and VP4 typing.

Table 1. VP7, VP4 and NSP4 primers used for amplification and genotyping

Primers	Sequences	Positions	Genes
Beg9 (+)	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1036–1062	VP7
End9 (–)	GGTCACATCATACAATTCTAATCTAAG	1–28	VP7
Con2 (–)	ATTTCGGACCATTTATAACC	887–868	VP4
Con3 (+)	TGGCTTCGCTCATTTATAGACA	11–32	VP4
NSP4-1a (+)	GGCTTTTAAAAGTTCTGTTCCG	1–12	NSP4
NSP4-2b (–)	GGTCACATTAAGACCGTTCC	750–731	NSP4
NSP4-Kun-1a (+)	ATTGATAGTGCGATCGACTGG	439–460	NSP4-A
NSP4-Wa-1a (+)	GGCTGGATATAAAGAGCAGG	286–306	NSP4-B
NSP4-RRV-1a (+)	AACGATTGGGCTGAAAGGTG	508–528	NSP4-C

RESULTS

From 124 stool samples evaluated 69 were positive for RV (55.6%), for at least one of the methods used. Amongst these 69 positive samples, 63 (88.4%) were considered group A (RV-A), as demonstrated by serological methods, being positive either by ICG or LA. Additionally, four strains were also defined as RV-A by electrophoretic pattern by PAGE. Therefore, overwhelmingly, a total of 67 strains out of 69 (97.1%) were RV-A. Individually, the performance of the methods used accounted for the following results (Table 2): For LA, 66 samples were analyzed and 23 were positive (34.8%). For ICG, 40 samples were positive out of 64 (62.5%). For PAGE, out of 124 samples 47 were positive (37.9%), being 26 strains, 55.2% (26/47), with short pattern electropherotypes and 20, 42.5% (20/47), with long pattern. For EM, 47 samples were tested and 11 were positive (23.4%). As far as genotyping is concerned, out of the 124 samples, 44 samples (35.4%) were amplified as following.

Table 2. Rotavirus strains with the respective methods of detection/typing

Strain	Age (yrs./ mos.*)	LA	ICG	E-type[a]	EM	Genotypes[b]		
						VP7	VP4	NSP4
ASS08/001	1	ND[c]	-	-	+	NA[d]	NA	NA
ASS08/003	4	ND	+	L	+	NTD[e]	NTD	A
ASS08/004	2	ND	-	-	+	NA	NA	NA
LON08/016	2	+	ND	S	ND	G2	P[4]	A
LON08/018	2	+	ND	S	ND	G2	P[4]	A
LON08/019	3	-	ND	S	ND	NTD	NTD	A
LON08/022	7	+	ND	S	+	NTD	NTD	NTD
PGR05/001	1	-	ND	-	-	NTD	NTD	B
PGR05/002	3	+	ND	L	ND	G1	NTD	B
PGR05/006	3	+	ND	-	-	NTD	NTD	NTD
PGR05/010	11	-	ND	L	+	G1	P[8]	B
PGR05/011	6	+	ND	L	ND	NTD	P[8]	B
PGR05/012	1	+	ND	L	ND	NTD	NTD	NTD
PGR05/013	10	+	ND	-	-	NA	NA	NA
PGR05/015	1	+	ND	L	ND	G1	P[8]	B
PGR05/016	12	+	ND	L	ND	G1	P[8]	B
PGR05/019	14	-	ND	-	-	NTD	NTD	B
PGR05/020	2	+	ND	L	ND	NTD	NTD	B
PGR05/021	9	+	ND	-	-	NA	NA	NA
PGR05/023	4*	+	ND	L	ND	NTD	NTD	NTD
PGR05/024	8*	-	ND	L	ND	NTD	NTD	B
PGR05/025	1	+	ND	-	ND	NTD	NTD	NTD
PGR05/026	14	+	ND	S	ND	NTD	P[8]	B
PGR05/027	9*	+	ND	L	ND	G1	P[8]	B
PGR05/030	1	-	ND	L	ND	NTD	NTD	NTD
PGR05/031	7	+	ND	L	ND	NTD	NTD	NTD
PGR05/032	7	+	ND	L	ND	NTD	NTD	NTD
PGR05/033	4	+	ND	L	ND	G1	P[8]	B
PGR05/034	1	+	ND	-	ND	G1	NTD	B
PGR05/039	2	+	ND	L	+	G1	P[8]	B
PGR05/042	58	+	ND	L	+	G9	P[8]	B
PGR05/043	46	+	ND	L	ND	NTD	P[8]	B
PGR05/045	22	+	ND	NTD	ND	NTD	NTD	B
PGR05/046	8*	+	ND	L	ND	G9	P[8]	B
PGR05/049	3	+	ND	L	+	G1	P[8]	NTD
PGR08/001	4	ND	+	S	ND	NTD	P[4]	A
PGR08/002	81	ND	+	S	ND	G2	P[4]	A
PGR08/003	5	ND	+	-	-	NA	NA	NA
PGR08/004	4	ND	+	S	ND	G2	P[4]	A
PGR08/005	2	ND	+	-	-	NA	NA	NA
PGR08/006	1	ND	+	S	ND	NTD	P[4]	A
PGR08/007	3	ND	+	S	ND	NTD	P[4]	A
PGR08/008	3	ND	+	-	-	NA	NA	NA
PGR08/009	10	ND	+	-	-	NA	NA	NA
PGR08/010	6	ND	+	-	-	NA	NA	NA
PGR08/011	1	ND	+	S	ND	G2	P[4]	A
PGR08/012	6	ND	+	-	-	NA	NA	NA
PGR08/013	22	ND	+	S	ND	G2	P[4]	A
PGR08/014	2	ND	+	-	-	NA	NA	NA
PGR08/015	2	ND	+	S	ND	NTD	NTD	A
PGR08/016	1	ND	+	S	ND	NTD	P[4]	A
PGR08/017	52	ND	+	S	ND	NTD	NTD	A
PGR08/018	21	ND	+	S	+	G2	P[4]	A
PGR08/019	3	ND	+	-	-	NA	NA	NA
PGR08/020	3	ND	+	-	-	NA	NA	NA
PGR08/021	4	ND	+	S	ND	NTD	P[4]	A
PGR08/022	5	ND	+	S	ND	G2	P[4]	A
PGR08/023	4	ND	+	S	ND	NTD	P[4]	A
PGR08/024	3	ND	+	S	ND	G2	P[4]	A
PGR08/025	30	ND	+	S	ND	G2	P[4]	A
PGR08/026	7	ND	+	-	-	NA	NA	NA
PGR08/027	63	ND	+	S	+	NTD	NTD	A
PGR08/028	6	ND	+	-	-	NA	NA	NA
PGR08/029	45	ND	+	S	ND	G2	NTD	A
PGR08/030	1	ND	+	S	ND	G2	P[4]	A
PGR08/031	4	ND	+	-	-	NA	NA	NA
PGR08/032	4	ND	+	S	ND	G2	P[4]	A
PGR08/033	1	ND	+	S	+	NTD	NTD	A
PGR08/034	1	ND	+	-	ND	NA	NA	NA

[a]Eletropherotype: L - long, S - short.
[b]Antigenic specificity carried by indicated proteins.
[c]ND, not done.
[d]NA, not amplified.
[e]NTD, not determined.
+ positive/- negative.

Eleven samples amplified, individually, either for NSP4-A (6) or NSP4-B (5). Thirteen samples amplified for the double combinations G1/NSP4-B (2), G1P[8] (1), G2/NSP4-A (1), P[4]/NSP4-A (6), and P[8]/NSP4-B (3). Twenty samples amplified for the triple combinations G1P[8]/NSP4-B (6), G2P[4]/NSP4-A (12) and G9P[8]/NSP4-B (2). Genotypes G1, G2 and G9 accounted for 37.5% (9/24), 54.1% (13/24), and 8.3% (2/24) of the strains detected, respectively. In our study genotyping VP4 demonstrated that P[4] was prevalent in 56.2% (18/32) in comparison to 43.7% (14/32) of P[8]. Genotyping of NSP4 accounted for 56.8% (25/44) for type A and 43.2% for type B.

According to the ages of the patients, out of 124 stool samples 57 came from children and young children (≤ twelve years of age) (82.6%, 57/69). Positive cases in adults were detected in twelve patients over 12 years, including an 81-year-old male patient, overall representing 17.4% (12/69) (Table 3).

Table 3. Distribution of rotavirus positive cases according to the age of the patients

Age group (year)	n (%)
≥ 2	17 (24.6)
2-12	40 (58.0)
> 12	12 (17.4)
Total	69 (100)

DISCUSSION AND CONCLUSION

This study demonstrated the outstanding importance of RV as the causative of acute diarrheal disease, accounting for a positivity of 55.6%, particularly because all the subjects studied were hospitalized patients. Overwhelmingly, most of the RV positive stool samples were from children under six years of age. This epidemiological feature has been taken for granted, but, increasingly, adults have been affected by the disease. Infection of RV in adults seems to be common and has been reported mainly among geriatric patients, disabled individuals, health attendants and those living in households with diseased children.[22-25] In the present work, we found that 17.4% of RV positive samples were detected in adults from 12 years of age, including an 81-year old patient. It is suggested that the number of adults infected by RV would be greater, and studies involving them should be encouraged. It has been suggested that symptomatic or asymptomatic adults could serve as source of infection to children.[26,27] In this study RV-A, as a rule, accounted for 97.1% of the cases, as most of the world reports have demonstrated.[28-30] The electropherotypes of these strains of RV-A showed a slight predominance of short pattern in comparison to the long-pattern strains, albeit, unusual, this has been reported in India.[31] In general, the predominance of long e-types has been reported throughout the world,[32-36] however, variation has been shown according to some genotypes. As far as VP7 genotyping is concerned, our study revealed greater incidence of G2 secondly by G1. Although in general, greater incidence has been attributed to G1 worldwide, in Brazil, however, temporally/seasonally there has been a change of prevalence between G1 and G2, as demonstrated elsewhere.[29,37] In our study, the greater incidence of G2 may be explained by geographical features or due to the small sampling. Nevertheless, these results demonstrated the occurrence of G types common in most of the countries, including Brazil[9,10,37] combined with P[4] or P[8]. For VP4 genotyping, similar prevalence profile has been found in other countries and in Brazil, and the analysis underlying varied epidemiological features is also possible, between the two major genotypes presently found, P[4] and P[8]. As for NSP4 genotyping, we found that type A accounted for the most strains detected. B type NSP4 has been shown the most common in the world.[11,12] However, in Brazil, this is true for the southeastern area,[38] but not for the northern region, where A type is prevalent.[39] The data reported in our work demonstrated similar profile of the wild circulating strains of RV, as compared to data obtained elsewhere in the world and in our country. Molecular and serological nuances are mainly attributed to factors, such as RV genotypic and phenotypic variability; interspecies barriers crossing; host immunity pressure - naturally or artificially acquired, as well as, geographical and seasonal features. The apparent increase of adult infection and the increasing number of untyped strains, albeit, positive by other tests, as we found in our work, may be a demonstration of a constant genetic variability. Therefore, the emergence of new RV strains should be expected. The effect of RV vaccination recently launched in Brazil is of major benefit for preventing the disease. However, changing in RV genotypes might also be expected as a result of a selective evolutionary process. In concluding, the threat posed by RV still represents a heavy sanitary and economical burden and has to be carefully treated.

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