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Original article

Determination of antibiotic susceptibility pattern in *Campylobacter jejuni* and *Campylobacter coli* isolated from children with acute diarrhea

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Background: The emergence of antimicrobial resistant Campylobacter populations, which become an important consideration on the use of antimicrobial agents, especially in veterinary and human medicine, has become a serious concern worldwide. For monitoring the drug resistance, there is a need to develop reliable and reproducible laboratory techniques. There are several methods including disk diffusion, broth micro-dilution, agar dilution, and E-test to determine in-vitro susceptibility profiles of Campylobacter to a range of antimicrobial agents.

Objectives: Study the *Campylobacter jejuni* and *Campylobacter coli* from children with diarrhea and determine the antimicrobial susceptibilities of the isolates to clinically relevant antimicrobials.

Materials and methods: Two hundred twenty stool samples of children with diarrhea were cultured on Preston agar and the isolated campylobacter species were identified by further standard identification test. Susceptibility testing was carried out using Kirby-Bauer disk diffusion method and E-test.

Results: Fourteen Campylobacter strains were isolated (6.36%), of which nine (64.3%) were identified as *C. jejuni* and five (35.7%) as *C. coli*. Using disk diffusion, all the campylobacter isolates were fully resistant to cephalothin, oxacillin, and ampicillin followed by ceftazidime with resistance rate of 71.42%. Gentamicin and ciprofloxacin were the most effective antibiotics against both isolated campylobacter species. According to E-test results, Campylobacter isolates demonstrated the greatest resistance to cephalothin (92.85%), oxacillin (92.85%), and ampicillin (78.57%).

Conclusions: Our study reveals a high-level correlation between the E-test and agar disk diffusion method in evaluating the resistance of Campylobacter species to tested antimicrobial agents. This study also suggests disk diffusion is a reliable and cost effective technique for determining the prevalence of resistance among Campylobacter isolates.

Keywords: Antibiotic resistance, campylobacter, disk diffusion, E-test

Campylobacter is the most common cause of bacterial acute gastroenteritis in human beings. Campylobacter species are carried in the intestinal tracts of food animals, especially poultry, and they are often present in food of animal origin through fecal contamination during processing [1]. The genus comprises 14 species, out of which, in particular *Campylobacter jejuni* and *Campylobacter coli* are among the most common causes of bacterial diarrhea in people, world-wide [2, 3]. The importance of these species has increased since a food-borne infection caused by Campylobacter species was first described [4]. Campylobacter infections can occur in all age groups. Studies show a peak incidence in children younger than one year and in persons aged 15-29 years. The age-specific attack rate is highest in young children, but the rate of fecal cultures positive for Campylobacter species is greatest in adults and older children [5].

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Although most cases of Campylobacter enteritis do not require treatment and they are of short duration, clinically mild and self-limiting, a small percentage requires medical intervention. In those cases, antimicrobials may be indicated for treatment of the more severe Campylobacter infections such as systemic infections, infections in immunosuppressed patients, and severe or long-lasting infections [6]. In such cases, several antimicrobials are often recommended [3, 7]. Macrolides, in particular erythromycin, have been considered the first drug of choice for treating *Campylobacter jejuni* gastrointestinal infections [8]. Alternative antimicrobials recommended for the treatment of severe Campylobacter enteritis include ciprofloxacin and tetracycline. Chloramphenicol and ampicillin have also been indicated for systemic infections [9, 10]. However there are reports on development of resistance in Campylobacter species to erythromycin, tetracylines, and fluoroquinolones from developed and developing countries [11-13]. In the early 1990s, fluoroquinolone resistant strains of Campylobacter rapidly became prevalent in Thailand, increasing from 0% to 84% of isolates from 1990 to 1995 [14].

Due to serious consideration of antimicrobial resistance among Campylobacter isolates [15], several laboratory methods including disk diffusion, broth micro-dilution, agar dilution, and Epsilometer-test (E-test) have been used to determine in-vitro susceptibility profiles of Campylobacter to a range of antimicrobial agents [16, 17]. The aim of present study was isolation of *Campylobacter jejuni* and *Campylobacter coli* from children with diarrhea and determination of antimicrobial susceptibilities of the isolates to clinically relevant antimicrobials by using disk diffusion and E-test.

Materials and methods

The study was performed on 220 stool samples of children up to the age of fifteen years, admitted with diarrhea/dysentery in Abuzar children's hospital, Ahvaz, Iran during 2007-2008.

The samples were collected in clean containers with screw caps, containing charcoal transport medium for their transport to the Microbiology Laboratory, School of Medicine. Inclusion criteria were children of either sex with watery diarrhea more than three times per day with less than one to two week(s) duration. Children on antibiotics, three days prior to sample collection were excluded. The samples were inoculated into Preston agar (Hi-Media, Mumbai, India) containing 7% lysed horse blood, and campylobacter selective supplement (Mast Co., UK) comprising vancomycin, trimethoprim and polymixin B, and incubated for 72 hours at 42°C in anaerobic jar (Merck, Darmstadt, Germany) under a microaerophilic atmosphere provided by Gas pack C (Merck, Darmstadt, Germany) containing 5% O2, 10% CO2, and 85% N2. The agar plates were then examined for typical campylobacter colonies by characteristic morphology in gram staining, motility and oxidase test. The organism was identified to species level by catalase production, growth at 25 and 43°C, indoxyl acetate hydrolysis, urea and hippurate hydrolysis, and H2S production [16, 18].

Susceptibility testing was carried out on lysed horse blood Muller-Hinton agar using Kirby-Bauer disk diffusion method as recommended by the Clinical Laboratory Standard Institute (CLSI) [19], against ampicillin (10 μg), erythromycin (15 μg), tetracycline (30 μg), gentamycin (10 μg), trimethoprim/sulphamethoxazole (25 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), oxacillin (1 μg), cephalothin (30 μg), ceftazidime (30 μg) and cefotaxime (30 μg). Briefly, three to five well-isolated colonies were selected from surface of Preston agar medium and transferred into trypticase soy broth (Hi media, Mumbai, India). After incubation at 42°C for 24 hours under microaerobic conditions, the suspension turbidity was adjusted to 1.0 McFarland. A sterile cotton swab was dipped into the suspension and streaked on the entire surface of Mueller-Hinton agar. The antibiotic disks were placed on the plate. The plates were incubated at 42°C under microaerophilic conditions generated in a similar manner as for primary isolation. The diameter of the inhibition zone was measured. Zones of growth inhibition were evaluated according to the CLSI.

For performing E-test, a total of 0.1 ml of the pre-prepared 1.0 McFarland suspension was plated on a Mueller-Hinton agar plate supplemented with 7% lysed horse blood. When the surface of each plate had dried, one E-test strip (AB Biodisk, Solna, Sweden) was put on each plate. The plates were incubated with the lid side up at 42°C for 48 hours under microaerophilic atmosphere as previously mentioned. The minimal inhibition concentrations (MICs) were read directly from the E-test strip according to the instructions of the manufacturer, where the elliptical zone of inhibition intersected with the MIC scale on the strip.

Statistical analysis: Antimicrobial resistance among the Campylobacter isolates in this study to each of the antimicrobials using both the agar disk diffusion and E-test methodologies was compared using Fischer exact two-tailed analysis with significance defined at the 95.0% level ($P \leq 0.05$).

Results

From the stool samples screened, 122 (55.45%) were obtained from male patients and 98 (44.5%) from females. Fourteen Campylobacter species strains were isolated (6.36%), of which eight cases were isolated from male patients samples (57.1%) and six cases were isolated from female patients samples (42.9%).

Based on the information from culture and identification tests, nine cases (64.3%) were identified as *C. jejuni,* four of which isolated of children in age group of less than one year, and five cases (35.7%) were *C. coli,* three of which were isolated from children in age group of one to three years. **Figure 1** represents the isolated campylobacter species according to patients' age group.

The results of susceptibility testing using the disk diffusion method for each antibiotic are shown in **Figure 2**.

Since there were no recommended antimicrobial susceptibility interpretive criteria for Campylobacter, resistant isolates were identified based on the CLSI interpretive criteria for Enterobacteriaceae. Based on this interpretation, all the campylobacter isolates were fully resistant to cephalothin, oxacillin and ampicillin followed by ceftazidime with resistance rate of 71.42%. Gentamicin and ciprofloxacin were the most effective antibiotics against both isolated campylobacter species as shown in **Table 1**. **Table 2,** represents the antibiotic resistance profile for *C.jejuni* and *C.coli.*

Based on the susceptibility results from E-test, MICs for erythromycin ranged between 0.24 and 3.0 μg/ml, those for gentamicin ranged between 0.64 and 32 μg/ml, for ciprofloxacin ranged between 0.008 and 1.0 μg/ml, for sulfamethaxazole ranged between 0.032 to 0.19 μg/ml among sensitive strains and the rest were resistant. Among highly resistant antibiotics, the MIC ranges for minority sensitive strains were as follow: cephalothin one sensitive strain with MIC 0.064 μg/ml, oxacillin one sensitive strain with MICs of 0.48 μg/ml, and ampicillin three sensitive strains with MICs of 0.19, 0.75 and 6.0 μg/ml. According to E-test results based on the standard breakpoints recommended by CLSI (**Table 3**). Campylobacter isolates demonstrated the greatest resistance to cephalothin (92.85%), oxacillin (92.85%) and ampicillin (78.57%). The resistance patterns of isolated campylobacter species using E-test are presented in **Table 4**.

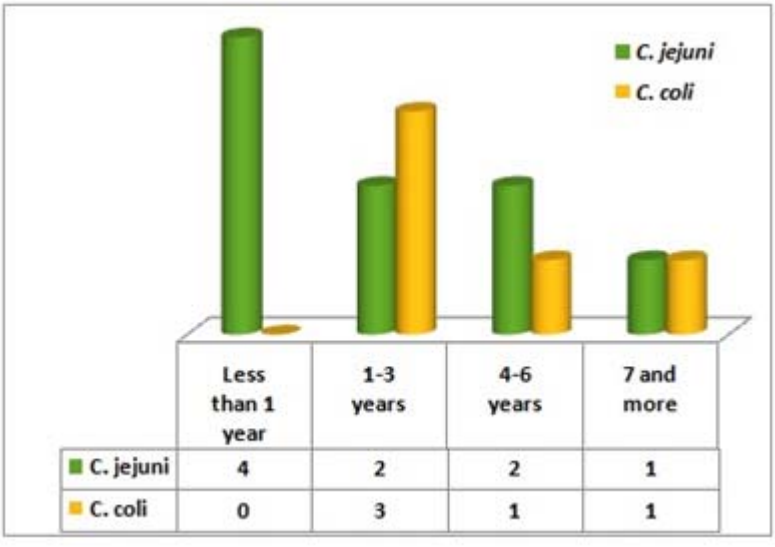

Figure 1. The number of isolated *Campylobacter jejuni* and *Campylobacter coli* according to patients' age group.

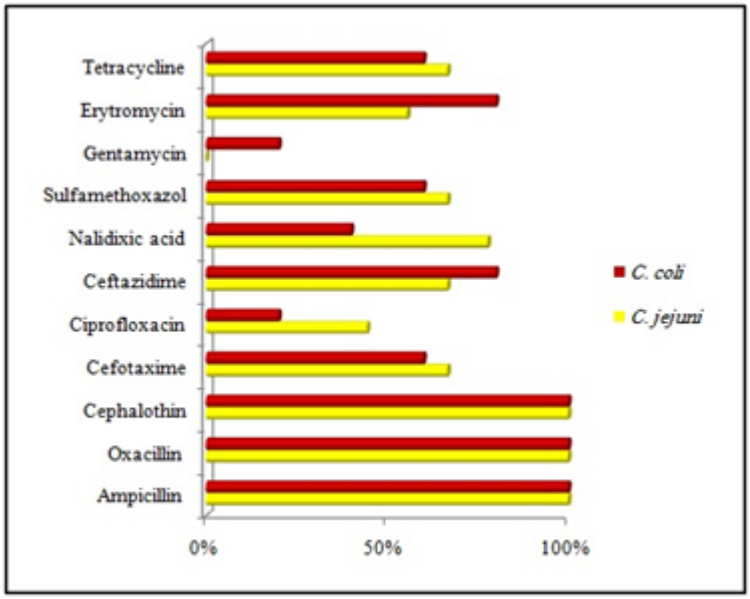

Figure 2. The percentage of antibiotic resistance among isolated *C. jejuni* and *C. coli* obtained in disk diffusion method.

Table1. Antimicrobial susceptibility pattern of campylobacter species identified by the disk diffusion method

Antimicrobial agent	Number of Campylobacter isolates			% of resistant isolates
	S	I	R	
Ampicillin	0	0	14	100
Oxacillin	0	0	14	100
Cephalothin	0	0	14	100
Tetracycline	3	2	9	64.28
Erythromycin	3	2	9	64.28
Ceftazidime	2	2	10	71.42
Nalidixic acid	3	2	9	64.28
Sulfamethaxazole	3	2	9	64.28
Cefotaxime	5	0	9	64.28
Ciprofloxacin	9	0	5	35.71
Gentamicin	12	1	1	7.14

Table 2. The antibiotic resistance profile of isolated *C. jejuni* and *C. coli* in present study

Antimicrobial agent	*C. jejuni* (No./%)	*C. coli* (No./%)
Ampicillin	9/100	5/100
Oxacillin	9/100	5/100
Cephalothin	9/100	5/100
Tetracycline	6/66.66	3/60
Erythromycin	5/55.55	4/80
Ceftazidime	6/66.66	4/80
Nalidixic acid	7/77.77	2/40
Sulfamethaxazole	6/66.66	3/60
Cefotaxime	6/66.66	3/60
Ciprofloxacin	4/44.44	1/20
Gentamicin	0/0	1/20

Table 3. Breakpoints of the E-test as per CLSI recommended interpretation guideline

Antimicrobial agent	Breakpoint MICs (µg/mL)	
	S	R
Ampicillin	≤8	≥32
Oxacillin	≤8	≥32
Cephalothin	≤8	≥32
Tetracycline	≤4	≥16
Erythromycin	≤8	≥32
Gentamicin	≤4	≥16
Ciprofloxacin	≤1	≥4
Sulfamethaxazole	≤8	≥16

Table 4. Antimicrobial susceptibility pattern of isolated campylobacter species based on E-test.

Antimicrobial agent	No. of campylobacter isolates		% of resistant isolates
	S	R	
Cephalothin	1	13	92.85
Oxacillin	1	13	92.85
Ampicillin	3	11	78.57
Sulfamethaxazole	4	10	71.42
Tetracycline	6	8	57.14
Erythromycin	6	8	57.14
Ciprofloxacin	9	5	35.71
Gentamicin	12	2	14.28

Discussion

For monitoring drug resistance among the campylobacter isolates, there is a need to create reliable and reproducible laboratory techniques. Unfortunately, similar to certain fastidious bacteria, Campylobacter present difficulties in antimicrobial susceptibility testing due to both unique growth requirements and test conditions [20]. In present study, the E-test, an agar-based stable concentration gradient method for MIC determination has been used in addition to traditional disk diffusion method to determine the susceptibility profiles of Campylobacter isolates. The methodology has proven to be a relatively accurate method to test antimicrobial susceptibilities of fastidious organisms, including Campylobacter [21, 22].

Based on the results from present study, despite the low prevalence of campylobacter among children with diarrhea (6.36%), the percentage of resistant isolates to clinically relevant antibiotics using both techniques was high, ranged from 92.8 to 100% for cephalothin and oxacillin, 78.5 to 100% for ampicillin, 57.14 to 64.28% for erythromycin and tetracycline, and 35.7% for ciprofloxacin. Besides, we found a higher prevalence of flouroquinolone resistance among *C. jejuni* strains. This was 44.44% for ciprofloxacin and 77.77% for nalidixic acid compared to a similar study [22]. The lowest range of resistance belonged to gentamicin. It was 7.14 to 14.28%. In general, these resistance rates were higher than those found by other investigators for disk diffusion [22, 23]. The higher prevalence of resistance in our study may be due to empirical therapy for diarrheal diseases in children without performing antimicrobial susceptibility testing and mis- and over-use of antibiotics, which increase the risk of drug resistance among campylobacter species.

There was a good correlation between disk diffusion and E-test methods for susceptibility testing of Campylobacter species to cephalothin, oxacillin, sulfamethaxazole, and ciprofloxacin with agreement higher than 90%. Besides, there was 88.8% agreement for erythromycin and tetracycline, and 78.5% agreement for ampicillin. Only a weak correlation was found between two susceptibility methods for gentamicin with low agreement of 50% in present

study (**Figure 3**). No significant difference was found between the antimicrobial resistance prevalence determined using the two methods (P=0.5), with eight of the antimicrobials tested, except for resistance to gentamicin (P<0.05). Similar conclusions have been drawn by other authors when they compared agar disk diffusion to a standardized agar dilution method for antimicrobial susceptibility testing in 686 Campylobacter poultry isolates [24]. These authors reported a high level of correlation between the methods for ciprofloxacin, erythromycin, tetracycline, and nalidixic acid. However, the criteria for the breakpoints of some antibiotics, particularly erythromycin, tetracycline, and ampicillin, needed to be adjusted. Previous studies compared disk diffusion, broth microdilution, agar dilution, and the E-test in the validity and accuracy of determining Campylobacter susceptibilities to antimicrobial agents. The overall conclusion from these reports was that the E-test was a preferable method for susceptibility testing of Campylobacter [20, 21]. During this study, a few Campylobacter isolates on E-test plates, caused some difficulty in interpreting the E-test results. However, by re-testing of these isolates, we gained satisfactory results. However, the correlation in our study was not affected by the species of the organism tested, i.e. *C. jejuni* versus *C. coli.*

Our study suggested that ciprofloxacin and gentamicin might be more effective against Campylobacter than erythromycin. *C. coli*, in particular, displayed significantly higher resistance rates to erythromycin, and ceftazidime. Erythromycin resistance in *C. jejuni* was moderate and this antibiotic is still relatively effective for treating this campylobacter, which comprised the majority of our isolates. As other investigators pointed out, *C. jejuni* accounts for about 95% of human Campylobacter infections [25]. Multidrug resistance has been observed in all Campylobacter isolates in present study. Several investigators have reported the increasing incidence of human *C. jejuni* and *C. coli* infections in many parts of the world for the last decade with higher multidrug resistance [20]. Since *C. jejuni* and *C. coli* demonstrate different susceptibility profiles, it is important to differentiate Campylobacter at the species level, and to provide antimicrobial susceptibility data for each species, in order to monitor better the trend in antimicrobial resistance among Campylobacter isolates and to ensure effective treatment of Campylobacter infections.

In conclusion, our study reveals a high-level correlation between the E-test and agar disk diffusion method in evaluating the resistance of Campylobacter species to tested antimicrobial agents. This study also suggests that while E-test based antimicrobial susceptibility testing has the advantage of providing a quantitative result (MIC), which may be useful for clinicians selecting appropriate treatments, disk diffusion is a reliable and more cost effective technique for determining the prevalence of resistance among Campylobacter isolates.

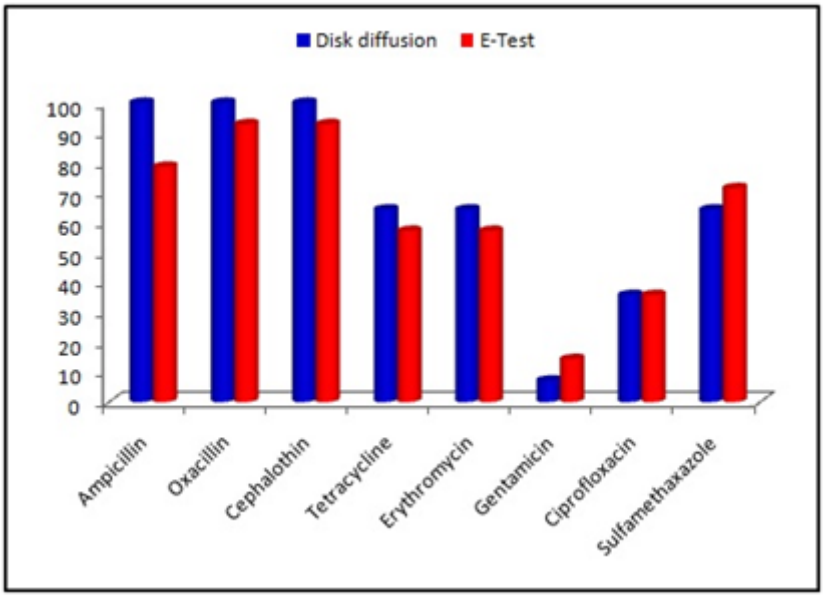

Figure 3. Comparison of the antibiotic resistance patterns of Campylobacters using Disk diffusion and E- test methods

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