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Inaccuracy of routine susceptibility tests for detection of erythromycin resistance of *Campylobacter jejuni* and *Campylobacter coli*

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Abstract

In The Netherlands, both an increase in and regional differences in erythromycin resistance of *Campylobacter jejuni* and *Campylobacter coli* have been reported. To determine the accuracy of routine tests for erythromycin resistance, 48 erythromycin-resistant isolates from various laboratories that participate in the Dutch surveillance of *Campylobacter* infections were reinvestigated. Initial susceptibility testing for erythromycin had been performed by disk diffusion in six and MIC-based methods in two laboratories. Reinvestigation was carried out using broth microdilution as a reference standard, as well as *E*-test and genetic resistance testing. Of 36 *C. jejuni* isolates reported by the initial laboratories as erythromycin-resistant, four (11%) and five (14%) were confirmed as erythromycin-resistant using broth microdilution according to CLSI and EUCAST resistance criteria, respectively. Erythromycin resistance was found in eight of 12 (67%) *C. coli* isolates according to both criteria. Results of *E*-tests were in accordance with these results in all isolates. Resistance-associated mutations in the 23S rRNA gene (A2059G and A2058T) were found in all isolates showing high-level resistance, whereas none were found in susceptible isolates. Routine determination of the erythromycin resistance of *C. jejuni* and *C. coli* shows unacceptable interlaboratory variation. In the absence of standardized protocols and interpretive criteria for disk diffusion, and while we await the development of easily applicable and reliable methods for molecular resistance testing, the use of broth microdilution remains the best method.

Keywords: 23S rRNA gene, *Campylobacter coli*, *Campylobacter jejuni*, erythromycin resistance, susceptibility testing
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Introduction

Campylobacter contributes to considerable childhood morbidity in developing countries and is one of the most common causes of bacterial gastroenteritis in industrialized countries [1,2]. Additionally, travellers' diarrhoea is often caused by *Campylobacter* species [3,4]. In industrialized countries the most frequently encountered *Campylobacter* species isolated from diarrhoeal stools are *Campylobacter jejuni* and *Campylobacter coli*, which account for > 98% of laboratory-confirmed infections [4,5]. *Campylobacter* infections are usually self-limiting, but early treatment with erythromycin has been shown to shorten the duration of diarrhoeal illness in children [6]. In cases of severe or persistent illness and in immunocompromised patients, *Campylobacter* infections can cause substantial morbidity for which rapid and effective antibiotic treatment is required. The treatment of choice is a macrolide antibiotic, such as erythromycin, as fluoroquinolone resistance has rapidly increased during the last decade [5].

In *Campylobacter*, erythromycin resistance is mainly caused by alterations of the erythromycin binding site in the 23S rRNA unit of the ribosome, similar to those observed in *Helicobacter* isolates [7]. Other mechanisms of resistance, such as alterations of efflux pumps, can also be found in *Campylobacter* isolates and have recently been reported to act in synergy with the 23S rRNA mutation to confer high-level erythromycin resistance [8,9]. In the Netherlands and in most industrialized countries, resistance rates for erythro-

mycin in *Campylobacter* spp. have remained stable at relatively low levels, whereas higher resistance rates are reported in southern Europe and some countries in Asia [10]. Infections with erythromycin-resistant isolates have been reported in association with a five-fold increased risk of invasive illness or death in a Danish study [11].

A recent report on *Campylobacter* laboratory surveillance in the Netherlands indicated that erythromycin resistance levels among *C. jejuni* and *C. coli* apparently increased from stable levels of 1.5% and 2.0%, respectively, in 2002, to 3.9% and 6.3%, respectively, in 2003 [4,12]. Furthermore, regional differences in the prevalence of erythromycin resistance were reported [13]. A national survey demonstrated that disk diffusion was the test most frequently applied to determine *Campylobacter* resistance in surveillance laboratories, but results were not confirmed by MIC-based methods (van der Beek *et al.*, submitted for publication). Therefore, we hypothesized that the apparent increase in erythromycin resistance and the regional differences in resistance levels merely reflected inaccurate resistance testing. To study this, erythromycin-resistant *C. jejuni* and *C. coli* strains from a 2002–2003 case-control study nested in the routine laboratory surveillance of *Campylobacter* were reinvestigated using MIC-based methods and molecular resistance testing.

Materials and Methods

Campylobacter isolates

All *Campylobacter* isolates included in the present study were derived from a case-control study carried out in 2002–2003 (CaSa Study) [14,15] and from routine laboratory surveillance of *Campylobacter* and *Salmonella*. In total, 18 regional public health laboratories collected 3169 *Campylobacter* isolates. The participating laboratories performed species identification and susceptibility testing according to local procedures. Confirmation of species identification was performed by PCR-restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA and 23S rRNA genes at a central laboratory [16,17].

Among 2823 isolates tested for erythromycin susceptibility, 112 (4%) were reported to be resistant [12]. Among all resistant isolates, a selection of 48 *C. jejuni* and *C. coli* isolates from 12 laboratories were available for further analysis at the Leiden University Medical Centre (LUMC). In the LUMC, susceptibility for erythromycin was reinvestigated using broth microdilution and *E*-test. An erythromycin-resistant *C. jejuni* isolate (MIC value > 256 mg/L) that had been isolated from a diarrheic stool sample at the Clinical Microbiology Laboratory at the LUMC (isolate 6534) was included as a resistant control isolate. *C. jejuni* ATCC 33560 was included as an erythromycin-susceptible control isolate.

In vitro susceptibility tests

Broth microdilution was performed using custom-made microtitre plates from Trek Diagnostic Systems (East Grinstead, UK) according to CLSI guideline M45-A [18]. The two-fold dilution range of erythromycin concentrations in the plates was 0.5–64 mg/L. Briefly, colonies from 24–48-h cultures were suspended in PBS 0.9% to a turbidity of 0.5 McF and subsequently diluted 200 times in cation-adjusted Mueller–Hinton broth with 2–2.5% lysed horse blood. Fifty microlitre of this suspension was added to 96-well microtitre plates containing antibiotics. Plates were incubated in a micro-aerobic atmosphere at 37 °C. MIC values were determined after 48 h of incubation.

All isolates were also tested for susceptibility to erythromycin using *E*-test (AB Bomérieux, Solna, Sweden). Colonies from 24–48-h cultures were suspended in PBS 0.9% to a turbidity of 0.5 McF. This suspension was plated on a Mueller–Hinton agar plate with 5% sheep blood (Biomérieux BV, Boxtel, the Netherlands) and an *E*-test strip was put on each plate after the surface of the plate had dried. Plates were incubated in a micro-aerobic atmosphere at 37 °C. MIC values were determined after 48 h of incubation.

Classification of isolates as resistant (*R*) was based on MIC values after 48 h of incubation using criteria from the CLSI (*R*: MIC ≥ 32 mg/L) and EUCAST (*R*: MIC > 4 mg/L for *C. jejuni* and wildtype ≤ 16 mg/L for *C. coli*; http://www.srga.org/eucastwt/MICTAB/index.html) [18].

Erythromycin resistance testing by 23S rRNA sequencing

Detection of single nucleotide polymorphisms in the 23S rRNA sequence was performed by sequence analysis of the nucleotides 1909–2257 (position of nucleotides reported as the *E. coli* equivalents) in the 23S rRNA as previously described [19]. The 23S rRNA fragment was amplified using primers Ar69fw and Ery23sr (Ar69fw: GTAACTATAACGGTCCTAAG nt. 1909–1928; Ery23sr: GACCGCCCCAGTCAAACT nt. 2257–2227). Cycle sequencing reactions were performed using the same primers and an ABI Prism 3100 Genetic Analyser (Applied Biosystem, Inc. Foster City, CA, USA).

Results

Using broth microdilution and applying CLSI criteria for resistance, four of the 36 (11%) *C. jejuni* isolates previously reported as erythromycin-resistant were found to be erythromycin-resistant (Table 1). According to EUCAST

criteria, five of the 36 (14%) isolates were erythromycin-resistant. The isolate that was classified differently by CLSI and EUCAST criteria (isolate 1306) had an MIC value of 16 mg/L and was classified as intermediate-susceptible according to CLSI criteria and resistant according to EUCAST criteria. Of the 12 *C. coli* isolates reported to be resistant, eight (67%) were resistant according to both CLSI and EUCAST criteria using broth microdilution (Table 1).

Using *E*-test, CLSI criteria revealed four (11%) and EUCAST criteria revealed five (14%) of the 36 *C. jejuni* isolates to be erythromycin-resistant (Table 1). The isolate that was classified differently by CLSI and EUCAST criteria had an MIC value of 12 mg/L and was classified as intermediate-susceptible according to CLSI and resistant according to EUCAST. Of the 12 *C. coli* isolates reported to be resistant, eight (67%) were resistant according to CLSI and EUCAST criteria using *E*-test (Table 1).

The MIC values as determined by *E*-test differed on average two-fold from the MIC values established by broth microdilution, but the classification as resistant was identical using both methods (Table 1). MIC values determined after 24 h of incubation of the *E*-test resulted in 1.5- to two-fold lower MIC values in 12 isolates (25%) compared with results after 48 h of incubation (data not shown).

TABLE 1. Results of MIC-based erythromycin susceptibility tests for 50 *Campylobacter* isolates

Sample	Species	Broth microdilution (MIC, mg/L)	Interpretation according to CLSI[a]	Interpretation according to EUCAST[b]	*E*-test (MIC, mg/L)	Interpretation according to CLSI[a]	Interpretation according to EUCAST[b]
1095	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
1122	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
1132	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
1141	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
1142	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
1219	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
2824	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
5065	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
5689	*C. jejuni*	≤ 0.50	S	S	0.50	S	S
1109	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
1145	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
1147	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
1292	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
2195	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
2205	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
2274	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
2892	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
4215	*C. jejuni*	≤ 0.50	S	S	0.75	S	S
1143	*C. jejuni*	≤ 0.50	S	S	1.0	S	S
1174	*C. jejuni*	≤ 0.50	S	S	1.0	S	S
5044	*C. jejuni*	≤ 0.50	S	S	1.0	S	S
5129	*C. jejuni*	≤ 0.50	S	S	1.0	S	S
1221	*C. jejuni*	≤ 0.50	S	S	1.5	S	S
1768	*C. jejuni*	1.0	S	S	0.50	S	S
1144	*C. jejuni*	1.0	S	S	0.75	S	S
0998	*C. jejuni*	1.0	S	S	1.0	S	S
3553	*C. jejuni*	1.0	S	S	1.0	S	S
1043	*C. jejuni*	1.0	S	S	1.5	S	S
1146	*C. jejuni*	1.0	S	S	1.5	S	S
1031	*C. jejuni*	2.0	S	S	1.5	S	S
5052	*C. jejuni*	2.0	S	S	4.0	S	S
1306	*C. jejuni*	16.0	I	R	12.0	I	R
2704	*C. jejuni*	> 64	R	R	> 256	R	R
3715	*C. jejuni*	> 64	R	R	> 256	R	R
4222	*C. jejuni*	> 64	R	R	> 256	R	R
4713	*C. jejuni*	> 64	R	R	> 256	R	R
3576	*C. coli*	2.0	S	S	2.0	S	S
1190	*C. coli*	2.0	S	S	3.0	S	S
1034	*C. coli*	2.0	S	S	4.0	S	S
5131	*C. coli*	4.0	S	S	6.0	S	S
0328	*C. coli*	> 64	R	R	256	R	R
0878	*C. coli*	> 64	R	R	256	R	R
3629	*C. coli*	> 64	R	R	256	R	R
4188	*C. coli*	> 64	R	R	> 256	R	R
4312	*C. coli*	> 64	R	R	> 256	R	R
5127	*C. coli*	> 64	R	R	> 256	R	R
5675	*C. coli*	> 64	R	R	> 256	R	R
5708	*C. coli*	> 64	R	R	> 256	R	R
ATCC 33560	*C. jejuni*	1.0	S	S	1.0	S	S
Leiden 6534	*C. jejuni*	> 64	R	R	> 256	R	R

S, susceptible; I, intermediate-susceptible; R, resistant.
[a]CLSI breakpoints for *C. jejuni* and *C. coli*: S ≤ 8 mg/L, R ≥ 32 mg/L.
[b]EUCAST breakpoints for *C. jejuni*: S ≤ 4 mg/L, R > 4 mg/L; for *C. coli* only wild-type distribution given: wild-type ≤ 16 mg/L.

TABLE 2. Results of phenotypical and genotypical susceptibility tests for 28 selected *Campylobacter* isolates

Sample	Species	Broth dilution microdilution (MIC: mg/L)	*E*-test (MIC: mg/L)	Genotype (nt 2058–2059)[a]
5689	*C. jejuni*	≤ 0.50	0.50	AA
2274	*C. jejuni*	≤ 0.50	0.75	AA
2195	*C. jejuni*	≤ 0.50	0.75	AA
5129	*C. jejuni*	≤ 0.50	1.0	AA
5044	*C. jejuni*	≤ 0.50	1.0	AA
1768	*C. jejuni*	1.0	0.50	AA
0998	*C. jejuni*	1.0	1.0	AA
3553	*C. jejuni*	1.0	1.0	AA
1043	*C. jejuni*	1.0	1.5	AA
1031	*C. jejuni*	2.0	1.5	AA
5052	*C. jejuni*	2.0	4.0	AA
1306	*C. jejuni*	16.0	12.0	AA
4713	*C. jejuni*	> 64	> 256	AG
4222	*C. jejuni*	> 64	> 256	AG
3715	*C. jejuni*	> 64	> 256	AG
2704	*C. jejuni*	> 64	> 256	TA
3576	*C. coli*	2.0	2.0	AA
1190	*C. coli*	2.0	3.0	AA
1034	*C. coli*	2.0	4.0	AA
5131	*C. coli*	4.0	6.0	AA
3629	*C. coli*	> 64	256	AG
0878	*C. coli*	> 64	256	AG
0328	*C. coli*	> 64	256	AG
5708	*C. coli*	> 64	> 256	AG
5675	*C. coli*	> 64	> 256	AG
5127	*C. coli*	> 64	> 256	AG
4312	*C. coli*	> 64	> 256	AG
4188	*C. coli*	> 64	> 256	AG
ATCC 33560	*C. jejuni*	1.0	1.0	AA
Leiden 6534	*C. jejuni*	> 64	> 256	TA

[a]AA, wild-type; A2059G, mutant; A2058T, mutant.

To determine the presence of resistance-associated mutations, 23S rRNA genotyping was performed on a subset of 28 isolates comprising all the different MIC values found among the isolates upon retesting (Table 2). Nucleotide sequence analysis showed the wild-type genotype (AA at nucleotides 2058 and 2059) in all isolates that were susceptible in broth microdilution (n = 16) and in one *C. jejuni* isolate with an MIC value of 16 mg/L (intermediate-susceptible according to CLSI criteria and resistant according to EUCAST criteria).

An A2059G mutation was found in all resistant *C. coli* isolates (n = 8) and in three resistant *C. jejuni* isolates (all MIC > 64 mg/L). In one resistant *C. jejuni* isolate and in our resistant clinical control isolate (*C. jejuni* Leiden 6534) an A2058T mutation was found (both MIC > 64 mg/L).

Discussion

In this study, 48 *Campylobacter* isolates collected in a national surveillance survey and reported to be resistant to erythromycin were reinvestigated using broth microdilution as the reference standard in order to evaluate the efficacy of routine methods for determining erythromycin resistance. Depending on the application of CLSI or EUCAST criteria, only 11–14% of the *C. jejuni* isolates and 67% of the *C. coli* isolates were classified as resistant upon reinvestigation. This is worrisome because false-positive resistance tests may have important clinical consequences, particularly in cases of isolates with resistance to both erythromycin and ciprofloxacin, where physicians may be unnecessarily forced to choose less effective or more toxic antibiotics for the treatment of *Campylobacter* infections.

Several hypotheses can explain the discrepancies in the determination of erythromycin resistance. Firstly, differences in the methods applied for susceptibility testing can cause different susceptibility results. Most participating laboratories (eight of ten laboratories from which data were available) used varying protocols for disk diffusion as methods for routine susceptibility testing in *Campylobacter* isolates (van der Beek *et al.*, submitted for publication). The disk diffusion test is relatively inexpensive and easily applicable and has been investigated as a susceptibility test for *Campylobacter* with varying results [20–22]. The British Society for Antimicrobial Chemotherapy (BSAC) and the CLSI advise that disk diffusion should be used to screen for resistance and that results should be confirmed by MIC determined methods [18,23]. When applying disk diffusion, critical zone diameters adapted from those of non-fastidious organisms should be applied with caution because the incubation conditions of such organisms differ from those required for *Campylobacter*. The effect of a basic antibiotic such as erythromycin decreases in an environment with high CO_2 levels and can result in falsely increased MIC values [20,22,24]. Therefore, interpretation of *Campylobacter* susceptibility test results from disk diffusion, other than according to CLSI protocol, or without confirmation by MIC-based methods, remains unreliable. In a study by Mevius *et al.*, interpretative criteria were proposed in association with EUCAST epidemiological cut-off values based on disk diffusion and broth microdilution according to CLSI protocol on a limited collection of 89 isolates (Mevius DJ, Veldman K, van Pelt W, Heres L, Mulder B, Wagenaar JA. Determination of Interpretive Criteria for Susceptibility Tests of *Campylobacter* spp. 14th International Conference on *Campylobacter*, *Helicobacter* and Related Organisms, 2007; p. 28.).

The *E*-test is an MIC-based technique that is readily available and easy to apply in many laboratories. In several studies, *E*-test results were found to agree with results of broth or agar dilution assays, but in none of these studies was the *E*-test compared with current CLSI-approved methods for broth microdilution [25–28]. In this study, the MIC values as determined by *E*-test were comparable with those found by broth microdilution. Susceptibility classification based on *E*-test results was in accordance with that of broth microdilution in all isolates. MIC values determined by *E*-test after

24 h of incubation (data not shown) were lower than those obtained after 48 h of incubation in 25% of the isolates and would lead to higher false susceptibility rates. This demonstrates that an adequate incubation period is important for reliable resistance determination.

Secondly, the use of different breakpoints may have played a role in causing the discrepancies. The two laboratories that used MIC-based methods (*E*-test and broth microdilution, respectively) used lower critical MIC values than the CLSI and EUCAST criteria.

Finally, instability of erythromycin resistance may have played a role in the discrepancy between our study findings and the initial susceptibility testing results. The stability of macrolide resistance after subculturing *Campylobacter* isolates has been studied previously. Restoration of susceptibility was observed in one of seven erythromycin-resistant isolates after 55 subcultures in the absence of antibiotic selection pressure [7]. In *Helicobacter pylori* isolates, instability rates of 0%, 10% and 45% were reported in three different studies [29–31]. Instability may have led to an *in vitro* underestimation of resistance as we tested after more subculture rounds than the original laboratories. However, the number of subcultures was not very high (< 20) in this study, making instability and reversion to susceptibility not very likely.

Campylobacter isolates displaying resistance caused by the presence of mechanisms other than 23S rRNA mutations seem to constitute a minority of all resistant isolates, although a recent report indicated that an efflux mechanism can act in synergy with the 23S rRNA mutation to confer high-level erythromycin resistance [7–9,32]. The most common resistance-associated 23S rRNA mutations in this and other studies have been detected at nucleotide positions 2058 (A $\rightarrow$ C, A $\rightarrow$ T) and 2059 (A $\rightarrow$ G) of the 23S rRNA. They lead to cross-resistance to macrolide and lincosamide antibiotics [7,10,33]. In this study, resistance-associated mutations were found in all resistant isolates except one *C. jejuni* isolate with an MIC value of 16 mg/L. All investigated susceptible strains showed a wild-type genotype. The A2058T mutation that was detected in two *C. jejuni* isolates has been previously associated with low-level resistance to macrolides in a *C. coli* isolate (MIC 8 mg/L), but this isolate was heterozygous for this mutation [34].

The isolates in this study appeared to carry only a single mutation and had MIC values > 64 mg/L. It is possible that in the near future genotypic detection of resistance will come into use more commonly than phenotypical tests. For erythromycin resistance in *Helicobacter*, less cumbersome methods than sequence analysis have been described for detection of the most frequently occurring resistance-associated mutations, such as PCR-RFLP analysis and real-time PCR, even on stool samples [34–37]. For *Campylobacter*, such methods have recently been introduced for isolates originating from clinical samples [38], but not yet for direct application on stool samples. In comparison with phenotypic tests, determination of genotypic resistance is relatively fast, especially for slow-growing isolates. In addition, less interlaboratory or interassay variability is expected when using this method. However, more knowledge of resistance mutations may be necessary before this method can be routinely applied.

This study had some limitations. It represents a retrospective analysis and it included only those isolates reported as resistant by local laboratories. Nevertheless, because a relevant proportion of isolates appeared to be susceptible to erythromycin using broth microdilution, our selection included isolates with varying levels of susceptibility to erythromycin.

In conclusion, the determination of the erythromycin resistance of *C. jejuni* and *C. coli* is inaccurate according to current practice. The standardization of frequently used susceptibility testing methods, such as disk diffusion, must be mandatory if we are to obtain reliable results for fastidious and micro-aerophilic organisms such as *Campylobacter*. In the absence of standardized protocols and interpretive to criteria for disk diffusion, broth dilution remains the best method.

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Transparency Declaration

All authors declare that there are no conflicting or dual interests.

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