Viral, Bacterial and Parasitic Etiology of Pediatric Diarrhea in Gaza, Palestine

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Key Words

Polymerase chain reaction • Rotavirus • Diarrhea • Gaza • Enteropathogens • *Salmonella* • *Shigella* • *Campylobacter* and parasites

Abstract

Objectives: To determine the etiology of acute diarrhea in Palestinian children under 5 years of age and to improve knowledge of the etiology of gastrointestinal pathogens using traditional and molecular diagnostic techniques. **Materials and Methods:** Various common enteropathogens (viral, bacterial and parasites) associated with diarrhea were investigated by conventional and molecular techniques (PCR) in 150 children less than 5 years of age admitted to the Central Pediatric Hospital, Gaza Strip, Palestine. **Results:** The occurrence of enteropathogens identified was as follows: rotavirus 42/150 (28%), *Entamoeba histolytica/dispar* 23/150 (15%), *Shigella* spp. 9/150 (6%), *Campylobacter coli/jejuni* and *Escherichia coli* O157:H7 7/150 (5%) each, *Salmonella* spp. 3/150 (2%), *Giardia intestinalis* 1/150 (1%), and *Strongyloides stercoralis* 1/150 (1%) of the samples. *Shigella* and *Salmonella* isolates were tested for their susceptibility to common antimicrobial agents and most of the isolates were resistant to ampicillin and trimethoprim/sulfamethoxazole. **Conclusion:** This study demonstrated that rotavirus, *E. coli* O157:H7 and *Campylobacter*, which are not routinely screened for in Gaza Strip, were significant enteropathogens. The results highlight the value of using a combination of traditional and PCR techniques in the diagnosis of enteropathogens related to gastroenteritis.

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Introduction

Acute gastroenteritis is one of the leading causes of illness and death in infants and children throughout the world, especially in developing countries. An estimated 2.5 million gastroenteritis deaths occur each year in children less than 5 years of age [1, 2]. Diarrhea is also one of the leading causes of death among the population in Gaza Strip [3]. In the United Kingdom, approximately 9% of all hospitalizations of children younger than 5 years are due to diarrhea and dehydration [4].

Investigations on diarrheal diseases in young children in the Gaza Strip demonstrated that *Salmonella* spp., *Cryptosporidium* spp., *Campylobacter* spp. and rotavirus were the major pathogens, and overcrowding was linked with an increased risk of diarrhea [5, 6]. However, there has been no comprehensive study describing the prevalence of viral, bacterial and parasitic enteropathogens in Gazian children since 1994 [5, 7].

In Palestine, diarrhea is one of the main causes of outpatient visits and hospitalizations [8]. Routine identification of enteropathogens in Gaza Strip health laboratories is done only for *Salmonella* and *Shigella* spp. by culture, biochemical and serological assays. Routine tests are not performed for rotavirus, *Campylobacter*, and *Escherichia coli* O157:H7 [8]. In the present study, both conventional (culture for bacterial pathogens, direct microscopic examination for parasites and immunochromatographic assay for rotavirus) and molecular diagnostic (polymerase chain reaction, PCR) techniques were used for analyzing stool samples collected from

children under the age of 5 years with acute diarrhea for the presence of *Salmonella* spp., *Shigella* spp., *Campylobacter coli/jejuni*, and *E. coli* O157:H7, parasites and rotavirus.

Materials and Methods

Study Population and Sample Collection

During the peak of the diarrheal season (May–August of 2005), 150 children less than 5 years old (table 1), who were admitted with acute diarrheal diseases to El Nasser Pediatric Hospital, Gaza, were enrolled in the study. The patients were selected randomly from all diarrhea admissions in the hospital over the 24-hour period, every day from May to August of 2005. One of the limitations of the present study was that all diarrhea patients could not be examined because some of them were discharged before the collection of stool specimens. About 84% of these children were aged between 1 and 24 months, and the majority were males (59%, 89/150). For the hospitalized patients the typical symptoms at presentation were diarrhea together with vomiting and fever, and in some cases dehydration.

After obtaining informed consent, one fecal sample was collected from each patient as soon as the children were admitted to the hospital. All specimens were processed within 2 h of collection.

Bacterial Detection

All collected stool specimens were tested for *Salmonella* spp., *Shigella* spp., and enterohemorrhagic *E. coli* (*E. coli* O157:H7). Fresh stool samples were plated onto *Salmonella*-*Shigella* (SS) agar medium (Difco, USA), Hektoen enteric (HE) agar (Difco), Xylose Lysine Deoxycolate (XLD) agar (Difco) and Sorbitol MacConkey agar (Himedia, India), and the plates were incubated for 18–24 h at 37°C. Approximately 1 g of each sample was inoculated into 10 ml of selenite cysteine broth (Himedia) and 10 ml Gram-negative broth (Himedia), and the broth was incubated for 18–24 h at 37°C. Approximately 0.5 ml of selenite cysteine broth and Gram-negative broth were subcultured onto SS agar, HE agar and XLD agar after 18–24 h of incubation. Suspected colonies on the primary or subculture plates resembling those of *Salmonella*, *Shigella* and *E. coli* O157:H7 were selected for further identification by standard laboratory procedures according to the biochemical and serological procedures recommended by the World Health Organization [9, 10].

Antibiotic susceptibility of the *Salmonella* and *Shigella* isolates was determined using the disk diffusion method, using Mueller-Hinton agar (Sanofi Diagnostic Pasteur, France), as described by the National Committee for Clinical Laboratory Standards [10]. Antibiotics tested included ampicillin, piperacillin, cephalixin, cefuroxime, ceftazidime, ceftriaxone, amikacin, gentamicin, doxycycline, sulfamethoxazole/trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, cefaclor and meropenem.

Rotavirus Detection

Stool samples were analyzed for group A rotavirus using RotaStick one-step test kit for determination of rotavirus in human feces (Novamed Ltd., Jerusalem) following the manufacturer's instructions. Stool specimens of 0.1 g were added to 0.6 ml of buffer

Table 1. Age group distribution of the study subjects

Age group	Subjects	
	n	%
1–12 months	95	63.3
13–24 months	31	20.7
25–36 months	8	5.3
37–48 months	7	4.7
49–60 months	9	6.0
Total	150	100

solution in a test tube. The content of test tube was then mixed vigorously by vortex to suspend the specimen. After sedimentation of large particles to the bottom of the tube (2–5 min), the dipstick test strip was placed vertically into the sample tube and removed after 10 s or when the fluid had reached the middle of the test area of the dipstick.

Parasite Detection

A smear of fecal specimens with 0.9% saline was examined microscopically for the presence of leukocytes, red blood cells and parasites. In addition, stool samples were examined in iodine wet mounts. Slides were scanned by light microscopy at $\times 100$ and $\times 400$ magnifications.

Polymerase Chain Reaction

DNA was extracted from all the stool specimens using the AccuPrep Stool DNA Extraction Kit (Bioneer, Korea) following the manufacturer's instructions. The PCR primers used are detailed in table 2. For the detection of bacterial enteropathogens by PCR, 5 μ l (~200 ng) of the prepared DNA template was added to 45 μ l of PCR reaction mixture in 0.2-ml thin-walled microfuge tube. The reaction mixtures used in the PCR steps contained $\times 1$ PCR buffer, 2.0 mM $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate, 1.0 μ M of forward primer, 1.0 μ M of reverse primer (table 2), and 2 U of Taq DNA polymerase (Euroclone, Italy). DNA amplification was carried out in an Eppendorf Mastercycler Personal thermocycler using an initial denaturation step of 95°C for 3 min, followed by 35 cycles of amplification with denaturation at 95°C for 60 s, annealing (as indicated in table 2) for 60 s, and extension at 72°C for 90 s, ending with a final extension at 72°C for 10 min. Upon completion of PCR, the products were analyzed by ethidium bromide-stained 2% agarose gel electrophoresis.

Results

Bacterial Enteropathogens

Using a combination of traditional and molecular diagnostic techniques, bacterial enteropathogens were detected in 26 (17%) cases (table 3). *Shigella* spp. were found in 9 (6%) by PCR, and in 6 (4%) by bacteriological culture

Table 2. Sequence of primers used in PCR

Organism	Primer name	Sequence 5'-3'	Annealing temperature, °C	Expected product size, bp	Ref.
<i>Shigella</i> sp.	ipaH (F)-H8/15 ipaH (R)-H8/3	5'-GTTTCCTTGACCGCCTTTCCGATAC-3' 5'-GCCGGTCAGCCACCCTA-3'	59	700	11
<i>Salmonella</i> sp.	16S rDNA (F) 16S rDNA (R)	5'-TGTTGTGGTTAATAACCGCA-3' 5'-CACAAATCCATCTCTGGA-3'	56	574	12
<i>E. coli</i> O157:H7	UidA PT2 UidA PT3	5'-GCGAAAACGTGTGGAATTGGG-3' 5'-TGATGCTCCATAACTTCCTG-3'	55	252	13
<i>C. coli/jejuni</i>	16S rDNA CCCJ609F 16S rDNA CCCJ1442R	AATCTAATGGCTTAACCATTA GTAAGTAGTTTAGTATTCCGG	58	854	14

F = Forward; R = reverse. All primers were synthesized by Operon Biotechnologies, Germany.

Table 3. Enteropathogens identified in the 150 specimens studied

Microorganism	Positive samples by							
	PCR ¹		culture		immuno-chromatography		microscopic examination	
	n	%	n	%	n	%	n	%
<i>Salmonella</i> spp.	3	2.0	3	2.0				
<i>Shigella</i> spp. ²	9	6.0	6	4.0				
<i>E. coli</i> O157:H7	7	4.7	6	4.0				
<i>C. jejuni/coli</i>	7	4.7	ND					
Rotavirus					42	28.0		
<i>E. histolytica/dispar</i>							23	15.3
<i>G. intestinalis</i>							2	1.33
<i>S. stercoralis</i>							1	0.7

¹ PCR detected all *Salmonella* spp., *Shigella* spp., *E. coli* O157:H7 culture-positive pathogens; 3 cases of *Shigella* spp. and 1 of *E. coli* O157:H7 were detected by PCR but not by culture.

² Three cases of *S. flexneri*, 2 *S. sonnei*, and 1 *S. boydii*; no case of *Shigella dysenteriae* was encountered.

ND = Not determined.

(3 *S. flexneri*, 2 *S. sonnei*, and 1 *S. boydii*). *Salmonella* spp. were found in 3 (2%) by both PCR and bacteriological culture. *E. coli* O157:H7 was found in 7 (5%) by PCR, and in 6 (4%) by bacteriological culture. *Campylobacter* was only identified by PCR in 7 (5%).

Rotavirus, Parasites, Antibiotic Susceptibility

Rotavirus was detected in 28% (42/150), and the majority of the positive cases (90%, 38/42) were under 2 years old (table 4). Microscopy identified parasites in 23 cases (15%, *Entamoeba histolytica/dispar*), *Giardia intestinalis* in 2 cases and *Strongyloides stercoralis* in 1 case (table 3).

The *Salmonella* and *Shigella* isolates were frequently resistant to doxycycline (89%), ampicillin and Septrin (78%) (fig. 1). Not all antibiotics tested were used in the treatment of diarrhea in children.

Discussion

Diarrhea remains one of the most common illnesses of children and one of the major causes of infant and childhood mortality in developing countries. Considering the usually scanty resources available in developing

Fig. 1. Antibiotic resistance found in *Salmonella* and *Shigella* isolates.

countries, a reduction in diarrhea-related mortality may be possible by identifying high-risk subjects and targeting them for intensive intervention.

Rotavirus was the dominant pathogen detected in over a quarter of diarrhea admissions (28%). Our findings are similar to those reported previously in the neighboring countries, approximately 25–35% of infants and young children under 5 years of age with diarrhea were infected with rotavirus [15, 16]. Also, as shown in previous studies, a majority (90%) of rotavirus admissions were children under 2 years of age. The prevalence of rotavirus infection in this age group emphasizes the importance of rotavirus vaccines, which after extensive field trials for several years are now becoming available [17, 18].

E. histolytica/dispar was the second most common enteric pathogen found in the present study (15%). Previous local studies in Gaza found *E. histolytica* 18% in school-children [19], and *E. histolytica/dispar* in 11% of children studied who were less than 4 years old [20]. In our study the isolation rate for both *G. intestinalis* and *S. stercoralis* were only 1%.

Infections with bacterial pathogens peak during the summer months. *Shigella* was the most common bacterial enteric pathogen accounting for 6% of diarrhea admissions during this period. *S. flexneri* was followed by *S. sonnei*, showing a similar trend to reports from other developing countries such as India, Bangladesh, Brazil, Tanzania, Egypt, and Thailand [21]. The prevalence of *Shigella* in our study is consistent with reports from neighboring countries such as Saudi Arabia, Jordan, Leb-

Table 4. Distribution of rotavirus infections detected in 0- to 60-month-old children with acute diarrhea

Age	Patients examined n	Rotavirus antigen			
		positive		negative	
		n	%	n	%
0–12 months	95				
13–24 months	31	13	41.9	18	58.1
25–36 months	8	2	25.0	6	75.0
37–48 months	7	1	14.3	6	85.7
49–60 months	9	1	11.1	8	88.9
Total	150	42	28	108	72.0

anon, Kuwait, and Israel [22]. Only 3 cases (2%) of *Salmonella* were identified, which is lower than in other reports from neighboring countries where detection rates ranged from 2 to 18% [22].

E. coli O157:H7 was found in 6 (4%) of diarrhea admissions. This organism is not routinely sought but has been associated with 10–15% cases of bloody diarrhea [23, 24]. Children with gastrointestinal infections caused by *E. coli* O157:H7 are at risk for the hemolytic-uremic syndrome that can be fatal because it may lead to acute kidney failure. In our study *C. coli/jejuni* was detected in 5% by PCR assay only. Conventional identification of *Campylobacter* is known to be problematic, principally because of their complex taxonomy, biochemical inertness, and fastidious growth requirements [25].

The results of the present study showed that PCR is more rapid, sensitive and specific than conventional culture methods. Our results showed this to be true for *Shigella* spp. (4% culture vs. 6% PCR) and *E. coli* O157:H7 (4% culture and 5% PCR). In total, we found pathogens in 21% (4/19) specimens only by PCR but not by routine cultures. PCR is a selective, sensitive, and specific assay that can detect a small number of culturable as well as nonculturable organisms. Such detection is especially important for *Shigellae*, since they can produce disease by as few as 10–100 organisms. Moreover, the time required for this analysis (~4 h) is shorter when compared to that of the culture technique (48–72 h). Hence, this PCR-based method contributes to the speed of diagnosis of enteric bacterial infections, also yielding higher detection rates of causative agents.

The assay was extremely reliable, being able to detect 100% of culture-confirmed bacterial infections in the study specimens. Furthermore, it also detected 4 culture-negative clinically important gastroenteritis cases, indicating the high level of efficiency of the assay system. Thus, PCR may be judged as superior for its rapidity and sensitivity in the detection of *Shigellae*. Moreover, since this method is applied without cultivation of the organism on synthetic media, nonculturable populations of *Shigella* and *Campylobacter* spp. can also be detected by this method. However the cost of culture methods is about 50% lower than the cost of the PCR method, and the latter does not give information about antimicrobial resistance.

The antibiograms of *Salmonella* and *Shigella* isolates showed that the most frequent patterns of resistance were exhibited towards doxycycline (89%), ampicillin and trimethoprim/sulfamethoxazole (SXT, 78% each). Ceftazidime, ceftriaxone, and ciprofloxacin had the low resistance pattern, whereas all the isolates were sensitive to meropenem, and amikacin. Several studies have indicated the high resistance of *Salmonella*, *Shigella* to ampicil-

lin, SXT and doxycyclines as observed in our study [26–28].

Shigella is becoming more resistant to the commonly used antibiotics especially in developing countries [22]. However, the prevalence of resistance to the same antibiotics is lower in developed countries [29, 30]. This could be due to the more prudent usage of antibiotics in the developed as compared to the developing countries, where for the treatment of diarrhea ampicillin, chloramphenicol and SXT are widely used because of their low cost and ready availability. Most children in our community are treated with antibiotics that had been purchased from private drug outlets. Misuse of antibiotics has resulted in increased resistance to most of these commonly used drugs for treatment. Therefore, increased regulation of the use of antimicrobials may have to be considered.

The current study highlights the necessity for continuous monitoring of antibiotic resistance in diarrhea-related bacterial pathogens. It is recommended that hospital and private laboratories in Gaza Strip routinely examine all diarrheal stool samples for *E. coli* O157:H7 and *Campylobacter* and use PCR technique to augment the conventional culture techniques and improve the quality of detection of bacterial enteropathogens.

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