Calibration of the disk diffusion test for trovafloxacin susceptibility testing of four anaerobic species

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Objectives To study trovafloxacin susceptibility among clinical isolates of four anaerobic bacterial species using minimum inhibitory concentrations (MIC) determinations, E test assays and disk diffusion test results and to calibrate the disk diffusion method for these species using single strain regression analysis (SRA).

Methods One-hundred and eighty-seven clinical isolates of four anaerobic bacterial species were included. Trovafloxacin MIC determinations were performed using the agar dilution technique and MIC estimations using the E test. The disk diffusion test was performed according to Swedish Reference Group for Antibiotics standardization. NCCLS limits for susceptibility categories were applied. SRA was performed using 1, 3, 10, 30, and 100 μg trovafloxacin disk contents and ATCC control strains. The regression lines obtained permitted the calculation of zone equivalents to MIC limits as well as an evaluation of various disk potencies.

Results Trovafloxacin susceptibility (S + I) was noted in 98.9, 100, 100, and 97% of Bacteroides fragilis, Bacteroides thetaiotaomicron, Clostridium perfringens, and Peptostreptococcus magnus strains, respectively, as judged by MIC determinations. Agar dilution and E test estimations gave the same results, but E test values were consistently lower than MIC values by the reference method. Regression lines calculated for the four species using SRA showed different equation constants indicating species-related differences. Interpretive zone diameter breakpoints were calculated for the four species and used for the interpretation of susceptibility.

Conclusions The disk diffusion test was successfully calibrated for trovafloxacin susceptibility testing of four anaerobic species using single strain regression analysis, SRA. There was a good agreement between the results of MIC-tests and disk testing. Interpretive errors of type I are prone to occur among Bacteroides isolates and might require species-related MIC limits. SRA calculations permitted the testing of the effect of different disk potencies on inhibition zones produced at the interpretive MIC limits. Criteria for the selection of a minimal disk content showed that 5 μg trovafloxacin is sufficient, but a 10 μg disk will safeguard against residual laboratory variation without producing too large inhibition zones for very susceptible strains.

Keywords antibiotic susceptibility testing, disk diffusion test, calibration, regression line, interpretive zone breakpoint, trovafloxacin susceptibility, quality control

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INTRODUCTION

Among the newer fluoroquinolones, trovafloxacin (CP 99,219) shows improved efficacy not only against Gram-positive and Gram-negative aerobic organisms [1–3] but also against anaerobic bacteria [4–6]. With relatively few side-effects and a rather complete antibacterial coverage, trovafloxacin therefore seems to be a very promising fluoroquinolone with a wide range of indications, including serious invasive infections [6]. Because of the broader antibacterial spectrum of trovafloxacin, in-vitro antibiotic susceptibility test results for other fluoroquinolones cannot always be used as guidelines also for trovafloxacin. Susceptibility testing might therefore be recommended with trovafloxacin.

Disk diffusion methods for susceptibility testing of anaerobes to various antibiotics are not always standardized or recommended and the disk diffusion method is therefore not used as
Materials and Methods

Bacterial strains

Clinical isolates of anaerobic bacteria were collected from routine specimens received at the Clinical Microbiology Laboratory, University Hospital of Lund, Lund, Sweden. The 187 strains included 89 isolates of Bacteroides fragilis, 22 isolates of Bacteroides thetaiotaomicron, 41 isolates of Clostridium perfringens, and 35 strains of Peptostreptococcus magnus. Species identification followed established procedures [9,10]. All strains were subcultured and stored on Fastidious anaerobe agar (Lab M, Topley House, Wash Lane, Bury, England) in Gas Pack jars (BBL) at room temperature until testing. The strains had experienced 2–4 passages before testing. Control strains Bacteroides fragilis, ATCC 25285, Bacteroides thetaiotaomicron, ATCC 29741, Clostridium perfringens, ATCC 13124, and Peptostreptococcus magnus, ATCC 29328, were included for MIC determinations and disk diffusion tests and also for single-strain regression analysis (SRA) to calibrate the disk test.

MIC determinations

The MIC of trovafloxacin for clinical isolates of anaerobic pathogens and for control strains was determined using the agar dilution method [11]. MICs were determined by incorporating the antibiotics in PDM agar medium (AB Biodisk, Solna, Sweden) supplemented with 5% horse blood, vitamin K, and hemin. Supplemented PDM agar plates containing two-fold dilutions of trovafloxacin were prepared and used on the same day. The inoculum was prepared by suspending colonies from a 48 h culture plate into Fastidious anaerobe broth (F.A.B. Lab 71; Lab M, ) to a visible turbidity of a no. 0.5 McFarland standard. Using a Steers’ replicator, 10 μL of the bacterial suspension was applied to the plate with a final inoculum of approximately 10^8 CFU per spot. The plates were incubated at 35–37 °C in an anaerobic cabinet and evaluated after 24 and 48 h. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth.

MIC limits for interpretation of susceptibility were S ≤ 1.0, R ≥ 4.0 mg/L for trovafloxacin according to Swedish Reference Group for Antibiotics (SRGA) or S ≤ 2.0, R ≥ 8.0 mg/L according to NCCLS recommendations. The German DIN guidelines recommend S ≤ 1.0, R ≥ 4.0 mg/L, but other German studies favour S ≤ 2.0, R ≥ 8.0 mg/L [12]. MIC_{50} and MIC_{90} values for populations of strains represent the MIC of 50% and 90% of the strains, respectively. When MIC-values constituted a single homogeneous population and included more than two dilution values, the MIC_{50} and MIC_{90} values were also calculated using probit analysis of the distribution for more exact interpolation [13]. These values are called MIC_{50} and MIC_{90}.

E test

For the E test (AB Biodisk), prereduced supplemented PDM plates were used. The inoculum (approximately 10^6 CFU/mL) was the same as used for the agar dilution tests. The inoculum was applied with a sterile cotton swab streaked in two crossing directions and then allowed to be absorbed for 10 min before the antibiotic strip was applied. The plates were incubated at 35–37 °C in an anaerobic cabinet and evaluated after 24 and 48 h. The MIC was read from the scale at the intersection of the zone with the strip according to the instructions of the manufacturer.

Disk diffusion antibiotic susceptibility testing

The antibiotic susceptibility of clinical isolates was determined by the disk diffusion method according to SRGA [11] with interpretations adjusted for species groups when applicable [14,15]. Suspensions of the bacterial isolates were inoculated on prereduced supplemented PDM agar using cotton swabs streaked on the medium in two crossing directions. The inoculum (approximately 10^8 CFU/mL) was the same as used for the agar dilution tests and the E tests. After absorption of the inoculum, the antibiotic disks were applied followed by pre-incubation at room temperature for 15–20 min. The plates were then incubated at 35–37 °C in an anaerobic cabinet and evaluated after 24 and 48 h.

Both SRGA and NCCLS recommend a trovafloxacin disk content of 10 μg whereas German DIN propose a 5-μg disk [12]. Trovafloxacin disks were provided by Pfizer AB (Täby, Sweden). Inhibition zone diameter values were read with a pair of calipers, in millimetres to one decimal place. Zone diameter values were plotted species-wise for the different antibiotics as histograms with zone values (as integers) on the X-axis and per cent strains on the Y-axis [16,17].

Single strain regression analysis

Antibiotic disks were produced with five different trovafloxacin disk contents: 1, 3, 10, 30, and 100 μg. The production of these disks followed methods used since the 1960s in the Karolinska hospital laboratory [18]. The four control strains were tested widely as it deserves [7] provided it can be calibrated properly [8]. The present studies were performed to evaluate a calibration method for trovafloxacin susceptibility testing of anaerobic isolates and to study trovafloxacin susceptibility among rapidly growing anaerobes in Sweden using minimum inhibitory concentration (MIC) testing with the agar dilution method, MIC estimation using the E test and the calibrated disk diffusion method.

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Evaluation of zone breakpoints

The evaluation of inhibition zone breakpoints for the susceptibility categories, S, I, and R, was performed using error rating according to NCCLS. Minor discrepancy is defined as an intermediate result by one method and either susceptible or resistant by the other. Major discrepancy is defined as a true susceptible result but resistance according to the other method. A very major discrepancy is a true resistant result which is read as susceptible by the disk diffusion method. The rates of these discrepancies were calculated for the different species and the MIC values from agar dilution tests compared with interpretations using the calculated zone breakpoints. Plotting of inhibition zone diameter values against 10-log MIC for the clinical isolates was performed species-wise according to mathematical principles with the independent variable MIC on the X-axis [21].

Evaluation of optimal disk content of antibiotic for disk diffusion tests

SRA permits the calculation of zone diameters for various MIC values and different disk contents. The effect of different disk contents for routine testing was therefore performed. A rational definition of criteria for such an optimal disk content has been presented as the lowest disk content of antibiotic which will distinguish resistant strains of any bacterial species from strains of the intermediate or susceptible category [22,23]. The consequence of such a definition is that the disk chosen must produce inhibition zones for all strains belonging to the intermediate or susceptible category. It is possible to test this criterion using SRA.

RESULTS

Antibiotic susceptibility of clinical isolates of anaerobic bacteria

Trovafloxacin MIC determinations were performed on 89 isolates of B. fragilis, 22 B. thetaiotaomicron, 41 C. perfringens, and 35 P. magnus, a total of 187 anaerobic strains (Table 1). Using the NCCLS interpretive MIC limits for trovafloxacin, S ≤ 2.0, R ≥ 8.0 mg/L, the resistance rate was 1.1% among B. fragilis (1/89), no resistance was noted among B. thetaiotaomicron (0/22), and C. perfringens (0/41), and 3% resistance among P. magnus (1/35). The MIC$_{50}$ and MIC$_{90}$ values were also very favourable (Table 1). E test estimations of MIC values gave the same resistance rates. The numerical values of the E test results were lower than the MIC-values determined by the standardized agar dilution method by a factor of (mean values) 0.52,
0.59, 0.70, and 0.83, for isolates belonging to the four species, respectively.

Calibration of the disk diffusion method

Since the disk diffusion test is a more practical alternative to MIC determinations in clinical microbiology, we applied SRGA standardization of the disk diffusion method for testing anaerobic bacterial isolates included in our studies. The 10-µg trovafloxacin disk diffusion test was first calibrated as follows. SRA calculations were performed for the four reference strains of the four anaerobic species included. These experiments showed that the regression lines for the four reference strains were slightly different (Figure 1). *Bacteroides fragilis* and *C. perfringens* gave slope constants which were lower than the slope constants for the other two species, *B. thetaiotaomicron* and *P. magnus* (Table 2).

The zone diameter equivalents of MIC values around the MIC limits for the susceptibility categories were then calculated (Table 2). Corresponding to the differences in regression lines, the calculated zone breakpoints differed between the four species by up to 5–6 mm (Table 2). In order to apply these zone breakpoints to the present studies, the inhibition zone diameter values from disk testing of the 187 clinical isolates were plotted against the corresponding MIC values. For the *Bacteroides* isolates the number of strains having MIC values close to the MIC limits was fairly high. *Bacteroides fragilis* and *B. thetaiotaomicron* showed 21.4 and 31.8% of the strains with an MIC value of 1 mg/L, and 4.5 and 13.6% at 2 mg/L, respectively. This will obviously give rise to interpretive errors of type I [24]. The SRGA interpretive MIC limits for trovafloxacin are more prone to such errors than the NCCLS MIC limits. Trovafloxacin susceptibility according to uncorrected disk diffusion test results can therefore be expected to give rise to interpretive errors among *Bacteroides* isolates.

**Disk diffusion test results**

Using the NCCLS limits, the application of the calculated zone breakpoints for a 10-µg trovafloxacin disk (Table 2) to the *Bacteroides* isolates gave a correct interpretation in 92.1 and 68.2% of *B. fragilis* and *B. thetaiotaomicron*, respectively. For *C. perfringens* and *P. magnus* the interpretation was correct in 100 and 91.4%, respectively. There were no very major errors for the four species tested against trovafloxacin. Major errors were recorded in 2.2% for *B. fragilis*, in 4.5% (1/22 strains) for *B. thetaiotaomicron*, and in no cases for the isolates belonging to the other two species. Minor errors were seen in 5.6, 27.3, and 8.6% for *B. fragilis* and *B. thetaiotaomicron* and *P. magnus*, respectively. The expected interpretive errors among *Bacteroides* iso-

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**Table 2**  Single strain regression analysis (SRA) constants and inhibition zone equivalents of minimum inhibitory concentration (MIC)-values 1–8 mg/L. The interpretive zone diameter breakpoints according to NCCLS and SRGA MIC limits are shown as calculated according to Forsberg et al. [20]

<table>
<thead>
<tr>
<th>Species</th>
<th>Constant A</th>
<th>Constant B</th>
<th>1 mg/L</th>
<th>2 mg/L</th>
<th>4 mg/L</th>
<th>8 mg/L</th>
<th>S¹</th>
<th>R¹</th>
<th>S²</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>329.1</td>
<td>429.2</td>
<td>27.5</td>
<td>26.7</td>
<td>23.7</td>
<td>21.5</td>
<td>25</td>
<td>22</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td><em>B. thetaiotaomicron</em></td>
<td>446.6</td>
<td>280.6</td>
<td>27.0</td>
<td>24.3</td>
<td>21.4</td>
<td>18.0</td>
<td>23</td>
<td>19</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>265.8</td>
<td>282.6</td>
<td>23.4</td>
<td>21.6</td>
<td>19.7</td>
<td>17.6</td>
<td>21</td>
<td>18</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td><em>Peptostreptococcus magnus</em></td>
<td>536.2</td>
<td>369.8</td>
<td>30.1</td>
<td>27.3</td>
<td>24.1</td>
<td>20.5</td>
<td>26</td>
<td>22</td>
<td>29</td>
<td>25</td>
</tr>
</tbody>
</table>

¹ NCCLS limits for trovafloxacin susceptibility, S ≤ 2.0, R > 8.0 mg/L.
² SRGA limits for trovafloxacin susceptibility, S ≤ 1.0, R > 4.0 mg/L.
lates were therefore confirmed in these disk diffusion tests. For \textit{B. thetaiotaomicron} the errors are not acceptable and the MIC limits have to be corrected for this species, in a direction determined by relevant clinical trials and correlations to MIC data, tentatively providing zone breakpoints 3 mm lower than the present calculated ones [24]. However, the calculation of interpretive breakpoints using SRA provided an accurate reflection of the present interpretive MIC limits and the method therefore offers a new tool for the individual laboratory to set up a standardized and calibrated disk diffusion method also for anaerobic bacteria.

**Choice of disk content for routine disk diffusion testing**

The SRA calculations also permit a theoretical consideration of various disk contents for testing of anaerobes in clinical microbiology. The basic criterion is given above under Materials and Methods and it requires that measurable zones are produced around the disk by all species for all S and I isolates [22]. The inhibition zones corresponding to the NCCLS limit for resistance, 8 mg/L, are shown in Figure 2 for the four species and for different disk contents. It is clear that neither \textit{B. fragilis} nor \textit{C. perfringens} isolates would pose any problems (Figure 2). However, the more steep regression lines for the other two species (Figure 1) give almost no zones for MIC 8 mg/L with a 2 µg trovafloxacin disk (Figure 2). The 5 µg disk would be able to give zones for intermediate strains (4 mg/L) of \textit{B. thetaiotaomicron} taking into account a methodological variation of ± 1 MIC dilution steps. \textit{Peptostreptococcus magnus} isolates would be less critical. A 10-µg disk would be safer without introducing too large zones for more susceptible isolates. For testing anaerobes belonging to the species studied, both the 5 and the 10 µg disks should produce accurate results.

**DISCUSSION**

Trovafloxacin is a fluoroquinolone antibiotic which is active against anaerobic human pathogens [5,6,25]. This was also shown in the present studies where 98.9, 100, 100, and 97% of \textit{B. fragilis}, \textit{B. thetaiotaomicron}, \textit{C. perfringens}, and \textit{P. magnus} strains, respectively, were susceptible (S + I) as judged by MIC determinations. Trovafloxacin compares well with other potent antibiotic drugs, but has a broader range of action than most other fluoroquinolone drugs. It might be used as monotherapy in intra-abdominal infections, which often start off with aerobic \textit{Enterobacteriaceae} such as \textit{E. coli}, and then switch to obligate anaerobes such as \textit{B. fragilis} in a second phase. Its proper place in the anti-infective arsenal remains to be seen.

Standardization of antibiotic susceptibility testing of non-fastidious aerobic isolates using the disk diffusion method has been well supported by several reference authorities over the years. Similar efforts have been largely lacking when it comes to disk diffusion testing of anaerobes. However, studies have shown promising results for disk testing of rapidly growing anaerobes [7,8]. Also, trovafloxacin disk diffusion tests of anaerobes has been described [25]. Since the major problem with disk testing is associated with deviations from the reference laboratory in methodology as well as in performance of individual bacterial species, the accuracy of the disk test can be improved with laboratory-specific regression lines and species-related interpretations [14,15,17,24]. We have therefore used SRA to calibrate the disk diffusion test for the four different anaerobic species studied [15,19]. The SRA experiments permitted the calculation of interpretive zone breakpoints according to recommended MIC limits (NCCLS) and according to species-specific regression lines.

When a new antibiotic is introduced it is necessary to determine which disk content should be used for routine disk diffusion tests in microbiology laboratories. Usually, different contents of antibiotic are tested and their performance evaluated using a panel of clinical isolates. However, no clear criteria for the optimal choice have been defined. We have used a recently presented definition of the optimal disk content of antibiotic — the lowest disk content of antibiotic which will distinguish resistant strains from those of the intermediate or susceptible

![Figure 2](image-url)
category [22]. This definition is possible to apply with the aid of SRA calculations. Our results showed that it would be possible to use a 5-μg trovafloxacin disk. However, considering some residual variation in laboratory performance, the 10-μg trovafloxacin disk would be safer without giving rise to too large inhibition zones for susceptible isolates. Both disk contents have been advocated by others [12,25].

The trovafloxacin MIC results for Bacteroides isolates showed that MICs and MIC90s 0.5 and 1–2 mg/L, respectively, were close to the interpretive limits, S ≤ 2.0, R ≥ 8.0 mg/L. It was therefore postulated that interpretive errors of type I might occur when performing disk diffusion tests [24]. Such was actually the case and it was concluded that the MIC limits for Bacteroides isolates should be species-related. However, even without such corrective measures, which also have been advocated and implemented by SRGA, the susceptibility figures were in line with the favourable activity of trovafloxacin according to MIC values (Tables 1, 2) [14,24]. Another situation which might require species-related interpretive MIC-limits, is not of a strict methodological nature. Clostridium perfringens isolates showed an MIC50 value of 0.125 mg/L, which is much lower than the MIC limit of S ≤ 2.0 mg/L. If some resistance mechanism gives a 10-fold rise in the MIC, then the strain will still be labelled trovafloxacin susceptible. It is not advisable to recommend the drug in such a situation. A lowering of the MIC limit for this bacterial species might safeguard against such abuse.

A small but consistent discrepancy between MIC results from agar dilution tests and E test estimations was noted. The E test values were always lower, down to 50% of the reference MIC values. Such discrepancies have been described by several authors for other antibiotics and aerobic as well as anaerobic isolates [26–28], both towards higher values [29–31] and lower values [32,33]. Basically, the E test is a diffusion method and it was originally described and patented by Professor Olof Vestergård. It consists of a continuous gradient of antibiotic and it is widely used for susceptibility testing of anaerobes [26–28], both towards higher values [29–31] and lower values [32,33]. Basically, the E test is a diffusion method and it was originally described and patented by Professor Olof Vestergård. It consists of a continuous gradient of antibiotic and it is widely used for susceptibility testing of anaerobes [26–28], both towards higher values [29–31] and lower values [32,33].

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