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Detection and identification of bacterial enteropathogens by polymerase chain reaction and conventional techniques in childhood acute gastroenteritis in Gaza, Palestine

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Enteropathogens

Summary
Background: Acute gastroenteritis and diarrhea are common and costly problems that cause significant morbidity and mortality in children worldwide. In Palestine, diarrhea is one of the major causes of outpatient visits and hospitalizations.
Methods: To improve knowledge on the etiology of gastroenteritis and diarrhea in our patient population, stool specimens from 150 children under 5 years of age suffering from acute gastroenteritis were investigated for various common bacterial enteropathogens by conventional and molecular techniques.
Results: Bacterial enteropathogens were detected in 17.3% of the diarrheal samples. *Shigella spp* was the most common bacterial pathogen (6.0%), followed by *Campylobacter coli/jejuni* (4.7%), *Escherichia coli* O157:H7 (4.7%), and *Salmonella spp* (2.0%). *Shigella* and *Salmonella* isolates were tested for their susceptibility to common antimicrobial agents and most of the *Shigella* isolates were resistant to ampicillin, trimethoprim/sulfamethoxazole and doxycycline and most of the *Salmonella* isolates showed resistant to ampicillin, trimethoprim/sulfamethoxazole, doxycycline and nalidixic acid.
Conclusions: The results highlight the value of using a combination of traditional and molecular techniques (PCR) in the diagnosis of bacterial gastroenteritis. Furthermore, this study demonstrated that *E. coli* O157:H7 and *Campylobacter*, which are not screened for routinely in the Gaza Strip, were significant enteropathogens.

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Introduction

Acute gastroenteritis is one of the leading causes of illnesses and death in infants and children throughout the world, especially in developing countries. This is so in Asia, Africa and Latin America, where an estimated 2.5 million deaths occur each year in children under 5 years of age.[1,2] Diarrhea is also one of the leading causes of death among the population of the Gaza Strip.[3]

Gastroenteritis is characterized by painful abdominal cramps and frequent defecation of blood and mucus, attributed to penetration and destruction of colonic epithelia by invasive microorganisms. Symptoms can include nausea, vomiting, diarrhea, fever, abdominal cramping and/or pain and a general feeling of tiredness. 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 demonstrated that *Salmonella spp*, *Cryptosporidium spp*, *Campylobacter spp* and rotavirus were the major pathogens in the Gaza Strip, and overcrowding was linked with an increased risk of diarrhea.[5,6]

Worldwide, the most common bacterial pathogens that cause this disease are: *Salmonella spp*, *Shigella spp*, *Campylobacter spp*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Vibrio cholerae*, and *Yersinia enterocolitica*.[7]

These pathogens can cause potentially serious diseases, which may be fatal, especially in children. The common route of infection by these pathogens is the ingestion of contaminated foods and drinks.[8]

In Palestine, diarrhea is one of the major causes of outpatient visits and hospitalizations. The identification and diagnosis of bacterial gastroenteritis in the Gaza Strip health laboratories is carried out only for *Salmonella* and *Shigella* species, through culture, biochemical and serological assays; however, stool samples are not routinely screened for *Campylobacter* and *E. coli* O157:H7.[9]

Data from the health laboratories all over the Gaza Strip show that the detection rate of *Salmonella spp* is very low (about 0.4% in the year 2004) and even lower for *Shigella spp* (0.4, 1.2 and 0.12%, in the respective years 2002, 2003 and 2004). Moreover, data concerning cases of *Campylobacter* and *E. coli* O157:H7 and their relation to infection in Palestinian children are extremely scarce.[9–11]

One of the modern techniques for identifying enteropathogens relies on PCR amplification assays with specifically designed nucleotide primers. PCR is suggested by many investigators to be safer, more sensitive and more rapid than the ordinary culture methods for the diagnosis of bacteria or viruses.[12] The use of PCR for detecting and identifying enteric pathogens is rapid and easy while the conventional identification methods are not only time consuming, but also require an experienced laboratory technician to isolate and identify bacterial colonies accurately. Studies in several parts of the world have shown that the sensitivity and specificity of a direct PCR method for the detection of enteric pathogens in stool samples is quite high when compared to conventional methods.[13–15]

In the present study both conventional and molecular diagnostic (PCR) techniques were used for analyzing stool samples collected from children under 5 years of age with acute diarrhea, for the presence of the four common bacterial enteropathogens: *Salmonella spp*, *Shigella spp*, *Campylobacter coli/jejuni*, and *E. coli* O157:H7. *Shigella spp* and *Salmonella spp* isolates were tested for their susceptibility to common antimicrobial agents.

Materials and methods

Study population

During the peak diarrheal season (May–August 2005), 150 children up to 5 years of age who were admitted to El Nasser Pediatric Hospital Gaza (the central pediatric hospital in the Gaza Strip) with acute diarrheal diseases were enrolled in the study.

Sample collection

After informed consent was obtained, fecal samples (one per subject) from children were collected as soon as they were admitted to the hospital with the help of their parents. All specimens were processed within 2 hours of collection.

Bacteriological investigation

All collected stool specimens were tested for *Salmonella spp*, *Shigella spp*, and diarrheagenic *E. coli* (*E. coli* O157:H7). Fresh stool samples were plated onto salmonella shigella (SS) agar medium (Difco, USA), Hecktoen enteric (HE) agar (Difco, USA), xylose lysine deoxycholate (XLD) agar (Difco, USA), and sorbitol MacConkey agar (SMAC) (Himedia, India); 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, India) and 10 ml Gram-negative (GN) broth (Himedia, India), and the broth was incubated for 18–24 h at 37 °C.

Subculture was done only for *Salmonella* and *Shigella*, approximately 0.5 ml of selenite cysteine broth and GN 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.[16,17]

Identification of *E. coli*, *Salmonella* and *Shigella*

Isolated bacteria were identified by their biochemical reaction profile using Hy.enterotest (Hy.laboratories Ltd, Jerusalem) and the API-20E test kit (bioMerieux, France). *E. coli* O157 strains were also tested for the presence of glucuronidase enzyme using a Violet Red with MUG media.[18]

The biochemical assays were supplemented with testing for agglutination using the specific antisera: anti-*Shigella* agglutination sera (BioRad, France), anti-*Salmonella* agglutination sera (Difco, USA) and anti-*E. coli* O157:H7 latex test kit (Plasmatic, UK).

Antimicrobial susceptibility testing by disk diffusion

Antibiotic susceptibility of *Salmonella* and *Shigella* isolates were determined using the disk diffusion method using

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Table 1 Sequence of the primers used in PCR

Organism	Primer name	Sequence 5′ to 3′	Annealing temperature	Expected product size	Reference
Shigella spp	ipaH (F)—H8/15 ipaH (R)—H8/3	5′-GTTCCTTGACCGCCTTTCCGATAC-3′ 5′-GCCGGTCAGCCACCCTA-3′	59 °C	700 bp	13
Salmonella spp	16S rDNA (F) 16S rDNA (R)	5′-TGTTGTGGTTAATAACCGCA-3′ 5′-CACAAATCCATCTCTGGA-3′	56 °C	574 bp	21
E. coli O157:H7	UidA PT2 UidA PT3	5′-GCGAAAACTGTGGAATTGGG-3′ 5′-TGATGCTCCATAACTTCCTG-3′	55 °C	252 bp	22
Campylobacter coli/jejuni	16S rDNA CCCJ609F 16S rDNA CCCJ1442R	AATCTAATGGCTTAACCATTA GTAACTAGTTTAGTATTCCGG	58 °C	854 bp	23

F: forward; R: reverse. All primers were synthesized by Operon Biotechnologies, Germany.

Muller—Hinton agar (Sanofi Diagnostic Pasteur, France) as described by the National Committee for Clinical Laboratory Standards (NCCLS). The isolates were tested against the following antibiotics: ampicillin, piperacillin, cephalexin, cefuroxime, ceftazidime, ceftriaxone, amikacin, gentamicin, doxycycline, trimethoprim/sulfamethoxazole, ciprofloxacin, nalidixic acid, chloramphenicol, cefaclor and meropenem.[19,20]

DNA extraction

Approximately 0.2 g of each sample was used for DNA extraction. DNA was extracted from all the stool specimens using the AccuPrep stool DNA extraction kit (Bioneer, Korea) following the manufacturer's instructions.

PCR primers

Oligonucleotide primers for the PCR are shown in Table 1.[13,21—23]

Detection of bacterial enteropathogens by PCR

Five microliters (~200 ng) of the prepared DNA template was added to 45 μl of PCR reaction mixture in 0.2 ml thin walled microfuge tube. The reaction mixtures used in the PCR steps contained 1× PCR buffer, 2.0 mM $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate, 1.0 μM of forward primer, 1.0 μM of reverse primer (as indicated in Table 1), and 2 U of Taq DNA polymerase (Euroclone, Italy).

DNA amplification was carried out in a 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 1) 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

The study focused on detection and identification of the commonly reported bacterial enteric pathogens in 150 children under 5 years of age in Gaza, Palestine. The results of the study are summarized below.

Detected bacterial enteropathogens

Using a combination of traditional and molecular diagnostic techniques, we detected a bacterial enteropathogen in 26 cases (17.3%) of the diarrhea specimens examined. Table 2 shows the distribution of pathogens found in stools of the 150 children with diarrhea.

Shigella species were found in nine cases (6.0%) by PCR (Figure 1(a)) and in only six cases (4.0%) by bacteriological culture, with a higher frequency of *S. flexneri* (3/6) in relation to *S. sonnei* (2/6), and *S. boydii* (1/6). *Salmonella spp* were found in three cases (2.0% of diarrheal cases) as revealed both by PCR (Figure 1(b)) and bacteriological culture. *E. coli* O157:H7 was found in seven cases (4.7%) by PCR

Table 2 Bacterial enteropathogens identified in the 150 studied specimens

Microorganism	Number and percentage of positive samples			
	By PCR		By culture	
	n	(%)	*n*	(%)
Salmonella spp	3	(2.0)	3	(2.0)
Shigella spp	9	(6.0)	6	(4.0)[a]
E. coli O157:H7	7	(4.7)	6	(4.0)
Campylobacter coli/jejuni	7	(4.7)	ND[b]	

[a] Three cases of the *Shigella spp* were *S. flexneri*, two were *S. sonni*, and one was *S. boydii*; no cases of *S. dysenteriae* were encountered.
[b] Not determined.

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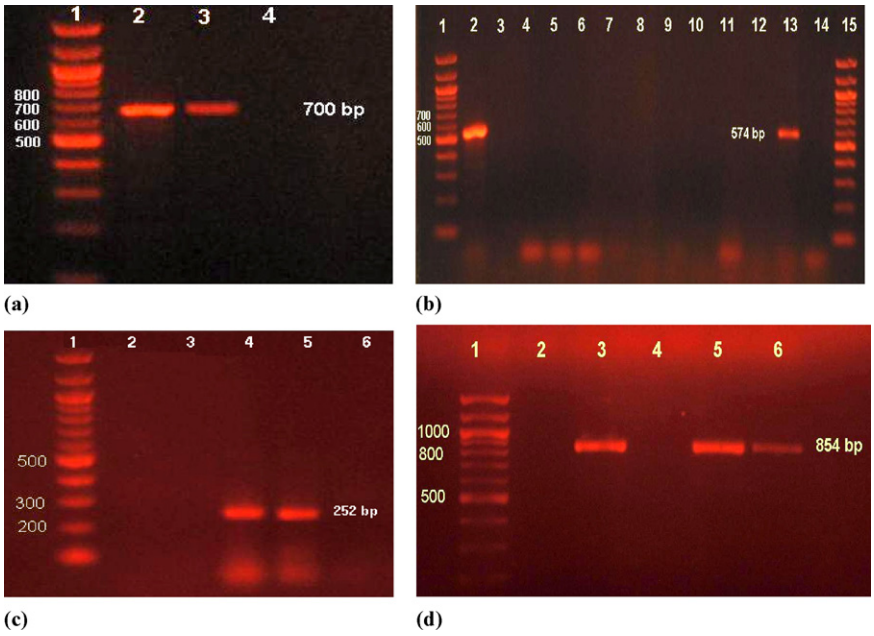

Figure 1 Specific amplification of (a) *Shigella spp*, (b) *Salmonella spp*, (c) *E. coli* O157:H7, and (d) *Campylobacter coli/jejuni* by PCR; examples of some positive results. Amplified products of each reaction were analyzed by electrophoresis on ethidium bromide-stained 2.0% agarose gels. The bands in each figure correspond to the specific PCR product for each bacterial enteropathogen. (a) Specific amplification of *Shigella* specific invasion plasmid antigen H locus DNA by PCR. Lane 1: 100-bp DNA ladder; lane 2: positive control; lane 4: negative control; and lane 3: PCR amplified DNA extracted from a stool sample positive for *Shigella* showing the 700-bp product. (b) The 16S rDNA gene PCR assay specific for *Salmonella spp*. Lanes 1 and 15: 100-bp DNA ladder; lane 2: positive control; lanes 3 and 14: negative control; lanes 4—13: tested sample; and lane 13: a stool sample positive for *Salmonella* showing the 574-bp product. (c) PCR for identification of *E. coli* O157:H7 targeting *uidA*. All positive samples yielded an amplicon size of 252 bp. Lane 1: 100-bp DNA ladder; lane 2: a negative control; lane 3: a blank; lane 4: a positive control; lane 5: an amplicon (252 bp) from DNA extracted directly from a stool sample; and lane 6: stool samples showing negative sample. (d) 16S rDNA-based PCR assay specific for *C. jejuni* and *C. coli*. PCR assay yields an 854-bp product. Lane 1: 100-bp DNA ladder; lane 2: negative control; lane 3 positive control; lanes 4—6 stool samples showing positive samples in lanes 5 and 6 with the 854-bp product.

(Figure 1(c)) and in six (4.0%) of the diarrheal cases by bacteriological culture, while *Campylobacter* was evident in seven (4.7%) of the diarrheal cases as revealed by PCR only (Figure 1(d); Table 2).

PCR results

The main objective of this study was the specific identification of the most common cause of bacterial gastroenteritis.

The extracted DNA of the fecal samples was specifically subjected to amplification for: *Shigella*-specific *ipaH* gene using H8/15, H8/3 primers, *16S rDNA* gene for *Salmonella* species, *uidA* gene for *E. coli* O157:H7 using PT2, PT3 primers and *16S rDNA* gene for *C. jejuni* and *C. coli* using CCCJ609, CCCJ1442 primers.

Of the 150 samples tested in our study, 124 samples were negative for bacteria by PCR and 26 samples were positive by PCR identification.

Figure 1a—d represent specific amplification of PCR results for *Shigella spp*, *Salmonella spp*, *E. coli* O157:H7, and *Campylobacter coli/jejuni*, respectively.PCR detected all 100% for *Salmonella spp*, *Shigella spp*, and *E. coli* O157:H7 culture-positive pathogens and we detected three cases of *Shigella spp* and one case of *E. coli* O157:H7 that were not found by culture.

Antimicrobial susceptibility of *Shigella* and *Salmonella* isolates

The susceptibility of isolated *Salmonella* and *Shigella* species to various antimicrobial drugs was determined by the disk diffusion method following the recommendations of the NCCLS. The antimicrobial agents used and the susceptibility results of the *Shigella* and *Salmonella* isolates are shown in Figures 2 and 3, respectively.

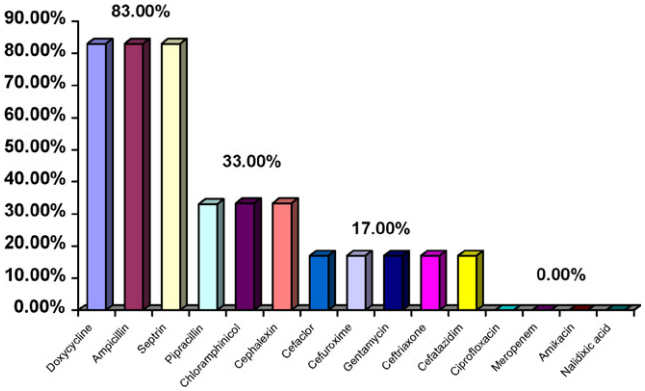

Figure 2 Antibiotic resistance for *Shigella* isolates.

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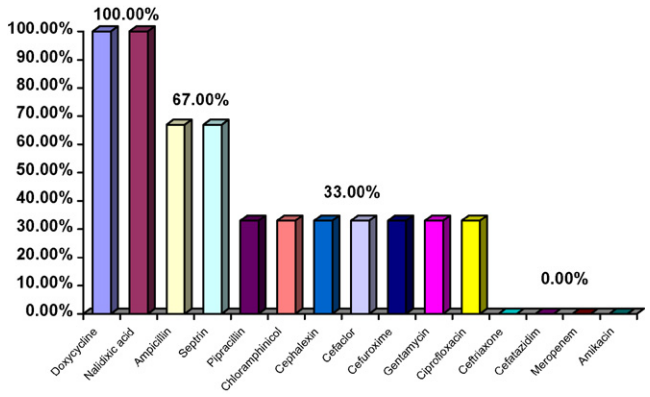

Figure 3 Antibiotic resistance for *Salmonella* isolates.

It is important to note here that some of the antibiotics used were employed to characterize the isolates, but do not have any clinical utility in the treatment of diarrhea in children.

Antimicrobial susceptibility testing of *Shigella* strains showed that 83% of the isolates were resistant to ampicillin, trimethoprim/sulfamethoxazole and doxycycline, 33% to piperacillin, cephalexin and chloramphenicol, and 17% to cefuroxime, ceftazidime, ceftriaxone, cefaclor and gentamicin. All isolates were sensitive to meropenem, nalidixic acid, ciprofloxacin and amikacin (Figure 2).

Antimicrobial susceptibility testing of *Salmonella* strains showed that 100% of the isolates were resistant to doxycycline and nalidixic acid, 67% to ampicillin and trimethoprim/sulfamethoxazole, and 33% to piperacillin, cefaclor, gentamicin, cefuroxime, cephalexin, ciprofloxacin and chloramphenicol. All isolates were sensitive to meropenem, ceftriaxone, ceftazidime and amikacin (Figure 3).

It is important to note here that doxycycline and ciprofloxacin are contraindicated for children.

Discussion

Diarrhea remains one of the most common illnesses in children and one of the major causes of infant and childhood mortality in developing countries. Considering the usually scanty resources available in third world countries, a reduction in diarrhea-related mortality may be possible by identifying high-risk subjects and targeting them for intensive intervention.

The etiology of diarrhea is multifactorial (viral, parasites and bacterial pathogens); viral infection is the most common cause of diarrhea. Infections with bacterial pathogens peak during the summertime when there are suitable conditions such as humidity and high temperature, which facilitate bacterial growth and dissemination.

Shigella was the first most common enteric pathogen identified in the present study with an isolation rate of 6.0%. *Shigella flexneri* was found with the highest prevalence (50%) among the isolated *Shigella spp* emphasizing the importance of this pathogen in the epidemiology of childhood bacterial diarrhea in Gaza, followed by *S. sonnei*; this finding is consistent with other reports from developing countries such as India, Bangladesh, Brazil, Tanzania, Egypt, and Thailand.[24] The prevalence of *Shigella* recorded in this study is similar to that reported by other investigators in many neighboring countries such as Saudi Arabia, Jordan, Lebanon, Kuwait, and Israel.[25]

Three cases (2%) proved positive for *Salmonella* both by culture and PCR. Other reports from neighboring countries indicate that the detection rate of *Salmonella* ranges from 2% to 18%.[25]

E. coli O157:H7 was found in six (4.0%) diarrheal cases by bacteriological culture. This microorganism is not routinely analyzed in our clinical laboratories. *E. coli* O157:H7 has been associated with 10–15% of bloody diarrheas by several investigators.[26,27] Children with gastrointestinal infections caused by *E. coli* O157:H7 are at risk for the hemolytic-uremic syndrome (HUS), which can be fatal as it may lead to acute kidney failure.

Campylobacter coli/jejuni was detected in 4.7% (7/150) of the stool samples when analyzed by PCR assay. Conventional identification of *Campylobacter* is known to be problematic because of their complex taxonomy, biochemical inertness, and fastidious growth requirements.[28,29]

The results of the present study showed that PCR is more rapid, sensitive and specific than conventional culture methods: *Shigella spp* was detected in 4.0% and in 6.0% of the stool samples when analyzed by the bacteriological culture and PCR assay, respectively. By the use of PCR we found that three of the samples were *Shigella spp*-positive by PCR and not by culture. *E. coli* O157:H7 was detected in 4.0% and in 4.7% of the samples when analyzed by conventional culture method and PCR, respectively. By use of PCR we found one sample of *E. coli* O157:H7 that was positive by PCR while it was not resolved by culture. In total we found 4/19 (21.1%) enteropathogen-positive specimens by PCR that the bacteriological stool cultures failed to detect.

PCR is a selective, sensitive, and specific assay that can detect a small number of culturable as well as non-culturable organisms. Such detection is especially important for shigellae, since they can produce disease with as few as 10 to 100 organisms. In our study, the use of the PCR technique improved the rate of detecting shigellae in stool samples from six to nine positive samples, which is 33.3% (3/9) higher than the conventional culture method. Moreover, the time requirement of this technique (~4 hours) is shorter when compared to that of the culture technique (48–72 hours). Hence, this PCR-based method contributes to improvement of the rapid diagnosis of enteric bacterial infections, whilst 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 four 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, non-culturable populations of shigellae and *Campylobacter spp* can also be detected by this method.

In spite of the relatively small size of the study sample, we believe that our data provide useful information about the antimicrobial resistance of *Shigella* and *Salmonella* isolates. In order to help practitioners to choose an adequate antimicrobial drug to start empirical therapy in a

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patient with severe diarrhea without knowledge of a specific pathogen, we assessed the antimicrobial resistance patterns of *Shigella* and *Salmonella spp* that could be isolated. In most pediatric hospitals the empirical antimicrobial drugs used in the treatment of gastroenteritis and diarrhea patients is intravenous ampicillin, and the physicians usually prescribe trimethoprim/sulfamethoxazole after discharge from the hospital.

The antibiograms of *Shigella* isolates showed that 83% of the isolates were resistant to ampicillin, trimethoprim/sulfamethoxazole and doxycycline. Piperacillin, cephalexin and chloramphenicol, cefuroxime, ceftazidime, ceftriaxone, cefaclor and gentamicin had the low resistance pattern, whereas all the isolates were sensitive to meropenem, nalidixic acid, ciprofloxacin and amikacin. Antimicrobial susceptibility testing of *Salmonella* strains showed that 100% of the isolates were resistant to doxycycline and nalidixic acid, 67% to ampicillin and trimethoprim/sulfamethoxazole. Piperacillin, cefaclor, gentamicin, cefuroxime, cephalexin, ciprofloxacin and chloramphenicol had the low resistance pattern, whereas all the isolates were sensitive to meropenem, ceftriaxone, ceftazidime and amikacin.

Several studies have indicated the high resistance of *Salmonella* and *Shigella* to ampicillin, trimethoprim/sulfamethoxazole and doxycycline, which is in agreement with our study.[30–32]

Shigella is becoming more resistant to the commonly used antibiotics especially in developing countries.[25] However, the prevalence of resistance to the same antibiotics is lower in developed countries.[33,34] This could be due to the more appropriate usage of antibiotics in the developed as compared to the developing countries.

The high resistance of our isolates to trimethoprim/sulfamethoxazole and ampicillin observed may be because trimethoprim/sulfamethoxazole is the most commonly used antimicrobial drug for the treatment of gastroenteritis and diarrhea in primary health care in the Gaza Strip, while amoxicillin is the most frequently prescribed antibiotic for the treatment of upper respiratory tract infections (URTIs). As in developing countries, ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole are widely used to treat diarrhea because of their low cost and ready availability. Most children in our community are treated with antibiotics that are purchased from private drug outlets.

Misuse of antibiotics has resulted in increased resistance to most of the commonly used drugs for treatment. A call to regulate the use of antimicrobials may be necessary. According to our study, ampicillin, trimethoprim/sulfamethoxazole, and doxycycline should not be used as empirical treatment of diarrhea in children.

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 the Gaza Strip test all diarrheal stool samples for *E. coli* O157:H7 and *Campylobacter*, which are not routinely diagnosed.

We also recommend the introduction of PCR techniques in order to augment the conventional culture techniques and thus to improve the quality of detection of the bacterial enteropathogens. This will make clinical diagnosis better and help the pediatricians to treat children with diarrhea.

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Conflict of interest: No conflict of interest to declare.

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