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Killing of *Streptococcus pneumoniae* by azithromycin, clarithromycin, erythromycin, telithromycin and gemifloxacin using drug minimum inhibitory concentrations and mutant prevention concentrations

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ABSTRACT

Streptococcus pneumoniae continues to be a significant respiratory pathogen, and increasing antimicrobial resistance compromises the use of β-lactam and macrolide antibiotics. Bacterial eradication impacts clinical outcome, and bacterial loads at the site of infection may fluctuate. Killing of two macrolide- and quinolone-susceptible clinical *S. pneumoniae* isolates by azithromycin, clarithromycin, erythromycin, telithromycin and gemifloxacin against varying bacterial densities was determined using the measured minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC). For kill experiments, 10^6–10^9 CFU/mL were exposed to the drug and were sampled at 0, 0.5, 1, 2, 3, 4, 6, 12 and 24 h following drug exposure. The $\log_{10}$ reduction and percent reduction (kill) of viable cells was recorded. MICs and MPCs (mg/L) for azithromycin, clarithromycin, erythromycin, telithromycin and gemifloxacin were 0.063–0.125/0.5–1, 0.031–0.063/0.25–0.5, 0.063/0.25–0.5, 0.008/0.016 and 0.031/0.25, respectively. Killing 10^6–10^9 CFU/mL of bacteria by the drug MIC yielded incomplete killing, however $\log_{10}$ reductions occurred by 12 h and 24 h for all drugs. Exposure of 10^6–10^9 CFU/mL to MPC drug concentrations resulted in the following $\log_{10}$ reduction by 6 h of drug exposure: azithromycin, 1.3–3.9; clarithromycin, 1.9–5.8; erythromycin, 0.8–4.7; telithromycin, 0.3–1.7; and gemifloxacin, 1.8–4.2. Bacterial loads at the site of infection may range from 10^6 to 10^9, and kill experiments utilising a higher bacterial inoculum provided a more accurate measure of antibiotic performance in high biomass situations. Killing was slower with telithromycin. Kill was greater and fastest with MPC versus MIC drug concentrations.

1. Introduction

Streptococcus pneumoniae is a prevalent cause of community-acquired upper and lower respiratory tract infections and spans all age groups. Drug-resistant pneumococcal strains have increased in prevalence, and numerous reports have documented the selection of drug-resistant strains during therapy—that is, the organism isolated from the patient was initially susceptible to the treatment antibiotic but resistance or reduced susceptibility developed during therapy [1,2].

Clinical outcome and the relationship with antimicrobial resistance is not perfect, as patients infected with drug-resistant organisms and treated with the 'wrong' antibiotic may still clinically recover; moreover, patients with drug-susceptible organisms may clinically deteriorate despite adequate therapy. Despite this, we still consider susceptible versus resistant organisms an important distinction for choosing optimal therapy.

We have previously argued that the mutant prevention concentration (MPC) may more accurately reflect the true dynamics of drug–bacterial interactions as testing is based on bacterial inocula $\geq 10^9$ CFU, organism densities present during some infections [3]. Minimum inhibitory concentration (MIC) testing is insufficient for detecting mutant subpopulations that may be present in higher bacterial inocula: $>10^7$–10^{10} CFU present in patients with middle ear infections, ocular infections, urinary tract infections, meningitis and pneumonia [4–8]. As such, measurement of the MPC may more accurately reflect the true dynamics of high-density bacterial

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Table 1
Comparative minimum inhibitory concentrations (MICs) and mutant prevention concentrations (MPCs) (in μg/mL) for five antimicrobial agents tested against two clinical isolates of *Streptococcus pneumoniae* and the control strain *S. pneumoniae* ATCC 49619.

Drug	Strain					
	RUH-03-1		RUH-03-6		ATCC 49619	
	MIC	MPC	MIC	MPC	MIC	MPC
Azithromycin	0.063	0.5	0.125	1	0.125	2
Clarithromycin	0.031	0.5	0.063	0.25	0.031	0.25
Erythromycin	0.063	0.25	0.063	0.5	0.063	0.25
Gemifloxacin	0.031	0.25	0.031	0.25	0.016	0.125
Telithromycin	0.008	0.016	0.008	0.016	0.008	0.031

populations as it determines the antimicrobial drug concentration required to inhibit the growth of the most resistant bacterial cell present in the population [4]. As bacterial eradication has been linked to clinical outcome in respiratory tract infections, eliminating the infecting bacterial population appears to be of paramount importance for recovery from an infectious disease and to minimise the risk for resistance selection [9].

Determination of MIC and MPC drug concentrations involves the testing of pre-determined bacterial inocula (i.e. 10^5 CFU/mL or $\geq 10^9$ CFU, respectively) against a range of doubling dilution drug concentrations either in liquid or on solid media [10]. Following incubation, the minimum drug concentration that prevents growth is recorded as the MIC or MPC depending on the test method. Neither MIC nor MPC testing is a measure of bacterial killing but rather a measurement of inhibition of bacterial growth. In order to assess bacterial killing, the $\log_{10}$ reduction in viable organisms and determination of percentage kill have been previously reported by our laboratory with high-density bacterial inocula [11,12]. As previously reported, a reduction of ≥ 3 $\log_{10}$ is associated with bactericidal activity and reductions ≤ 2 $\log_{10}$ are defined as bacteriostatic [11]; values >2 but <3 are considered indeterminate. Traditional kill studies have tended to utilise bacterial inocula of 10^5–10^6 CFU/mL and against multiple drug concentrations of the MIC (1×, 5×, 10× MIC, etc.). Whilst these studies have provided useful information, they failed to account for the dynamics of higher density bacterial populations such as those seen in some human infections. In this study, the killing by azithromycin, clarithromycin, erythromycin, gemifloxacin and telithromycin against two clinical strains of *S. pneumoniae* over a range of bacterial inocula (10^6–10^9 CFU/mL) was determined using the measured MIC and MPC drug concentrations for each organism and antimicrobial compound.

2. Materials and methods

2.1. Bacterial strains

Two clinical isolates of *S. pneumoniae* were collected at the Clinical Microbiology Laboratory of Royal University Hospital (Saskatoon, Canada). Both strains were classified as susceptible to the studied drugs as MICs were below the susceptibility breakpoints (Table 1) recommended by the Clinical and Laboratory Standards Institute (CLSI) [10].

2.2. Antimicrobial compounds

Pure substance drugs were obtained from the following manufacturers: azithromycin (Pfizer, Kirkland, Quebec, Canada); clarithromycin (Abbott Laboratories, Montreal, Quebec, Canada); gemifloxacin (Oscient Pharmaceuticals, Waltham, MA); and telithromycin (Sanofi-Aventis, Laval, Quebec, Canada). Erythromycin was purchased commercially (Sigma, Oakville, Ontario, Canada). Powdered forms of each compound were dissolved according to the manufacturer's instructions. Stock solutions were used as fresh preparations or from samples stored for <1 month at −70 °C.

2.3. Minimum inhibitory concentration testing

Susceptibility testing to determine the MIC was carried out in accordance with the guidelines established by the CLSI [10]. Isolates were tested by microbroth dilution in Todd–Hewitt broth (THB) (Difco Laboratories, Detroit, MI) owing to robust growth in this medium. Briefly, THB containing two-fold concentration increments of antimicrobial agent was added to 96-well microdilution trays (Sarstedt Inc., Newton, NC). *S. pneumoniae* suspensions equal to a 0.5 McFarland standard were further diluted to achieve a final inoculum of 5×10^5 CFU/mL in trays. Cultures were incubated for 18–24 h in 5% CO_2 and the MIC was taken as the lowest concentration that inhibited growth. MICs for the five drugs tested are summarised in Table 1. The American Type Culture Collection (ATCC) control strain *S. pneumoniae* ATCC 49619 was also tested.

2.4. Mutant prevention concentration testing

The procedure for testing *S. pneumoniae* isolates by MPC was as previously described by Blondeau et al. [13]. Briefly, starter cultures of seven blood agar plates (tryptic soy agar containing 5% sheep red blood cells) (PML Microbiologicals, Richmond, British Columbia, Canada) per isolate were inoculated to produce confluent growth and were incubated overnight (18–24 h) at 35–37 °C in 5% CO_2. The next day, the complete contents of the inoculated plates were removed from the plates with sterile swabs and were transferred to 500 mL of THB and incubated overnight at 35–37 °C in 5% CO_2. Following incubation, cultures were estimated to have concentrations of 3×10^8 CFU/mL by turbidity measurements. Cultures were then concentrated by centrifugation at 5000×*g* for 30 min at 4 °C and were then re-suspended in 3 mL of fresh THB. Aliquots of 200 μL containing $\geq 10^9$ CFU/mL were applied to individual 100-mm blood agar plates. For each experiment, agar dilution plates were prepared by incorporating the agents tested over a range of seven different concentrations (in doubling dilutions) into the blood agar plates. Plates were stored at 4 °C and were used within 7 days of preparation. Each experiment included the fully susceptible control strain *S. pneumoniae* ATCC 49619. Inoculated plates were incubated for 24 h, examined and re-incubated for another 24 h (48 h in total) at 35–37 °C in 5% CO_2 and were then screened for growth. To confirm MPC values, colonies were subcultured on agar plates containing the same drug concentration that they were isolated from, incubated as described and examined for growth. The MPC was recorded as the lowest drug concentration that prevented growth.

2.5. Kill studies

The method for the kill studies was as previously reported [11,12]. *S. pneumoniae* isolates were grown overnight on blood agar plates as described. The next day, an inoculum was transferred to THB and was incubated for 2 h at 35–37 °C in 5% CO_2. Following incubation, spectrophotometric readings of ≥1.5 verified cell densities $\geq 10^9$ cells/mL [13]. Further adjusting of inocula to achieve cell densities ranging from 10^6 CFU/mL to 10^9 CFU/mL was undertaken in THB and then antimicrobial agent was added to each inoculum density. The drug concentrations used for the kill experiments were based on the measured MIC or MPC drug concentrations for each antimicrobial agent tested against each strain (Table 1). Measurement of kill ($\log_{10}$ reduction in viable cells and percentage of organism killed) was recorded at 0, 0.5, 1, 2, 4, 6, 12 and 24 h by