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Original Article

Pattern of Antibiotic Susceptibility in *Campylobacter jejuni* Isolates of Human and Poultry Origin

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SUMMARY: *Campylobacter jejuni* antibiotic resistance is rising with a variable geographical pattern; but there is limited data from the Arabian Gulf region. We assessed the sensitivity of human (117) and chicken (33) *C. jejuni* isolates to erythromycin, ciprofloxacin, tetracycline and trimethoprim-sulfamethoxazole by agar dilution, disc diffusion and the E test. Only 2 human isolates were resistant to erythromycin. In contrast, over 80% of chicken and human isolates were resistant to ciprofloxacin. A significantly higher proportion of chicken isolates than human isolates were resistant to tetracycline, with much higher MIC_{50} values ($P < 0.001$). The MIC_{90} for trimethoprim-sulfamethoxazole by agar dilution was 40 μg/ml. Comparison of the results of the agar dilution method and E test showed 1 major disagreement and 8 minor disagreements for erythromycin, 4 major disagreements for ciprofloxacin and 23 disagreements for tetracycline (19 were major disagreements). This was the first study to describe the pattern of antibiotic resistance in *Campylobacter* isolates in this region; the results indicate a high degree of erythromycin sensitivity that validates the continued use of this agent as a first-line therapy for Campylobacter enteritis. These findings have wide implications because of the large, highly mobile expatriate population in this setting. In addition, the correlation between agar dilution and disc diffusion supports the use of the latter as an alternative susceptibility testing method for *Campylobacter*.

INTRODUCTION

Campylobacter is a common cause of human enteritis in both developing and developed countries, with the ingestion of raw or undercooked chicken being an important source of this zoonotic infection (1,2). The spectrum of infection ranges from mild noninflammatory to severe bloody diarrhea, and extra-intestinal manifestations may be seen in immunocompromised patients. *Campylobacter jejuni*, which is the best characterized and the most clinically relevant species in this genus, is sensitive to several classes of antibiotics, including macrolides (especially erythromycin), which have been traditionally utilized as the first-line therapy, and quinolones such as ciprofloxacin (3). However, recent data indicate an upward trend of *C. jejuni* resistance to antibiotics, with varying patterns being seen in different countries and regions (1,3,4). In addition, there is growing concern that the widespread use of antibiotics in animal feeds (particularly in poultry) may select for resistant *Campylobacter* spp., which could then be transmitted to humans through the food chain (3,5). Therefore, from both clinical and epidemiological perspectives, susceptibility testing of *Campylobacter* isolated from humans and poultry with a view to clearly defining the pattern of antimicrobial resistance present in a particular population is crucial.

Several testing methods, such as disc diffusion, broth microdilution, agar dilution and the Epsilometer test (E test), have been used to assess the susceptibility of *Campylobacter* to antimicrobial agents (6-8), but there are no internationally accepted minimum inhibitory concentration (MIC) interpretive criteria or resistance breakpoints. Although the agar dilution method has now been approved by the Clinical and Laboratory Standards Institute (CLSI) as a standard susceptibility testing method for *Campylobacter*, this time-consuming method is rarely carried out in routine diagnostic laboratories (3). The disc diffusion and E test represent alternative methodologies which are easier to apply for routine work, but their usefulness in susceptibility testing for *Campylobacter* is yet to be fully established. In this study, we assess the antimicrobial sensitivity pattern of *C. jejuni* isolated from humans and poultry in the Kingdom of Bahrain, and compare the efficacy of three susceptibility testing methods.

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MATERIALS AND METHODS

Bacterial strains: One hundred and fifty *C. jejuni* isolated between January and December 2004 were tested. One hundred and seventeen were human clinical isolates from the stool of patients with diarrhea seen at the Salmaniya Medical Complex and Bahrain Defence Force Hospital, while 33 isolates were from the stool and liver of chickens obtained from slaughter houses. *Campylobacter* isolates were identified to the species level using routine biochemical tests (production of catalase, hippurate, indoxyl acetate), sensitivity to nalidixic acid and cephalotin, and temperature preferences for growth at 25°C, 37°C and 42°C (9). Isolates were preserved at −80°C in Trypticase soy broth (Becton Dickinson, San Jose, Calif., USA) supplemented with 20% glycerol (v/v) and further subcultures were carried out on chocolate agar (Difco, Surrey, UK) at 42°C for 48 h under microaerobic conditions (Campy*Gen*®; Oxoid, Basingstoke, UK). Antibiotic sensitiv-

ity testing was carried out using Mueller-Hinton agar plates supplemented with 5% sheep blood and incubated at 37°C for 48 h under microaerobic conditions. *Staphylococcus aureus* ATCC 25923 cultured in blood agar was used as a quality control (10).

Antimicrobial agents and susceptibility testing: Four antibiotics, namely, erythromycin (Abbott, Chicago, Ill., USA), ciprofloxacin (Bayer, Leverkusen, Germany), tetracycline (Merck, Darmstadt, Germany) and trimethoprim-sulfamethoxazole (fixed dose combination, 1.25/23.75 μg; Glaxo SmithKline, Ontario, Canada), were tested using the agar dilution method. Sensitivity testing for these four antibiotics was also carried out using the E test (AB Biodisk, Solna, Sweden) and disc diffusion methods. For the agar dilution test, serial twofold dilutions of antimicrobial agents with a concentration range of 0.015 - 62.5 μg/ml were used, except in the case of trimethoprim-sulfamethoxazole, which had a concentration range of 0.078 - 320 μg/ml. For the E test, the concentration range was 0.016 - 256 μg/ml for erythromycin and tetracycline and 0.002 - 32 μg/ml for ciprofloxacin and trimethoprim-sulfamethoxazole. The tentative breakpoints for resistance for both the agar dilution method and E test were: erythromycin, ≥8 μg/ml; ciprofloxacin, ≥4 μg/ml; tetracycline, ≥16 μg/ml; and trimethoprim-sulfamethoxazole, ≥80 μg/ml. For the disc diffusion method the antibiotic disc potency and cut-off zone diameter for resistance were as follows: erythromycin, 15 μg and ≤20 mm (Mast, Merseyside, UK); ciprofloxacin, 5 μg and ≤16 mm (Oxoid); tetracycline, 30 μg and ≤17 mm (Oxoid); and trimethoprim-sulfamethoxazole, 1.25/23.75 μg with no established cut-off zone diameter (BBL, Sparks, Md., USA).

Agar dilution: Agar dilution was carried out according to CLIS-recommended guidelines (11) using Mueller-Hinton agar plates supplemented with 5% sheep blood and containing serial dilutions of each antibiotic. As previously described (10), the inoculum for each bacterial strain was a 1:10 dilution prepared from a bacterial suspension made in Mueller-Hinton broth with a turbidity equivalent to 0.5 McFarland. Plates were inoculated using a multipoint inoculator that delivered spots of 4 μl of the diluted bacterial suspension, and plates without antibiotics were inoculated as viability controls for the bacterial strains.

Disc diffusion: Bacterial suspension in Mueller-Hinton broth (equivalent to 0.5 McFarland turbidity) was inoculated with sterile swabs on 90 mm agar plates. After the inoculates were dry, four antimicrobial discs were applied per plate and incubated in the inverted position.

E test: The agar plates were inoculated as described above for the disc diffusion method. After the surface of the plate was dry, one E test strip was placed on each plate and incubated in the inverted position under appropriate conditions. The MIC was read according to the manufacturer's instructions directly from the strip where the elliptical zone of inhibition intersected with the MIC scale on the E test strip.

Statistical analysis: SPSS statistical package version 12 was used for statistical analysis. Correlations between different tests were determined using Pearson's correlation coefficient, and statistical significance was calculated using Fisher's exact test. For comparison of the E test and agar dilution methods, major disagreement was defined as results differing by more than twofold dilution or a shift from sensitive by one test to resistant by the other test and vice versa (12,13). Minor disagreement was defined as results differing by more than one dilution and resulting in either an unchanged sensitivity/resistance report or a shift from sensitive to intermediate resistance (12).

RESULTS

Agar dilution, the only CLIS-approved methodology for *Campylobacter* antibiotic susceptibility testing, was used as the benchmark for comparison of the E test and disc diffusion methods. By all three methods, none of the chicken isolates and only two (1.7%) of the human isolates showed a high resistance to erythromycin (Table 1). However, one major disagreement and eight minor disagreements between the E test and agar dilution method were identified. The major disagreement was due to an isolate which was reported by the agar dilution method to have an MIC of 7.8 μg/ml for erythromycin but was found to be highly sensitive by the E test, with an MIC of 1.5 μg/ml. All eight minor disagreements were the result of a shift from sensitive to intermediate resistance between tests. Comparison of the disc diffusion and agar dilution methods showed good correlation ($r = 0.631$; $P < 0.001$).

There was a high level of resistance to ciprofloxacin, with all three tests identifying over 80% of chicken and human isolates as resistant to this antibiotic (Table 1). By both the E test and agar dilution methods, over 50% of isolates showed MIC values much greater than the breakpoint for resistance (Table 2). For 4 isolates, the MIC by agar dilution was found to more than twofold higher than the MIC by the E test, and these were counted as major disagreements. These MIC values ranged from 0.122 - 0.97 μg/ml by the agar dilution method and from 0.016 - 0.125 μg/ml by the E test. The correlation between the disc diffusion and agar dilution methods was 0.521 ($P < 0.001$).

A significantly higher proportion of chicken isolates than

Table 1. Proportion of tested strains found to be resistant to antimicrobial agents using three susceptibility testing methods

Antimicrobial agent	Agar dilution[1] *n* (%)		Disc diffusion[1] *n* (%)		E test[1] *n* (%)	
	Human	Chicken	Human	Chicken	Human	Chicken
Erythromycin	2 (1.7)	0	2 (1.7)	0	2 (1.7)	0
Ciprofloxacin	77 (82.8)	29 (87.9)	77 (82.8)	29 (87.9)	77 (82.8)	29 (87.9)
Tetracycline[2]	32 (32.6)	26 (78.8)	32 (32.6)	24 (72.7)	32 (32.6)	24 (72.7)
Trimethoprim-sulfamethoxazole	0	0	NA	NA	NA	NA

[1]: 117 human and 33 chicken *Campylobacter* isolates were tested.
[2]: Significantly higher proportion of chicken isolates were resistant to tetracycline ($P < 0.001$).
NA, not applicable.

Table 2. Comparison of MIC_{50} and MIC_{90} values for agar dilution and E test

Antimicrobial agent	Tentative breakpoint for resistance (μg/ml)	MIC (μg/ml)[1]							
		Agar dilution				E test			
		Human		Chicken		Human		Chicken	
		50%	90%	50%	90%	50%	90%	50%	90%
Erythromycin	≥ 8	1.95	62.5	0.488	3.9	1.0	2.0	0.4	0.2
Ciprofloxacin	≥ 4	31.25	62.5	7.8	62.5	32	>32	32	>32
Tetracycline	≥ 16	1.0	62.5	16.0	62.5	0.032	256	24	256
Trimethoprim-sulfamethoxazole	≥ 80	10	40	20	40	NA[2]		NA[2]	

[1]: MIC_{50} and MIC_{90} values indicate the MIC at which 50% and 10%, respectively, of the strains were inhibited by the antibiotic.
[2]: The concentration range of test strip used was lower than the tentative breakpoint.
NA, not applicable.

human isolates was determined to be tetracycline resistant by all three tests ($P < 0.001$) (Table 1). Furthermore, for chicken isolates the MIC_{50} values by agar dilution were much higher than the MIC_{50} values detected for human isolates (16.0 μg/ml versus 1.0 μg/ml). A similar pattern was observed by the E test (Table 2). Comparison of the two assays showed 23 disagreements, with 19 of these being major disagreements. For the human isolates, the major disagreement was due to a greater than twofold increment of MIC seen with the agar dilution test (MIC, 0.122 - 1.95 μg/ml) compared to the E test (MIC, 0.016 - 0.25 μg/ml). For the chicken isolates, the major disagreement was due to 2 isolates demonstrating resistance when tested by agar dilution but showing sensitivity by the E test (Table 1). The MIC values for these 2 isolates were 62.5 μg/ml by agar dilution versus 12 μg/ml and 8 μg/ml by the E test. Interestingly, these same isolates also proved sensitive by disc diffusion. Analysis of the disc diffusion and agar dilution methods for this antibiotic showed a significantly high level of correlation ($r = 0.827$; $P < 0.001$).

The MIC_{90} for trimethoprim-sulfamethoxazole as determined using the agar dilution method was 40 μg/ml. Susceptibility testing using the disc diffusion method could not be assessed, as the maximum disc potency of 25 μg/ml used was insufficient for susceptibility testing of *Campylobacter* isolates. There were difficulties in assessing the end point for antibiotic susceptibility using the E test, as the concentration range of the test strip used was lower than the tentative breakpoint. Several variations of the reading patterns were observed. These included the growth of micro-colonies inside the inhibition zone, resistant isolates showing homogenous growth around the test strip, the presence of diffuse colony edges, and the growth of colony subpopulations within the inhibition zone.

Multidrug resistance was observed with some isolates. There were 32 human isolates and 6 chicken isolates that were resistant to both ciprofloxacin and tetracycline. Only one isolate (human origin) was resistant to tetracycline, ciprofloxacin and erythromycin.

DISCUSSION

Although the occurrence of *Campylobacter* as a cause of diarrhea in the Arabian Gulf region has been shown to range from 1.6 to 28% (9,14,15), there is limited data on the pattern and extent of antibiotic resistance in *C. jejuni* isolates found in the region (16). Erythromycin is recommended for the treatment of Campylobacter enteritis, and the low resistance demonstrated here implies its continued usefulness as a first-line drug in our setting. However, proper dosing is essential because exposure to subinhibitory concentrations of this antibiotic could potentially enhance the toxigenic effect of *Campylobacter* cytolethal distending toxin (17).

The very high level of ciprofloxacin resistance (>80%) seen in this study is similar to reports from other countries (1). Available reports show that in different geographical areas, there has been a consistent increase in *Campylobacter* antibiotic resistance over the years (1) and the resistance patterns seen are influenced by various factors, possibly including pressure exerted by antimicrobial use. As stated in various reports, including a WHO document (5,18), introduction of fluoroquinolones for use in veterinary practice has been associated with a dramatic rise in *Campylobacter* strains showing resistance to these antibiotics. However, the issue of human-cross infections with *Campylobacter* remains debatable (19,20). The number of ciprofloxacin pills dispensed annually for human clinical use showed a 58% increase between 1999 and 2001 (unpublished data, Ministry of Health, Kingdom of Bahrain). This might be a contributory factor in explaining the very high level of ciprofloxacin resistance we have demonstrated in isolates of human and animal sources.

In contrast, resistance to tetracycline (also widely used for treatment or prophylaxis in animals reared for food) was significantly higher in chicken isolates compared to human isolates. This pattern is the consequence of the decreased popularity of tetracyclines among prescribing clinicians together with their persistent use in animals. Alternatively, although we can speculate that the isolates circulating in the two populations are different, validation of the clonal independence of the isolates was not carried out in this study. Further work using DNA fingerprinting techniques with a larger number of chicken and human isolates is therefore suggested.

We tested for trimethoprim-sulfamethoxazole because of its applicability to the treatment of enteric pathogens, and the finding of high sensitivity to this antibiotic was in contrast to other reports (4). Due to the prevalence of sickle cell disease and G6PD deficiency in our setting, judicious clinical use of trimethoprim-sulfamethoxazole is the norm. This practice might have reduced the selective pressure for bacterial resistance to this antibiotic. Indeed, we have shown a decrease in the resistance of *Shigella flexneri* to trimethoprim-sulfamethoxazole from 83.4% in 1994 to 54.7% in 2000 (21).

Our findings have demonstrated a good correlation between the disc diffusion and agar dilution methods for susceptibility testing of erythromycin, ciprofloxacin and tetracycline. This is in keeping with previous reports describing disc diffusion

as a reliable, easy and inexpensive method for use in diagnostic laboratories (6). However, this method could not be applied to trimethoprim-sulfamethoxazole, as the available disc potencies were below the tentative breakpoint for *Campylobacter* resistance.

The E test is a simple method with the added advantage of flexibility when testing the MIC of a few isolates against several antibiotics. Our findings demonstrated several major disagreements between the E test and the agar dilution method. These included a shift from a resistant profile by agar dilution to a sensitive profile for the same strain by E test. This shift is significant, as the ability to identify a resistant strain is crucial, and incorrect classification of the bacterial susceptibility profile can have serious clinical implications. Also, in a number of strains the MIC values by the agar dilution were more than twofold greater than those from the E test, leading to major disagreements. These findings are probably a reflection of the fact that the E test has not yet been standardized for *Campylobacter*, and they indicate the need for more rigorous testing.

Overall, this study provides insight into the pattern of *Campylobacter* antibiotic resistance and the continued usefulness of erythromycin as a first-line therapy for Campylobacter enteritis in our setting. The findings also have a wider implication in the context of the global epidemiology of *Campylobacter* antibiotic resistance patterns, particularly because of the large highly mobile expatriate population in the country. In addition, as both imported and locally reared poultry is widely consumed in our setting, there remains the potential for the transfer of resistant *Campylobacter* isolates to humans via the food chain, and thus constant surveillance of the susceptibility patterns of isolates of both human and chicken origin is recommended.

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