The Effect of Antibacterial Drugs on the Growth Kinetics of \textit{Bacteroides in Vitro}

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Abstract. A method is described to express the effect of antimicrobial drugs on the growth \textit{in vitro} of various \textit{Bacteroides} species during the first few hours of exposure. Drugs studied were clindamycin, chloramphenicol, metronidazole, and tinidazole. The effect of the various drugs is expressed at different time points as the log ratio, this being the logarithm of the ratio of the number of colony forming units in the control growth and that in the presence of the drug. For any given concentration, the course of the log ratio in time can be fitted to the equation

$$\log \text{ ratio} = i \left( t + e^{-t} - 1 \right),$$

where $i$ is a concentration-dependent parameter. Concentration-effect curves on the basis of values of $i$ show a different behavior of drugs with different modes of action. Those curves also show that the interpretation of the minimal inhibitory concentration after 24 h is different for different kinds of drugs.

The clinical significance of infections by anaerobic microorganisms, whether present alone or in combination with aerobic bacteria, is now well recognized [4]. To assess the possible usefulness of antibacterial drugs in this kind of infection, \textit{in vitro} testing is necessary.

While the minimal inhibitory concentration (MIC) may be useful for initial screening, it does not give insight on the time-related effect of those drugs on the growth of bacteria [1]. It is doubtful whether the MIC of anti-anaerobic drugs gives sufficient information for clinical purposes.

In previous studies we developed a mathematical approach to the short-term \textit{in vitro} growth of aerobes that enabled us to express antibacterial activity in precise quantitative terms [3]. For anti-anaerobic drugs the short-term effects on growth may also be relevant considering their pharmacokinetics \textit{in vitro}; therefore, the present study was undertaken to modify our technique for anaerobes.

Materials and Methods

\textbf{Microorganisms.} The strain of \textit{Bacteroides fragilis} (no. 1) tested for four antibiotics was a clinical isolate obtained from the Rijksinstituut voor Volksgezondheid (RIV), Utrecht, The Netherlands. Six other \textit{Bacteroides} strains were also clinical isolates, obtained either from the RIV or from patients in the University Hospital, Leiden.

\textbf{Media.} The cultures were maintained in prereduced brain-heart infusion broth (BHI-OXOID) (Oxoid, Basingstoke, England), supplemented with 1 g MgSO$_4$ 7 H$_2$O, 0.5 g cysteine hydrochloride, 1 ml Tween 80, and 5 mg haemin/l. Resazurin (4 mg/l) was added as an indicator of anaerobic condition. Eugonagar (BBL, Becton, Dickinson & Co., MD) plates supplemented with 12 mg/l haemin were used for the viable counts.

\textbf{Antibiotics.} Clindamycin (Upjohn, Ede, The Netherlands), chloramphenicol (Gist Brocades, Delft, The Netherlands), metronidazole (Rhône-Poulenc, France), and tinidazole (Pfizer, Karlsruhe, FRG) were used. Standard stock solutions of 1000 mg/l were prepared in sterile physiological saline and stored at 4°C for a maximum of one month.

\textbf{MIC determinations.} The MICs for all four antibiotics were determined against \textit{Bacteroides fragilis} (no. 1) with serial twofold dilutions of the antibiotics, by broth dilution as well as by agar dilution. The broth dilution method was performed in prereduced BHI under a 90% nitrogen/10% carbon dioxide flow (N$_2$/CO$_2$), with an inoculum of 50 ml from an 18-h culture of \textit{Bacteroides fragilis} in 10 ml, giving a final density of $10^6$ colony forming units (CFU) per ml. The rubber-stoppered tubes were then incubated for 24 h at 37°C.

The agar dilution method was performed using Eugonagar plates supplemented with haemin. The inoculum was a drop of 5 ml of a 1:100 dilution of an 18-h culture containing $10^6$–$10^7$ CFU
per ml. The plates were incubated anaerobically in GasPak (BBL) for 24 h at 37°C. The MIC was designated as the lowest concentration of antibiotic that completely inhibited visible growth. The MIC of metronidazole and of tinidazole were also determined for the six other Bacteroides strains.

Growth curve determinations. An 18-h culture in the logarithmic growth phase, containing about $10^9$ organisms per ml, was diluted 1:100 in a sterile 500 ml screw-capped bottle containing preduced BHI under a N₂/CO₂ flow. The diluted culture was allowed to generate in the logarithmic phase for two hours at 37°C in a waterbath. At that time the suspension contained between $10^7$ and $10^8$ CFU per ml. The culture was then distributed aseptically into nitrogenated sterile 100 ml screw-capped bottles and the appropriate antibiotic was added. The short-term antibacterial effect of each antibiotic was determined by colony counts of the surviving bacteria at regular intervals of 45 min to an hour, up to 4 h after addition of the specific antibiotic, along with the colony counts of a control (without antibiotic). Anaerobic conditions were maintained during sampling by use of the N₂/CO₂ flow into the specific bottles. The colony counts were performed by placing six drops of 10 µl of a series of tenfold dilutions of the cultures at the specified time intervals on Eugonagar plates. The plates were accumulated in a "holding" GasPak jar, which was flushed with N₂/CO₂ every time it was opened, and after that in a normal GasPak jar at 37°C for 48 h to ensure the formation of easily visible colonies. All tests were performed in duplicate on different days.

Mathematical analysis. In previous studies the effect of antibacterial drugs was expressed by a quadratic function of time [3]. Although this also gave a good fit in the present study, the following approach appeared to be satisfactory.

The effect of the antibacterial drug at any time was expressed as the difference of the logarithms of the numbers of CFU in the control and that in the presence of antibiotics (log ratio). The log ratio values could then be fitted by multiple regression analysis to the equation:

$$\log \text{ratio} = i_1 \times t + i_2 \times e^{-t} + i_3$$

In this equation $i_1$ represents the final slope of the log ratio curve and $(i_2 - i_3)$ the intercept at $t = 0$. The slope of the tangent of the log ratio curve at any time is given by the equation:

$$\frac{d \log \text{ratio}}{dt} = i_1 - i_2 \times e^{-t}$$

Because in most instances the values of $i_1$, $i_2$, and $i_3$ were similar, the equation could be simplified further to a one-parameter function:

$$\log \text{ratio} = i \times (t + e^{-t} - 1)$$

In this equation the slope of the tangent at $t = 0$ is zero. The values of the growth inhibition constant $i$ can be considered as a dose-dependent parameter of antibacterial effect. For metronidazole and tinidazole, this parameter was used to calculate the relative potency of those drugs against all seven Bacteroides strains, according to standard bioassay procedures [2].

Results

An example of the growth curve of B. fragilis in the presence of metronidazole is shown in Fig. 1a. The log ratio values derived from these data are shown in Fig. 1b, as well as the fitted line through zero as described under Materials and Methods. The growth inhibition constant $i$ represents the final slope of this line. Fig. 2 shows all calculated values of the growth inhibition constant $i$ for various antibiotics against one particular strain of B. fragilis (no. 1). Also shown are the MICs in broth for this particular strain. Table 1 gives the MIC values and the values of $i$ for metronidazole and tinidazole.