Causative pathogens of severe diarrhea in children

Abdulrahman Y. Ismaeel, *MSc, PhD,* ***Afaf E. Jamsheer,*** *MBBS, MSc,* ***Ahmed Q. Yousif,*** *AIBMS, MSc,* ***Mohammad A. Al-Otaibi,*** *BSc, MSc,* ***Giuseppe A. Botta,*** *MD, PhD.*

ABSTRACT

Objectives: To investigate the enteropathogens in children with diarrhea attending Salmanyia Medical Complex, Bahrain.

Methods: Fecal samples from 805 children up to 15 years were examined for parasites, ova and cysts by direct wet preparation, formol-ether concentration and modified Ziehl-Neelsen stain, during the period November 1998 through to June 2000. Samples were cultured for *Salmonella, Shigella, Campylobacter* and *Enteropathogenic Escherichia coli*. Antibiotic sensitivity tests were performed on the relevant clinical isolates by agar disk diffusion method. All stools from children below 3 years of age (653 samples) were processed for adenovirus and rotavirus using a commercially available latex agglutination test (Diarlex®). In addition, reverse transcription-polymerase chain reaction was performed on 200 randomly selected samples using oligonucleotide primers for Rotavirus A, B and C.

Results: Four subjects were found positive for parasites. Eighty-three (10.3%) samples were found positive for *Salmonella* (46 isolates), *Shigella* (26 isolates), *Campylobacter jejuni* (7 isolates), and *Enteropathogenic Escherichia coli* (4 isolates). Rotavirus was found in 91 (13.9%) samples and 4 samples (0.6%) were found positive for adenovirus. Out of 200 samples examined by reverse transcription-polymerase chain reaction, 73 (36.5%) were positive for group A rotavirus.

Conclusions: Rotavirus type A appeared to be the most common single agent in our pediatric population, followed by the classical bacterial pathogens. Adenovirus and parasites appeared to play a very minor role in diarrhea. Thus, we suggest the introduction of rotavirus diagnostic tests in microbiological examination of diarrheic stools of children below 3 years of age.

Keywords: Gastroenteritis, rotavirus, adenovirus, *Shigella, Salmonella*.

Saudi Med J 2002; Vol. 23 (9): 1064-1069

Infectious diarrhea is one of the major causes of morbidity and mortality worldwide. It is estimated that 3.3 million children younger than 5 years die each year from diarrhea.[1] Many etiological agents including viruses, bacteria and intestinal parasites are implicated as causative agents in infectious diarrhea. Viruses like Rotavirus, Norwalk virus, Calicivirus, enteric Adenoviruses, Astroviruses and Coronaviruses, play a significant role as etiological agent in diarrhea. Out of these viruses, Rotaviruses are considered as the most common diarrheal single agent. Several reports estimated that group A rotaviruses cause 140 millions cases of rotaviral gastroenteritis and one million deaths worldwide each year.[2-3] Rotaviruses are classified by genomic and serological properties into seven groups from A to G. The majority of human cases are attributable to group A.[4-5] Several methods are used for the detection

From the Department of Microbiology, Immunology & Infectious Diseases, (Ismaeel, Al-Otaibi, Botta), College of Medicine and Medical Sciences, Arabian Gulf University, Microbiology Laboratory, (Jamsheer), Salmaniya Medical Complex, Ministry of Health, *Bahrain* and Universita' di Udine, (Yousif), *Italy*.

Received 2nd March 2002. Accepted for publication in final form 26th May 2002.

Address correspondence and reprint request to: Dr. Abdulrahman Y. Ismaeel, Department of Microbiology, Immunology & Infectious Diseases, College of Medicine and Medical Sciences, Arabian Gulf University, PO Box 22979, *Bahrain*. Tel. +973 239648. Fax. +973 271090. E-mail: ismaeel@agu.edu.bh

of the virus in stools such as latex agglutination test (LAT), Enzyme Linked Immunosorbent Assay (ELISA) tests, Polymerase Chain Reaction (PCR) and electron microscopy (EM). The list of bacterial agents responsible for gastrointestinal infections is increasing probably due to the improvement of the diagnostic methods used for their detection. The most common bacteria related to diarrhea include *Shigella spp, Salmonella spp, Campylobacter spp, Aeromonas, Plesiomonas, Vibrio cholerae* and enterovirulent strains of *Escherichia coli*. In many pathogens antibiotic resistance is on the rise and at present, in parts of Africa and Asia, there are species like *Shigella dysenteriae*, which are highly resistant to all antibiotics commonly used in children. Investigations previously carried on in Bahrain on Shigellosis, showed that among the 1,019 Shigella strains, *Shigella sonnei* accounted for 48% of isolates in the period between 1984-1988.[6]

Intestinal parasites also are one of the health problems where relevance varies in different population groups. The incidence of these parasites is generally high in the tropical and subtropical countries affecting at greater extent in low socioeconomic groups.[7] Between 1978-1988, the prevalence of parasites was 32.4% in all fecal samples tested from Bahraini and non-Bahraini patients of all ages.[7] In addition, rotavirus were reported in Bahrain with an incidence of 21% in young children.[8] In this context, this work aimed to investigate the current status of the causative agents of diarrhea in children attending Salmaniya Medical Complex (SMC), Bahrain. Our experience with the application of PCR technique for the direct detection of groups A, B and C rotavirus is presented.

Methods. ***Fecal samples.*** During the period November 1998 through to June 2000, 805 fecal specimens were collected from children (483 males and 322 females up to 15-years of age including non-Bahraini) attending SMC complaining of diarrhea. SMC is the major governmental hospital serving the country.

Parasite detection. Fecal samples were examined for parasites, ova and cysts by direct wet preparation and formalin-ether concentration method. In addition, fecal smears were stained by modified cold Ziehl-Neelsen stain for *Cryptosporidium*.

Bacterial detection. The specimens were innoculated on deoxylate citrate agar (DCA), Selenite broth, subcultured on DCA and incubated at 37°C in air incubator for 24 hours. Suspected colonies were further inoculated to Indole, Urea, Mannitol Motility, triple sugar iron agar (TSI), Citrate and Lysine Decarboxylase home made media. Biochemically identified strains were serogrouped by the slide agglutination test using polyvalent antisera (Murex Biotech Limited, United Kingdom). In vitro antibiotic sensitivity testing was performed by the standard disc (Mast, United Kingdom) diffusion method. The quality control organism was *Escherichia coli* ATCC 25922. *Campylobacter* was cultured on Colombia blood agar, supplemented with ceftazidime (15 μg/ml), trimethoprim (5 μg/ml) and vancomycin (10 μg/ml). The identification of *Campylobacter* to specie level was carried out by using biochemical tests (oxidase, urease, catalase and hippurate test) and by growing them at different temperatures (25°C, 37°C and 42°C) in microaerophilic conditions. *Enteropathogenic E. coli (EPEC)* was cultured on MacConkey agar. Routine biochemical tests and serological techniques were utilized to identify *Salmonella* and *Shigella* species. *Enteropathogenic E. coli* were typed by polyvalent[2-4] commercially available sera (Murex). The sensitivity tests for selected antibiotics were performed, according to National Committee for Clinical Laboratory Standards (NCCLS). [9]

Viral detection. Latex agglutination test (LAT). All stools from children below three years of age (653 samples) were examined for Adenovirus and Rotavirus using Diarlex® Rota-Adeno (Orion Diagnostic, Finland) card agglutination test kit.

Ribonucleic acid extraction. Two hundred fecal specimen previously examined by LAT were processed for reverse transcription-polymerase chain reaction (RT-PCR). Stools (0.5g) were dissolved in 4.5 ml of PBS, and stored in -80°C, for later use. Ribonucleic acid (RNA) was extracted by using high pure viral nucleic acid kit (Roche cat. No. 1-858-874), 200μl of the fecal suspension was used in the extraction according to the kit protocol.

Reverse transcription-polymerase chain reaction and amplification. Five μl of the extract were used in the reverse transcriptase PCR (RT-PCR) by using Perkin Elmer EZ rTth kit (cat. No. N808-0179). RT-PCR was performed using GENEAMP PCR SYSTEM 9700 (Perkin Elmer). The double strand RNA was denatured at 95°C for 5 minutes then followed with 5 minutes on ice. Primers for group A, B, and C were used according to Gouvea.[10] Samples were incubated in thermal cycler for initial 45 min at 42°C; followed by 30 cycles, each of one minute, denaturation at 94°C, 2 minutes annealing at 42°C and one minute extension at 72°C.

Electrophoresis of viral ribonucelic acid. Reverse transcription-polymerase chain reaction products were run on 1.2% agarose gel, using a Power PAC 1000 (Bio-Rad), followed by staining with ethidium bromide. Finally gels were visualized on UV transilluminator (Bio-Rad).

Results. Using LAT on 653 fecal samples of children below 3 years, 91 samples (83 Bahraini and 8 non-Bahraini) were found positive for rotavirus (13.9%). Four samples (0.6%) were found positive

Table 1 - Number (%) of pathogens isolated from Bahraini and non-Bahraini patients.

Pathogens	Bahrani	Non-Bahrani	Total (%)
Bacteria	75	8	**83 (10.3)***
Rotavirus	83	8	**91 (13.9)+**
Adenovirus	4	0	**4 (0.6)+**
Parasites	2	2	**4 (0.5)***
Total	**164**	**18**	**182**

*total bacteria and parasites - 805, +total virus - 653

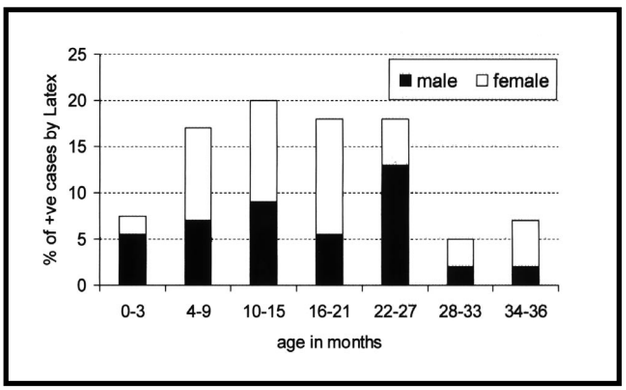

Figure 1 - Age distribution of children infected with Rotavirus. (+ve positive)

Table 2 - Species of pathogenic bacteria isolated from 805 fecal specimens.

Species	n (%)
Salmonella Group B	15 (1.9)
Salmonella Group C	12 (1.5)
Salmonella Group E	2 (0.5)
Salmonella Group G	1 (0.2)
Salmonella typhimurium	4 (0.5)
Salmonella species	12 (1.5)
Total *Salmonella*	**46 (5.7)**
Shigella flexneri	8 (1)
Shigella boydii	8 (1)
Shigella dysenteriae	2 (0.2)
Shigella sonnei	8 (1)
Total *Shigella*	**26 (3.2)**
Enteropathogenic *Escherichia coli*	4 (0.5)
Campylobacter jejuni (426 specimens tested)	7 (1.6)
Total	**83 (10.3)**

for adenovirus **(Table 1)**. Rotaviruses were observed in our selected population during the whole period of observation (data not shown). The age distribution of children infected with rotavirus was one-36 months with slight increase between 22-27 months. Males and females appeared to be equally affected **(Figure 1)**.

Out of 200 random samples examined by RT-PCR, 73 (36.5%) were found positive for rotavirus yielding 257 bp product of group A rotavirus. Group B and C rotaviruses were not detected. When LAT was applied to the same samples, it detected the viruses in 47 (23.5%) samples only. Chi square for PCR and LAT tests equals 8.048 (p value <0.05) which is highly significant.

Parasites were seen in 4 samples only; one was *Ascaris lumbricoides,* 2 *Entamoeba coli* and one *Giardia lamblia*. *Cryptosporidium* was not observed in any of our samples.

Cultures for clinically relevant bacteria were found positive in 83 (10.3%) samples from 75 Bahraini patients and 8 non-Bahraini **(Table 1)**. The majority of infections (76%) occurred in children below 3 years of age. Twenty-six (3.2%) *Shigella* strains were isolated: *S.flexneri* (8 isolates), *S.sonnei* (8 isolates), *S.dysenteriae* (2 isolates) and *S.boydii* (8 isolates). *Salmonella* were found in 46 (5.7%) samples and the following strains were isolated; group B (15 isolates), group C (12 isolates), group E (2 isolates), group G (1 isolates) *S.typhimurium* (4 isolates) and *Salmonella* species (12 isolates). *Enteropathogenic E. coli* was found in 4 patients and *Campylobacter jejuni* was found in 7 children **(Table 2)**. Mixed infection of bacteria and rotaviruses was seen in 4 patients only. The profile of antibiotic sensitivity **(Table 3)** shows that all *Shigella species* 23 were sensitive to ciprofloxacin, cefotaxime and ceftriaxone while many *Shigella* strains were resistant to ampicillin, chloramphenicol and co-trimoxazole, although significant differences were observed among the diverse species. Amid *Salmonella* the highest level of resistance was found for ampicillin.

Discussion. In interpreting our results some notes of caution should be kept in mind. It is likely that factors other than infectious ones might be responsible for causing diarrhea in our children population, most probably diet-related. Other microorganisms, which were not investigated here, are known to cause intestinal infections. Moreover, the population examined (hospitalized children) is more likely to suffer from severe gastro-enteritis and might not reflect the actual incidence of intestinal pathogens in the whole Bahrain population in this age range. A seasonal variability in the incidence of the various microorganisms should also be taken into account when examining our results, although it seems (data not shown) that in our epidemiological settings cases are reported year round.

Table 3 - Number of resistant bacterial strains.

Bacteria	Species	Ampicillin	Ceftriaxone	Cefotaxime	Chloramphenicol	Cotrimoxazole	Ciprofloxacin
Shigella	*Sonei* (8)	0	0	0	0	7	0
	Flexneri (8)	6	0	0	8	8	0
	Boydii (5)	1	0	0	1	2	0
	Dysenteriae (2)	0	0	0	1	1	0
Salmonella	Group B (15)	6	0	0	0	0	0
	Group C (11)	1	0	0	1	2	0
	Group E (2)	0	0	0	0	0	0
	Group G (1)	0	0	0	0	1	0
	Typhimurium (4)	0	0	0	0	0	0
	Spp (12)	9	2	2	2	2	0
E. Coli	*EPEC*	1	0	0	0	1	0

no resistance was found to Gentamicin and Meropenem, *E. coli - Escherichia coli, Spp - species, EPEC - Enteropathogenic E. coli*

Table 4 - Rotavirus in the Gulf Cooperation Council countries.

Country	Period	n of samples tested	Positive (%)	Reference
Bahrain	1984-1986	698	20.9 (LAT)	8
Kuwait	1984	343	40.2 (ELISA)	15
	1982	274	24.5 (ELISA)	16
	1980	274	15.3 (ELISA)	17
Oman	1990-1992	217	31 (ELISA)	11
	1996-1999	991	11.5 (ELISA)	12
Kingdom of Saudi Arabia	1988-1992	1242	42.2 (ELISA)	13
	1995	1726	41.3 (ELISA)	14

n - number, LAT - latex agglutination test, ELISA - enzyme linked immunosorbent assay

Rotaviruses are the most frequently detected pathogens in our population. The differences in the detection rate observed with LAT (23.5%) or with PCR (36.5%) are explained by the higher sensitivity of the latter method. Although RT-PCR is much more sensitive than agglutination test, ELISA and EM for detection of Rotaviruses it is time consuming, it requires well trained personnel and great care must be taken to remove inhibitory substances present in the stools. Thus, we do not presently recommend the RT-PCR to be used in routine clinical settings. However, this technique has other relevant applications, as the typing of viral strains using type-specific primers for epidemiological studies.

Studies have been conducted in other Countries in the Region such as Oman, Kuwait and the Kingdom of Saudi Arabia (KSA) (**Table 4**) to assess Rotavirus diffusion, however none of them used of RT-PCR. In Oman, Aithala et al[11] and Al-Dhahry et al[12] found an incidence of 31% using an ELISA method for antigen detection and 11.5% using the latex agglutination test. It is difficult to ascertain whether this difference is due to a changing epidemiology during the 10-year period or to a change in the

methodology used. In KSA a large study on 1242 children performed using ELISA test (a more sensitive assay than latex) showed a 42.2% positivity[13] while values of 41.3% were detected in similarly designed study.[14] Rotaviruses were also reported in Kuwait[15-17] and Bahrain.[8] In Bahrain, a previous report showed that 20.9% of children were positive for rotavirus by using LAT, a higher value than that observed here, likely linked to an epidemic outbreak. The parasitological findings in this study differ from those of Al-Hilli and Mirza.[7] They reported an incidence of parasites in 32.4% of 131,611 fecal samples of patients attending SMC between 1978-1988. This can be explained as of the large samples that were screened during a long period of time and it was including patients of all ages and not necessarily suffering from acute gastroenteritis. In addition, our finding might suggest that the health status in Bahrain is improving with decreasing incidence of traditional pathogens. The pattern of infection described here appears to be unique for several aspects, being similar to European countries in the rate of viral and parasitic infections while it differs significantly in the rate of bacterial infection and, more important, regarding the species of the isolated pathogens. On the other hand, the parasitic infection rate is lower than that of other neighboring tropical and subtropical countries where these pathogens are commonly encountered. Both worldwide distributed and tropical enteric pathogens have been detected in our population. *Salmonella enterica* with its major serovars was the most common pathogen (5.7%) immediately followed by *Shigella spp* (3.2%). *Campylobacter jejuni* was less frequently isolated, although this can reflect the lower number of specimens tested and the fact that this procedure was recently introduced in our laboratory. The profile of antibiotic sensitivity shows that many strains especially amongst *Shigella* were resistant to co-trimoxazole. Paradoxically, this is not a major therapeutical concern, due to the presence of sickle cell disease in Bahrain, pediatricians and family physicians do not routinely use this drug. Of greater concern is the resistance to chloramphenicol and ampicillin; for both drugs, the highest resistance level is apparent for *S flexneri*). From our experience since 1994 no *Shigella* isolates belonging to the 4 major species were found resistant to ciprofloxacine (manuscript in preparation). Thus, taking in consideration that ciprofloxacin is not recommended for pediatric use, 3rd generation cephalosporins appear to represent the best empirical therapy when antibiotics are needed (and this is a rare event as documented here.

The relevance of *Shigella flexneri* and *Shigella sonnei* isolated in this study has to be stressed as they are currently accounted for most cases of Shigella-associated seizures and encephalopathy.[18,19]

Rotavirus type A appeared to be the most common single agent in our pediatric population in addition to the classical bacterial pathogens; adenovirus and parasites showing a very minor role in our epidemiological context. Thus, we suggest the introduction of rotavirus diagnostic test to be included in the routine laboratory work up, especially in children below 3-years of age. However, before implementing this suggestion a more sensitive rapid test such as ELISA should be selected, in consideration of the poor sensitivity of the agglutination test as shown by the 13% of false negative samples. In addition, the availability of this diagnostic procedure in our hospital will guide and help the clinicians in selecting proper treatment and keeping a prudent use of antibiotics since bacterial infections appears to represent only a limited portion of pediatric cases.

Acknowledgment. Authors thank Mr. Vashisht Ravinder Pal and Mr. Ali Al Mahmeed for excellent technical support and Mr. Tomy Kaitharath for typing the manuscript. This study was in part supported by a grant from Arabian Gulf University to Professor Giuseppe A. Botta.

References

1. Rohner P, Pittet D, Pepy B, Nije-kinke T, Auckentahler R. Etiological agents of infectious diarrhea: Implications for requests for microbial culture. *J Clin Microbiol* 1997; 35: 1427-1432.
2. Kapikian AZ, Flores J, Joshino Y, Glass RJ, Midthun K, Gorziglia M et al. Rotavirus, the major etiological agent of severe infantile diarrhea may be controlled by a "Jennerian" approach to vaccination. *J Infect Dis* 1986; 153: 815-822.
3. Glass RI, Gentsch J, Smith JC. Rotavirus vaccines; success by reassortment? *Science* 1994; 265: 1389-1391.
4. Gray J, Desselberger U. Rotaviruses Methods and Protocols. Humana Press USA, 2000.
5. Estes MK. Advances in molecular biology. Impact on rotavirus vaccine development. *J Infect Dis* 1996; 174 (suppl 1): 37-46.
6. Aziz AY, Qarieballa A, Daniels M, Fernandes EL. Shigellosis species prevalence, incidence and drug resistance in Bahrain. *Journal Bahrain Medical Society* 1989; 2: 52-55.
7. Al-Hilli F, Mirza S. Pathogenic intestinal parasites in Bahrain. *Bahrain Medical Bulletin* 1990; 12: 8-12.
8. Dutta SR, Khalfan SA, Baig BH, Philipose L, Fulayfil R. Epidemiology of rotavirus diarrhea in children under five years in Bahrain. *Int J Epidemiol* 1990; 19: 722-727.
9. Ferraro MJ. Performance standards for antimicrobial disk susceptibility tests. M2/A7. 7th ed. Vol. 20. Wayme (PA): NCCLS; 2000. p. 1-26.
10. Gouvea V. PCR detection of rotavirus: Diagnostic molecular microbiology principles and applications. In: Persing DH, editor. Washington (DC): American Society of Microbiology; 199. p. 383-388.
11. Aithala G, Al-Dhahry SH, Elbualy MS. Epidemiological and clinical features of rotavirus gastroenteritis in Oman. *J Trop Paediatr* 1996; 42: 54-57.
12. El-Dhahry SH, Al-Tagi FS, Rajapakse W. Prevalence of rotavirus gastroenteritis at Sultan Qaboos University Hospital. Proceeding of 1st International Congress on Infectious and Tropical Diseases; 2000 March 27-30; Muscat, Oman. Muscat, Oman: The Ministry of Health & Sultan Qaboos University; 2000.

13. El-Assouli SM, Mohammed KA, Banjar ZM. Human rotavirus genomic RNA electropherotype in Jeddah, Saudi Arabia from 1988 to 1992. *Ann Trop Paediatr* 1995; 15: 45-53.
14. Milaat WA, El-Assouli SM. Epidemiology of diarrhea in two major cities in Saudi Arabia. *J Common Dis* 1995; 27: 84-91.
15. Sethi SK, Al-Nakib W, Khuffash FA, Majeed H. Acute diarrhea and rotavirus infection in young children in Kuwait. *Ann Trop Paediatr* 1984; 4: 117-121.
16. Khuffash FA, Majeed HA, Sethi SR, Al-Nakib W. Gastroenteritis in a regional hospital in Kuwait. *Ann Trop Paediatr* 1982; 2: 123-125.
17. Al-Nakib W, Christie H, Banatvala JE, Al-Sayegb F. Rotavirus and non-bacterial infantile gastroenteritis in Kuwait. *Int J Epidemiol* 1980; 9: 355-359.
18. Aki KF, Hamed R. Fulminating encephalopathy associated with Shigella flexneri. *British J Clin Pathol* 1989; 43: 305-306.
19. Ashkenazi S, Cleary KR, Pickering LK, Murray BE, Cleary G. The association of shiga toxin and other cytotoxins with the neurologic manifestations of shigellosis. *J Infect Dis* 1990; 161: 961-965.