Comparison of methods for the determination of antimicrobial resistance in *Campylobacter* spp. human and the food chain isolates

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ABSTRACT: With a microdilution method, using the commercial diagnostic test Sensititre Susceptibility Plates for Campylobacter MIC (Trek Diagnostic Systems, Cleveland, OH, USA), disk diffusion and agar dilution method, resistance to six antimicrobial agents were examined in a reference strain *Campylobacter jejuni* ATCC 33560 and 73 thermo-tolerant isolates of *Campylobacter* spp. For the microdilution method and all tested antimicrobial agents, our determined values of microbiological breakpoints of resistant strains were suggested as the minimum inhibitory concentration (MIC_R) for ciprofloxacin ≥ 0.5, erythromycin ≥ 4, gentamicin ≥ 4, nalidixic acid ≥ 32 and tetracycline ≥ 4 μg/ml. On the basis of our study results, strains resistant to clindamycin were MIC_R ≥ 2 μg/ml for the dilution methods and a zone $diameter_R$ ≤ 16 mm for the disk diffusion method. Comparison of the results of the resistance examination, a microdilution method and disk diffusion method with the reference agar dilution method, showed that all compared methods yielded identical results with the exception of the resistance determination in erythromycin and nalidixic acid. The errors were mostly the result of the interpretation criteria for MIC_R of agar dilution method and different conditions of cultivation used. However, the compared methods, provide results comparable with the reference method having greater convenience of measurement.

Keywords: dilution methods; disk diffusion method; resistance interpretation; clindamycin

Thermo-tolerant campylobacters, especially *Campylobacter jejuni*, belong among the most frequent etiological agents of food-borne diseases (Allos, 2001), the number of which has been increasing recently worldwide (EFSA, 2006). For example, in the Czech Republic, the number of reported cases approach the number of salmonella cases (Epidat, 2006).

The disorder usually recedes without antimicrobial therapy, however in more serious cases treatment is necessary (McDermott et al., 2005). The drug of choice is a macrolide (e.g., erythromycin) for the treatment of enteric campylobacter infections after microbiological diagnosis. However, for the empiric treatment of adults with suspected bacterial gastroenteritis, the drug of choice typically includes a fluoroquinolone (e.g., ciprofloxacin) because of their activity against almost all enteric bacterial pathogens (Allos, 2001; Engberg et al., 2004).

However, it has been shown that in the course of previous years there have been selected strains of *Campylobacter* spp. resistant to antimicrobial agents, especially to fluoroquinolones (Thakur and Gebreyes, 2005; Larkin et al., 2006). Antimicrobial drug resistance in campylobacter infections, in particular to quinolones, has increased dramatically in many countries (Engberg et al., 2001).

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Due to this, the need has developed for a standardised and fast method for determining resistance in this microorganism. The primary methods were agar dilution and disk diffusion for which the interpretation criteria of resistance for selected antimicrobial agents were determined (Communique, 2005). However, interpretation criteria for the evaluation of campylobacter resistance are a permanent problem, they are not unified in the standards and this problem is mentioned in CLSI (2006a).

The objective of this study was to assess the accuracy of three tests for determination of resistance to selected antimicrobial drugs: (1) the agar dilution method as reference, (2) the disk diffusion method, prepared according to the standard procedure in the laboratory and (3) microdilution method using the diagnostic test Sensititre Susceptibility Plates for Campylobacter MIC (Campylobacter MIC plate; Trek Diagnostic Systems, Cleveland, OH, USA). Interpretation criteria for the microdilution method were established and evaluated. Interpretation criteria for evaluation of the resistance to clindamycin for all three methods were suggested.

MATERIAL AND METHODS

Isolates

Isolates of *C. jejuni* and *C. coli* from humans and from food of animal origin tested in the study (73 isolates) were collected in 1995–2005. They were stored in Cooked Meat Medium (Hi-media, Mumbai, India) with 20% glycerol at –80°C. Prior to examination of resistance the isolates were resuscitated on blood agar with 5% defibrinated sheep's blood at 42°C under microaerophile atmosphere (5–10% O_2) for a period of 48 hours.

Antimicrobial susceptibility testing

The antimicrobial agents tested included ciprofloxacin (CIP), clindamycin (CLI), erythromycin (ERY), gentamicin (GEN), nalidixic acid (NAL) and tetracycline (TET) (Sigma-Aldrich, St. Louis, Missouri, USA). They were listed in the panel of agents for the examination of resistance of *Campylobacte*r spp. isolates (monitored in accordance with Article 7 of Directive 2003/99/EC EU) and they were also a part of the diagnostic test Campylobacter MIC plate. Diagnostics for the disk diffusion method (disk method) and agar dilution method (reference method) were prepared according to the standards (CLSI, 2006b). In the case of microdilution method (micromethod) a commercial diagnostic test Campylobacter MIC plate was used.

Dilution methods

For determination of the minimum inhibitory concentration (MIC) with the reference method, petri dishes with Mueller-Hinton agar (Oxoid, Basingstoke, UK) with 5% defibrinated sheep's blood supplemented with tested antimicrobial agents with the following lines of doubled dilution: CIP: 0.125–2 µg/ml, CLI: 0.125–4 µg/ml, ERY: 0.25–8 µg/ml, GEN: 0.25–8 µg/ml, NAL: 4–32 µg/ml, and TET: 0.25–8 µg/ml were used. The tested cultures were suspended in phosphate buffered saline and adjusted to an optical density McFarland standard of 0.5 and 2 µl of the suspension were inoculated onto the agar surfaces.

In a case of the microdilution method, the diagnostic test Campylobacter MIC plate was used. The procedure was conducted according to the instructions for use suggested by the producer of the diagnostics (Anonymous, 2004). Shortly: the same suspensions of tested cultures were diluted 100 times with Mueller-Hinton broth (Trek Diagnostics Systems) using 5% lysed horse's blood (Oxoid). The microplates with lines of double diluted and lyophilised antimicrobial agents were inoculated with 100 µl of the suspension with the desired inoculum concentration of 5.0×10^5 CFU/ml.

Both the petri dishes and microplates were incubated under microaerophile atmosphere at 37°C. MIC values were read after two days of incubation. The reference strain *C. jejuni* ATCC 33560 was used for the protocol quality control.

Disk diffusion method

To determine sensitivity with the disk method, petri dishes were used with Mueller-Hinton agar with 5% sheep's blood and disks with the given quantity of antimicrobial agents (Oxoid): CIP 5 µg, CLI 2 µg, ERY 15 µg, GEN 10 µg, NAL 30 µg and TET 30 µg. Above mentioned suspensions (adjusted to an optical density according to McFarland stan-

dard 0.5) were used for inoculation. Cultivation took place at 37°C for a period of 48 hours under microaerophile atmosphere. The diameters of the zones were measured with an accuracy of 1 mm.

Evaluation

The MIC_{90} and MIC_{50} values were calculated as the 90th and 50th percentile of the minimum inhibitory concentration values of the respective antimicrobial agents. The value of MIC_{mode} represents the most frequently recorded value of MIC for the appropriate antimicrobial agent.

Resistance to antimicrobial agents was interpreted for the reference and disk method based on our modified criteria provided in the standard Communique (2005) by including intermediate resistant categories to the resistant categories. This interpretation criterion, as well as other criteria used, are provided in Table 1.

RESULTS

The values of MIC for reference strain *C. jejuni* ATCC 33560 observed by dilution methods corresponded with the values provided by standard (CLSI, 2006a). For the disk method there were no required values for the reference strain provided in any of the available standards, but it was always possible to evaluate the strain, according to Communique (2005), as sensitive.

MIC_{mode}, MIC_{50} and MIC_{90} for individual antimicrobial agents determined with both dilution methods are provided in Table 2. In the examined group of 73 isolates resistant, by more than 10% (in MIC_{90}) or individually, were observed to all examined antimicrobial agents with both dilution methods. The values of MIC_{mode} and MIC_{50} obtained with the micromethod are lower than the values obtained with the reference method, in most cases they are within the range of approved tolerance of ± 1 dilution. The exception is the results of determining resistance to NAL by the micromethod when a minor error was recorded in 13.7% isolates (Table 3). The isolates were determined as resistant and thereby MIC_{mode} and MIC_{50} of this method achieved the category of resistant isolates.

Our determined values of the microbiological breakpoints for resistant isolates MIC_R (μg/ml): CIP ≥ 0.5; CLI ≥ 2; ERY ≥ 4; GEN ≥ 4; NAL ≥ 32; TET ≥ 4 were suggested for interpretation as results determined by the micromethod (Table 1). The values of microbiological breakpoints for resistant isolates to CLI were determined on the base analysis of dot plot of the MIC values and corresponding diameter zones: for the agar dilution method, MIC_R ≥ 2 μg/ml, for the disk method, zone $diameter_R$ ≤ 16 mm (Figure 1).

The difference in isolate classification as sensitive/resistant is based on the results of the methods used and the mentioned interpretation criteria, provided in Table 3. The greatest differences in the classification of isolates were observed for ERY and NAL. By a micromethod it was determined that 20.6%

Table 1. The interpretation criteria for resistance assessment by three compared methods in terms of minimum inhibitory concentration (MIC) and diameter of inhibition zones (mm) used in the study for classification of *Campylobacter* spp. isolates

Agent	Criteria for classification of isolates as sensitive/resistant for compared methods		
	MIC[a] (μg/ml)	MIC[b] (μg/ml)	diameter of inhibition zones[c] (mm)
Ciprofloxacin	≤ 0.5/≥ 1	≤ 0.25/≥ 0.5	≥ 25/≤ 22
Clindamycin	≤ 1/≥ 2	≤ 1/≥ 2	≥ 20/≤ 16
Erythromycin	≤ 2/≥ 4	≤ 2/≥ 4	≥ 22/≤ 17
Gentamicin	≤ 2/≥ 4	≤ 2/≥ 4	≥ 18/≤ 16
Nalidixic acid	≤ 8/≥ 16	≤ 16/≥ 32	≥ 20/≤ 15
Tetracycline	≤ 4/≥ 8	≤ 2/≥ 4	≥ 19/≤ 17

[a]determination by the agar dilution method
[b]determination by the microdilution method
[c]determination by the disk diffusion method

Table 2. Minimum inhibitory concentration (μg/ml) characteristics (MIC_{mode}, MIC_{90} and MIC_{50}) of antimicrobial agents for two dilution methods obtained on the bases of the examination of resistance in 73 field *Campylobacter* spp. isolates

	Agar dilution method						Microdilution method					
	CIP	CLI	ERY	GEN	NAL	TET	CIP	CLI	ERY	GEN	NAL	TET
MIC_{mode}	≤ 0.12	0.25	2	≤ 0.25	≤ 4	≤ 0.25	0.06	0.12	0.5	≤ 0.12	> 64	≤ 0.06
MIC_{50}	0.25	0.25	2	≤ 0.25	≤ 4	≤ 0.25	0.12	0.12	0. 5	≤ 0.12	64	0.25
MIC_{90}	> 2	2	8	1	> 32	> 8	8	2	2	0.5	> 64	> 64

CIP = ciprofloxacin; CLI = clindamycin; ERY = erythromycin; GEN = gentamicin; NAL = nalidixic acid; TET = tetracycline

Table 3. Analysis of accordance (%) of compared methods with reference agar dilution method in classification of 73 *Campylobacter* spp. isolates as resistant (R) or sensitive (S) including expression of the error of analysis

	Microdilution method						Disk diffusion method					
	CIP	CLI	ERY	GEN	NAL	TET	CIP	CLI	ERY	GEN	NAL	TET
Accordance (R)	39.7	13.7	8.2	5.5	38.4	17.8	37.0	15.1	11.0	5.5	35.6	16.4
Accordance (S)	54.8	80.9	69.9	93.2	42.5	76.7	57.5	76.7	69.9	91.8	48.0	75.3
Major Error	1.4	4.1	20.6	1.4	5.5	2.7	4.1	2.7	17.8	1.4	8.2	4.1
Minor Error	4.1	1.4	1.4	0.0	13.7	2.7	1.4	5.5	1.4	1.4	8.2	4.1

CIP = ciprofloxacin; CLI = clindamycin; ERY = erythromycin; GEN = gentamicin; NAL = nalidixic acid; TET = tetracycline

of the isolates were false sensitive and 1.4% were false resistant to ERY. For NAL, 5.5% of the isolates were determined as false sensitive and 13.7% of the isolates as false resistant. Based on the results of the disk method there were 17.8% of the isolates categorised as false sensitive to ERY and 1.4% as false resistant. Minor and major errors for NAL were observed consistently in 8.2% of the isolates.

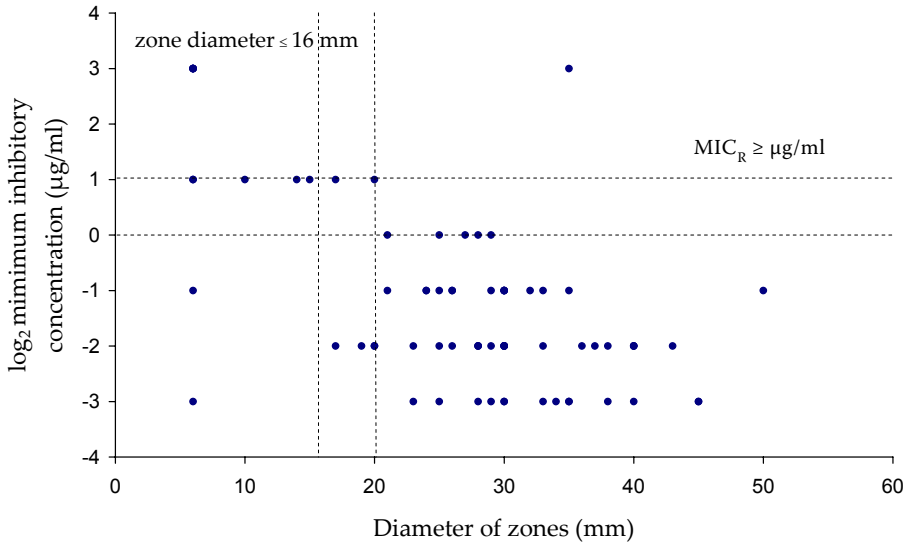

Figure 1. Dot plot of the MIC values and corresponding diameter zones used to determine microbiological breakpoints for campylobacter resistant to clindamycin (MIC_R) for agar dilution method and disk diffusion method

DISCUSSION

Diagnostics were tested on *C. jejuni* and *C. coli* isolates from humans and food of animal origin before implementation within a standard system of antimicrobial resistance monitoring. Interpretation of the assay of resistance with an agar dilution reference method was performed according to Communique (2005) with a modification when an intermediate category of resistance was included in the resistant category. The limit for sensitive isolates to ERY was changed here in comparison with Communique (2005), from $MIC_S \leq 1$ μg/ml to $MIC_S \leq 2$ μg/ml. The change complies with the values for a sensitive reference strain listed in CLSI (2006a). Criteria for the categorisation of resistant isolates by agar dilution method in the work by Hakanen et al. (2003) are different from our suggested criteria for ERY and CLI. If we used the criteria mentioned by those authors, all isolates would be detected as sensitive with this method, although other methods determined them as resistant.

For CLI, there were no interpretation criteria in the standards suggested by Communique (2005) for the agar dilution method provided. Therefore, our determined criteria was suggested on the basis of results by McDermott et al. (2005) and results from the agar and disk method in this study, this means: for the agar dilution method MIC_R for CLI ≥ 2 μg/ml. We thereby minimised a major error and maintained the categorisation of the reference strain as sensitive.

During the determination of interpretation criteria for the micromethod, we considered the work of McDermott et al. (2005), when the determined MIC values of the sensitive reference strain *C. jejuni* ATCC 33560 were the result of an assay of MIC repeated 300 times. Testing was performed with commercially prepared frozen panels (Trek Diagnostics Systems). The data were generated in a multi-laboratory study. On the basis of these results, one dilution lower than the maximum approved values of MIC for a sensitive reference strain we used strictly as a microbiological breakpoint for the categorisation of isolates as resistant, including resistant to CLI.

Interpretation of assay resistance with the disk method was performed according to Communique (2005), when an intermediate category of resistance was included in the resistant category. The microbiological breakpoint for the disk method in the case of CLI was determined by the error-rate bounded method (Metzler and DeHaan, 1974). Our newly determined microbiological breakpoint for resistant strains in this method is the zone with a $diameter_R \leq 16$ mm.

The comparison of results of the resistance examination using the micromethod and disk method with the agar dilution reference method shows that all the compared methods yielded the same results, with the exception of the resistance determination in ERY and NAL. These differences were manifested as major and minor errors during the determination of resistance, particularly in the micromethod. We suppose that the cause may be different cultivation conditions, which is different growth on the agar surface and growth in the liquid medium. We could verify this on "home made" microplates with brain heart infusion (BHI). Prolongation of the period of cultivation from 48 hours to 72–96 hours resulted in a shift to higher values of MIC (data not shown). Prolongation of the period of cultivation in the micromethod would probably result in a decline of major errors, especially in ERY.

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