Coinfection of Rotavirus Group A, Norovirus and Adenovirus in Egyptian Children with Gastroenteritis

Mona Z. Zaghloul[1], Samia F. El-Sahn[2] and Zeinab A. Galal[1]

[1]Clinical Pathology and [2]Pediatric Departments, Faculty of Medicine, Ain Shams University, Cairo, Egypt
monazaki_810@hotmail.com

Abstract: Background and aim: Acute gastroenteritis (AGE) is a common disorder that affects children worldwide. The aim of this work was determination of rotavirus A, norovirus, and adenovirus in stool samples of children with gastroenteritis by qualitative polymerase chain reaction and determination of coinfections between these viral agents. **Subjects and methods:** This study determined rotavirus A, norovirus subtypes I and II and adenovirus in 500 stool samples of children with gastroenteritis and 250 stool specimens from healthy control by qualitative polymerase chain reaction (PCR).**Results:** Rotavirus, norovirus and adenovirus were detected in 39%, 16.2% and 6.8% of the 500 stool specimens of the children with gastroenteritis. Regarding rotavirus, there were 155 (79.5%) cases with monoinfection and 40 (20.5%) with coinfection. For norovirus, there were 48 (59.3%) cases with monoinfection and 33 (40.7%) with coinfection. For adenovirus, there were 23 (67.6%) cases had monoinfection and 11(32.4%) cases with coinfection. Coinfection with rotavirus and norovirus was most common, and occurred in (5.6%) including coinfection with adenovirus. The detection rate of viral agents was most common in children aged from 1 to <3 years. **Conclusion:** Rotavirus A, norovirus and adenovirus could be diagnosed in stool samples of children with gastroenteritis by conventional polymerase chain reaction as a rapid technique. Rotavirus and norovirus were the most common coinfectious agents responsible for gastroenteritis.
[Mona Z. Zaghloul, Samia F. El-Sahn and Zeinab A. Galal. **Coinfection of Rotavirus Group A, Norovirus and Adenovirus in Egyptian Children with Gastroenteritis.** *Life Sci J* 2013;10(2):848-852]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 119

Keywords: coinfection -gastroenteritis- reverse transcription polymerase chain reaction - rotavirus.

1. Introduction

Acute gastroenteritis is a common disorder in young children, and the associated dehydration is a leading cause of admission to hospital in industrialized countries and a major source of mortality in developing countries [1]. Enteric viruses have been recognized as the most significant etiological agents of the disease, and four categories of viruses are being considered clinically relevant: group A rotavirus (family *Reoviridae*), norovirus (family *Caliciviridae*), adenovirus 40/41 (subgenus F), and astrovirus [2, 3, 4].

In children, group A rotavirus (RV) is the major etiologic agent of viral gastroenteritis and is responsible for 29 to 45% of hospitalizations worldwide [5, 6]. Previous work has showed that noroviruses are the second most frequent etiologic agents of viral gastroenteritis in children [7, 8].

The importance of these four viral agents as a cause of gastroenteritis outbreaks is well documented, but their role in sporadic acute severe gastroenteritis responsible for hospitalization or nosocomial infections remains to be assessed in developed countries [3, 8].

A variety of methodologies have been developed to diagnose the presence of rotavirus in diarrheic fecal samples. Virus isolation is used in some diagnostic laboratories [9] and is considered to be a very sensitive detection method when combined with fluorescent antibody staining. Electron microscopy has a relatively low detection limit (10^5 to 10^6 viral particles per gram of feces) [10]. Antigen capture enzyme-linked immunosorbent assay (ELISA), latex agglutination, and reverse transcription polymerase chain reaction (RT-PCR) have become more standard methods for the diagnosis of rotavirus infections [11-14].

In a study by Chinsangaram *et al.,* [15] combining RT-PCR with a post-PCR chemiluminescent hybridization assay resulted in a detection limit of 6×10^2 particles per ml of feces.

Aim of the work

The aim of this work was determination of rotavirus A, norovirus, and adenovirus in stool samples of children with gastroenteritis by qualitative polymerase chain reaction and determination of coinfections between these viral agents.

2. Subjects and methods

A total of 500 stool specimens obtained from Egyptian children < 15 years of age (median 3.7 years) attending the Pediatric outpatient clinic Ain Shams University Hospitals, Cairo, Egypt in the period from February 2012 to January 2013. A total of 154 (30.8%) patients were <1 years of age, 225 (45%) were 1- <5 years of age, 70 (14%) were 5- <10 years of age, and 51 (10.2%) were 10- <15 years of age.

Patients presented with symptoms of gastroenteritis such as diarrhea, colics, nausea and vomiting. In addition, 250 stool samples from healthy subjects matched in age with the patients as a control.

All patients and controls were subjected to the following after their written consent,

- Full history taking and thorough clinical examination.
- Chest plain X- ray.
- Abdominal ultrasound.
- Stool specimens of the patients and controls were subjected to microbiological examination to exclude bacterial or fungal causes of gastroenteritis, qualitative PCR for detection of rotavirus A, adenovirus and norovirus GI and GII subtypes. The stool samples were collected in sterile wide mouth covered universal containers and immediately processed upon receipt for PCR and microbiological examination.

I-Microbiological examination of fecal specimens [16]:

-Wet preparation by saline and eosin to exclude *Entamoeba histolytica, Giardia lamblia* and other cysts or ova of parasites.
-Basic fuchsin smears to exclude *Cambylobacter spp.*
-Methylene blue preparation to detect pus cells.
-Gram stained film and motility to exclude *Vibrio spp.*
-Culture on MacConkey agar, xylose lysine deoxycholate, sorbitol MacConkey agar and alkaline peptone and thiosulphate citrate bile sucrose (TCBS) media.

II-Rotavirus A determination by reverse transcription polymerase chain reaction (RT-PCR):

Primers amplifying a 294-bp fragment of the VP6 gene of type A rotavirus. RNA from fecal samples and reference stock virus preparations were extracted by using a QIAGEN RNeasy kit (QIAGEN, Inc., Valencia, Calif.). RT-PCR was performed with the Superscript One-Step RT-PCR System (Life Technologies, Rockville, Md.). Five microliters of extracted RNA was mixed with the primers (0.4 µ M) (table 1) and RNase-free water was added to a total volume of 24 µl. The mixture was heated at 95°C for 4 min and then quickly cooled to 4°C. Superscript 2 X-reaction mixes (25 µl) and RT-*Taq* mixes (1 µl) were then added. The RT-PCR conditions consisted of cDNA synthesis at 50°C for 30 min and denaturation at 94°C for 2 min, followed by 40 cycles of PCR amplification (94°C for 30 s, 52°C for 30 s, 72°C for 1 min) and a final extension at 72°C for 7 min. Amplification products were visualized in ethidium bromide-stained gels.

III-Norovirus determination by reverse transcription polymerase chain reaction (RT- PCR):

Stool specimens of the patients and controls were tested for the presence of norovirus genotypes I and II by reverse transcription PCR (RT- PCR).

Approximately 10% (Wt/vol) suspension of stool specimens was prepared with distilled sterile water and clarified by centrifugation at 3.000xg for 20 minutes [17].Viral RNA was extracted from 140 ul of the supernatant of the 10% stool suspension and also from positive control transcript by QIAamp Viral RNA kit (QIAGE N, Valencia, Calif.).Briefly:140 ul of each 10% stool suspension was denatured, adsorbed to a silica column, washed and then eluted with 60 ul of diethyl pyrocarbonate-treated water and kept at -80^{O}C until used in RT-PCR.

For cDNA amplification two primer pairs were used designed from genogroup I and II norovirus .The primers were listed in table (2) [18].Ten microliters of cDNA was added to 40 ul of PCR mixture containing 5 ul of 10x Ex Taq buffer, 2.5 mM magnesium chloride, 200 uM (each) d ATP, d GTP, d TTP and d CTP, 20 pmol of primers and 2.5 U of Ex Taq DNA polymerase [17].Conditions of PCR on the Gene Amp PCR system 9600 (Perkin –Elmer, Wellesly, Mass.) were as follows:

Initial denaturation at 95^{O}C for 10 minutes, forty amplification cycles each cycle consisted of denaturation at 95 ^{O}C for 30 sec, annealing at 48^{O}C for 30 sec and extension at 72 ^{O}C for 2 minutes and final incubation at 72 ^{O}C for 7 minutes. Electrophoresis of the amplified products was done on 2% agarose gel, stained with ethidium bromide and visualised by ultraviolet fluorescence. The molecular size marker gave different bands ranging from (100 bp-2000bp) (pharmacia Biotech, USA). The size of the amplified products of norovirus GI and GII corresponds to 213 bp [18].

IV-Adenovirus determination by nested polymerase chain reaction (nested PCR):

DNA was extracted from stool specimens by mixing them with an equal volume of phosphate-buffered saline. After centrifugation, DNA was extracted from the supernatant fraction by QIA-Amp extraction kit (Qiagen Valencia, Calif.) according to the manufacturer's recommendations. The primers used for conventional PCR (first round PCR) and nested PCR were specific for the detection of the hexon protein coding region of human adenovirus genome (Table 3). Amplification was carried out in a 50-µl reaction mixture containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate, 0.08 µM of each primers Adhex1F and Adhex2R (first round PCR), and 2 U of Ampli Taq DNA polymerase. Thermal cycling of the amplification mixture was performed in a programmable heat block (Gene Amp PCR System 2400; Perkin-Elmer) with an initial

denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 93°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. A final extension step at 72°C for 6 min was performed. Then, 1 µl of the first round PCR was transferred to a new batch of 50 µl of PCR mixture containing 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate,0.16 Mm of each nested primer (Adhex2F , Adhex1R) and 2 U of Ampli Taq DNA polymerase in a new 30-cycle amplification. First round PCR products and nested PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV illumination.

3.Results

Detection rates of infectious agents

There were 310/500 (62%) virus positive cases by PCR. Rotavirus was detected in 195/500 (39%), norovirus 81/500 (16.2%), and adenovirus 34/500 (6.8%).

Enteropathogenic *Escherichia coli* (EPEC) was found in 57/500 (11.4%) samples. *Salmonella spp.* was found in 7/500 (1.4%) and normal flora was reported in 126/500 (25.2%) samples. For the rotavirus, 155/195 (79.5%) cases had monoinfection, and 40/195 (20.5%) coinfection. For the norovirus, 48/81 (59.3%) cases had monoinfection, and 33/81 (40.7%) coinfection. Seventeen children had coinfection with Enteropathogenic *Escherichia coli* and rotavirus. Two children had coinfection with *Salmonella spp*. and norovirus. Among 34 cases with adenovirus, 23 (67.6%) cases had monoinfection and 11(32.4%) cases had coinfection (Table 4).

Adenovirus was not detected in stool specimens from the 250 controls, group A rotavirus was detected in 5 samples (2%) by RT-PCR ($p<0.001$), and norovirus was detected in 2 sample (0.8%) ($p<0.001$) among the controls.

As shown in (table 5) coinfection with another pathogen was observed in 45/500 (9%) cases. Coinfection with rotavirus and norovirus was the most common, and occurred in 28/500 (5.6%) including coinfection of adenovirus. In bacterial cultures, Enteropathogenic *Escherichia coli* in 7 children coinfected with rotavirus and *Salmonella spp.* was detected in 2 children coinfected with norovirus.

Age distribution of the viruses

Fifty (25.6%) children infected with rotavirus were less than 1 year of age, and 85 (43.6%) were from 1 to <3 years. Twenty nine (12.8 %) children infected with norovirus were less than 1 year of age and 31 (38.2%) were from 1 to <3 years. Adenovirus infection was higher in the children from 1 to <3 years compared to other age groups. The peak age for the three viruses was from 1 to < 3years (Table 6). In the controls, rotavirus was detected in 2 samples and norovirus was detected in 1 sample of children from 1 to < 3 years.

Table 1: RT- PCR primers for detection of type A rotavirus

Primer	Sequence (5′–3′)	Product (bp)
The upstream Primer	ACCACCAAATATGACACCAGC	294
The downstream Primer	CATGCTTCTAATGGAAGC	294

Table 2: RT- PCR primers for detection of norovirus.

Norovirus genogroup	Sequence (5′–3′)	Amplicon size (bp)
I	TGG ACI CGY GGI CCY AAY CA (RNA sense)	
	GAA SCG CAT CCA RCR GAA CAT (cRNA sense)	213
II	TGG ACI AGR GGI CCY AAY CA (RNA sense)	
	GGA YCT CAT CCA YCT GAA CAT (cRNA sense)	213

Table 3: Nested PCR primers used for detection of human adenoviral DNA in stool samples.

Primer	Sequence (5′–3′)*	Product (bp)
First round PCR: AdHEX1F AdHEX2R	AACACCTAYGASTACATGAAC KATGGGGTARAGCATGTT	473
Nested PCR: AdHEX2F AdHEX1R	CCCMTTYAACCACCACCG ACATCCTTBCKGAAGTTCCA	168

*Y = C + T, S = G + C, K = G + T, R = A + G, M = A + C, B = G + T + C.

Table 4: Incidence of viral infections in children with gastroenteritis

	Monoinfection	Coinfection	Total
RotavirusA	155 (79.5%)	40 (20.5%)	195/500 (39%)
Norovirus	48 (59.3%)	33 (40.7%)	81/500 (16.2%)
Adenovirus	23 (67.6%)	11(32.4%)	34/500(6.8%)

Table 5: Coinfectious viral agents and its incidence

Coinfection	No. of patients
Rotavirus A+Norovirus	25/45(55.5%)
Rotavirus A+Adenovirus	5/45(11.1%)
Rotavirus A+Norovirus+Adenovirus	3/45(6.7%)
Norovirus+Adenovirus	3/45(6.7%)
Rotavirus A+Enteropathogenic *E. coli*	7/45(15.5%)
Norovirus+ *Salmonella spp*.	2/45(4.4%)

Table 6: The age distribution of the three viruses in the patients.

Age (yr)	Rotavirus A	Norovirus	Adenovirus
<1	50(25.6%)	29(12.8%)	6(17.6%)
1- <3	85(43.6%)	31(38.2%)	12(35.3%)
3- <5	36(18.5%)	11(13.6%)	8(23.5%)
5- <10	15(7.7%)	6(7.4%)	4(11.8%)
10- <15	9(4.6%)	4(4.9%)	4(11.8%)
Total	195(100%)	81(100%)	34(100%)

4. Discussion

Acute nonbacterial gastroenteritis is one of the most important infectious diseases that severely affects infants and young children [19]. Morbidity rates worldwide and morbidity and mortality rates caused by diarrhea in developing countries remain high despite efforts to improve sanitary conditions, water quality, and the healthcare infrastructure (20).

In our study there was 310/500 (62%) virus positive cases by qualitative PCR among children with gastroenteritis. Rotavirus, norovirus, and adenovirus were detected in 39%, 16.2% and 6.8% of the children with gastroenteritis respectively. Enteropathogenic *E. coli* was found in 57/500 (11.4%) samples and *Salmonella spp*. was found in 7/500 (1.4%)

Cubitt et al., [21] reported that 28% of children with gastroenteritis had rotavirus, 6% adenovirus, 3% astrovirus, and 3% calicivirus in a 1985 study conducted in London. In France Bon *et al.,* [22] showed that the rotavirus group A was detected in 61% of cases, calicivirus in 14%, astrovirus in 6%, and enteric adenovirus in 3% of stool specimens from 414 children consulting for gastroenteritis between 1995 and 1998. Recently, Lee *et al.,* [23] studied the etiologic agents in 962 Korean children hospitalized with gastroenteritis that rotavirus, norovirus, adenovirus and astrovirus were detected in 25.7%, 13.7%, 3.0%, and 1.1% of the study population, respectively.

In our study coinfection with another pathogen was observed in 45/500 (9%) cases. Coinfection with rotavirus and norovirus was the most common, and occurred in 28/45(62.2%) including coinfection of adenovirus. In bacterial cultures, Enteropathogenic *Escherichia coli* in 7 children coinfected with rotavirus and *Salmonella spp.* was detected in 2 children coinfected with norovirus. Chung et al., [24] studied 812 Korean children with acute gastroenteritis, they reported that coinfection of viral agents was confirmed in 2.7% of the study population, most commonly with rotavirus and norovirus and with rotavirus and human astrovirus. Tran *et al.,* [25] reported that single infection cases were detected in 335 (34%) of the 973 study children, whereas mixed virus infections were detected in 32 (3.3%) of the same. The most frequent dual infections were rotavirus and norovirus (50% of 32), adenovirus and rotavirus (16%), rotavirus and astrovirus (13% of 32), norovirus and adenovirus (9% of 32), and norovirus and astrovirus (3%).

In our study fifty (25.6%) children infected with rotavirus were less than 1 year of age, and 85 (43.6%) were from 1 to <3 years. Twenty nine (12.8 %) children infected with norovirus were less than 1 year of age and 31 (38.2%) were from 1 to <3 years. Adenovirus infection was higher in the children from 1 to <3 years compared to other age groups. The peak age for the three viruses was from 1 to <3 years. Tamura *et al.,* [26] reported that in Vietnam, rotavirus and norovirus were detected in 87 (47.5%) and 12 (6.6%) of the 183 fecal specimens from children hospitalized with acute gastroenteritis, respectively. The majority of patients with rotavirus and norovirus were children younger than 2 years of age. While Ferreira *et al.,* (27) reported in their study to 84 rotavirus-positive samples from hospitalized patients at a teaching hospital in Southern Brazil analyzed by reverse transcription - polymerase chain reaction (RT-PCR), for the investigation of enteric adenovirus,

astrovirus, and norovirus that viral co-infection was more prevalent in children up to 18 months.

Conclusion:

Rotavirus A, norovirus and adenovirus could be diagnosed in stool samples of children with gastroenteritis by conventional polymerase chain reaction as a rapid technique. Rotavirus and norovirus were the most common coinfectious agents responsible for gastroenteritis.

Corresponding author:
Mona Zaki Zaghloul
Consultant of Clinical Pathology, Microbiology unit,Department of Clinical Pathology, Faculty of Medicine, Ain Shams University.
monazaki_810@hotmail.com

References

1. **Parkin PC, Macarthur C, Khambalia A, et al**. Clinical and laboratory assessment of dehydration severity in children with acute gastroenteritis. Clin Pediatr, 2009, 49:235-239.
2. **de Wit MA, Koopmans MP, Kortbeek LM, et al.** Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. Am J Epidemiol, 2001, 154:666-674.
3. **Levidiotou S, Gartzonika C, Papaventsis D, et al**. Viral agents of acute gastroenteritis in hospitalized children in Greece. Clin Microbiol Infect , 2009, 15:596-598.
4. **Oh DY, Gaedicke G and Schreier E.** Viral agents of acute gastroenteritis in German children: prevalence and molecular diversity. J Med Virol, 2003, 71:82-93.
5. **Parashar UD, Hummelman EG, Bresee JS, et al.** Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis 2003, 9:565-572.
6. **Parashar UD, Gibson CJ, Bresse JS, et al.** Rotavirus and severe childhood diarrhea. Emerg Infect Dis, 2006, 12:304-306.
7. **Iritani N, Seto Y, Kubo H, et al.** Prevalence of Norwalk-like virus infections in cases of viral gastroenteritis among children in Osaka City, Japan. J Clin Microbiol, 2003, 41:1756-1759.
8. **Junquera CG, de Baranda CS, Mialdea OG, et al.** Prevalence and clinical characteristics of norovirus gastroenteritis among hospitalized children in Spain. Pediatr Infect Dis J, 2009, 28:604-607.
9. **Al-Yousif Y, Anderson J, Chard-Bergstrom C, et al.** Evaluation of a latex agglutination kit (Virogen Rotatest) for detection of bovine rotavirus in fecal samples. Clin Diagn Lab. Immunol 2001, 8:496-498.
10. **Maes RK, Grooms DL, Wise AG, et al.** Evaluation of a human group a rotavirus assay for on-site detection of bovine rotavirus. J Clin Microbiol, 2003 Jan, 41(1):290-4.
11. **Benfield DA, Stotz IJ, Nelson EA, et al.** Comparison of a commercial enzyme-linked immunosorbent assay with electron microscopy, fluorescent antibody, and virus isolation for the detection of bovine and porcine rotavirus. Am J Vet Res, 1984, 45:1998-2002.
12. **de Beer M, Peenze I, de Costa Mendes VM, et al.** Comparison of electron microscopy, enzyme-linked immunosorbent assay and latex agglutination for the detection of bovine rotavirus in faeces. J S Afr Vet Assoc, 1997, 68:93-96.
13. **Nussbaum DJ, Salord JR and Rimmele DD.** Evaluation of quantitative latex agglutination for detection of Cryptosporidium parvum, E. coli K99, and rotavirus in calf feces. J Vet Diagn Investig, 1999, 11:314-318.
14. **Parwani AV, Rosen BI, Flores J, et al.** Detection and differentiation of bovine group rotavirus serotypes using polymerase chain reaction-generated probes to the VP7 gene. J Vet Diagn Investig, 1992, 4:148-158.
15. **Chinsangaram J, Akita GY, Castro AE, et al.** PCR detection of group A bovine rotaviruses in feces. J Vet Diagn Investig, 1993, 5:516-521.
16. **Cheesbrough M.** District laboratory practice in tropical countries,Cambridge University press, 2000 , part 2, page 104.
17. **Kageyama TS, Kojima S, Shinohara M, et al.** Broadly reactive and highly sensitive assay for Norwalk –like viruses based on real-time quantitative reverse transcription –PCR. J Clin Microbiol , 2003, 41:1548-1557.
18. **Richards GP, Watson MA, Fankhauser RL, et al.** Genogroup I and II Norovrus detected in stool samples by Real –time Reverse transcription –PCR using high degenerate universal primers. Appl Environ Microbiol , 2004, 70(12):7179-7184.
19. **Liu LJ, Liu W, Liu YX, et al.** Identification of norovirus as the top enteric viruses detected in adult cases with acute gastroenteritis. Am J Trop Med Hyg, 2010 Apr, 82(4):717-22.
20. **Sánchez-Fauquier A, Montero V, Moreno S, et al.** Human rotavirus G9 and G3 as major cause of diarrhea in hospitalized children, Spain. Emerg Infect Dis. 2006 Oct, 12(10):1536-41.
21. **Cubitt WD.** Historical background and classification of caliciviruses and astroviruses. Arch Virol Suppl , 1996, 12:225-235.
22. **Bon F, Fascia P, Dauvergne M, et al.** Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. J Clin Microbiol ,1999, 37:3055-3058.
23. **Lee JI, Chung JY, Han TH, et al.** Detection of human bocavirus in children hospitalized because of acute gastroenteritis. J Infec Dis, 2007, 196:994-997.
24. **Chung JY, Huh K, Kim SW, et al.** Molecular epidemiology of human astrovirus infection in hospitalized children with acute gastroenteritis. Korean J Pediatr Gastroenterol Nutr, 2006, 9:139-146.
25. **Tran A, Talmud D, Lejeune B, et al.** Prevalence of rotavirus, adenovirus, norovirus, and astrovirus infections and coinfections among hospitalized children in northern France. J Clin Microbiol , 2010 May, 48(5):1943-6.
26. **Tamura T, Nishikawa M, Anh DD, et al.** Molecular epidemiological study of rotavirus and norovirus infections among children with acute gastroenteritis in Nha Trang, Vietnam, December 2005- June 2006. Jpn J Infect Dis , 2010 Nov, 63(6):405-11.
27. **Ferreira CE, Raboni SM, Pereira LA, et al.** Viral acute gastroenteritis: clinical and epidemiological features of co-infected patients. Braz J Infect Dis. 2012 Jun, 16(3):267-72.