Bactericidal activity of levofloxacin, gatifloxacin, penicillin, meropenem and rokitamycin against *Bacillus anthracis* clinical isolates

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This study aimed to evaluate the bactericidal rates of levofloxacin, gatifloxacin, penicillin, meropenem and rokitamycin against seven isolates of *Bacillus anthracis* clinically isolated between 1960 and 1970. After determination of MIC and MBC, time–kill experiments were carried out. Antimicrobial activity was evaluated at concentrations equal to 1×, 2×, 4× and 8× MIC after 0, 3, 6, 12 and 24 h of incubation with the drugs. Bactericidal activity was defined as a decrease in bacterial count of at least 3 log_{10}. All the isolates were susceptible to all the antibiotics, by considering the antistaphylococcal breakpoints. Levofloxacin was bactericidal at 1×MIC after 24 h and at 4×MIC after 12 h, and gatifloxacin was bactericidal at 2×MIC after 24 h and at 8×MIC after 12 h. Meropenem, rokitamycin and penicillin also showed bactericidal activity at concentrations of 4× and 8×MIC, respectively, but only after 24 h incubation; after the same time, meropenem and rokitamycin showed a more marked killing than penicillin at 2×MIC.

Keywords: *Bacillus anthracis*, anthrax, fluoroquinolones, penicillin, meropenem, rokitamycin, macrolides, *in vitro* susceptibility testing

Introduction

*Bacillus anthracis*, the aetiological agent of anthrax, has gained new attention due to its recent use as an agent of biological warfare. *B. anthracis* is a rod-shaped Gram-positive bacterium. In humans, three forms of anthrax may occur. Cutaneous anthrax is the most common form, accounting for >95% of reported cases,1 and it is due to introduction of spores into the skin. In the past, cutaneous anthrax was considered as an occupational disease, since it naturally follows exposure to infected animals.2 Although self-limiting and fatal only if infection becomes systemic, antibiotic treatment is recommended.

Pharyngeal and gastrointestinal anthrax follow ingestion of insufficiently cooked contaminated meat. Oropharyngeal anthrax is less common than the gastrointestinal form, and generally has a more favourable prognosis, whereas the gastrointestinal form may result in high mortality due to intestinal perforation or anthrax toxemia.3

Inhalational anthrax was, until recently, a disease mainly of historical interest, with sporadic cases in persons in close contact with animals. It is contracted from breathing airborne anthrax spores and, as evidenced by the Sverdlovsk experience, is often fatal.4 Given the rapid course of symptomatic inhalational anthrax, early and appropriate antibiotic administration is essential for successful treatment of anthrax. Doxycycline and ciprofloxacin are the drugs of choice for the therapy of anthrax.5,6 Penicillins represent an alternative for antimicrobial prophylaxis for children and pregnant women and for complete treatment of cutaneous disease caused by penicillin-susceptible *B. anthracis*.1

Recently, *in vitro* development of resistance to some antimicrobial agents after serial passages has been reported for *B. anthracis* Sterne.7 This study suggested that serial subculturing using quinolones and macrolides led to selection of a subpopulation of bacteria with increasing MICs. This feature is particularly alarming because, in the last cases of...
exposure to anthrax spores, ciprofloxacin was chosen for post-exposure prophylaxis, and macrolides could represent potential alternative drugs.

A main concern in selecting anthrax therapy is the limited availability of clinical data on *B. anthracis* susceptibility to antimicrobials. In addition, the *in vitro* studies on susceptibility have been carried out by determination of MICs.

Time–kill methods represent a useful method for evaluating the kinetic interactions between bacteria and antimicrobial agents. Moreover, they generally appear to be more sensitive than MIC–MBC methods for evaluating antimicrobial activity. 6 Therefore, for a preliminary evaluation and comparison of antimicrobial compounds to be used as an alternative to the recommended primary therapeutic agents for treatment and prophylaxis of anthrax, knowledge of bactericidal activity may be useful. To the best of our knowledge, the killing rate of antimicrobials against this microorganism has never been studied.

Therefore, we planned to compare the killing kinetics of levofloxacin, gatifloxacin, rokitamycin and meropenem, possessing potential *in vitro* activity against *B. anthracis*, with that of penicillin G, which is one of the drugs proposed for anthrax treatment.

**Materials and methods**

**Microorganisms**

Seven human isolates of *B. anthracis* clinically isolated in the period 1960–1970 from pharmaceutical company collections were used for the study. Confirmation of identification was obtained by means of real-time PCR, carried out on the Light Cycler PCR System (Roche Diagnostic Co., Monza, Italy), amplifying a portion of the rpoB gene. 9 The primers were rpoBF1a (CCACCAACAGTAAAGAAATGCC) and rpoBR1a (AAATTTCAACAGTTTCTGGATCT). The amplification programme comprised an initial de-naturation at 95°C for 120 s, followed by 55 cycles of 95°C for 3 s, 63°C for 10 s and 72°C for 10 s. Detection was accomplished by hybridization of a pair of probes (BaP1, TCCAAAGCGCTATGATTGCAATTGTA-F; and BaP2, Cy5-GGTCGCTACAAGATCAACAAGTTAC-C) to the amplicons as they were formed.

**Drugs**

Gatifloxacin (Grunenthal-Formenti, Milan, Italy), levofloxacin (Gliaxo Smith Kline SpA, Verona, Italy), penicillin G (Sigma Chemical, St Louis, MO, USA), rokitamycin (Grunenthal-Formenti) and meropenem (AstraZeneca, Milan, Italy) were tested. Stock solutions (1.28 mg/mL) of gatifloxacin, meropenem and penicillin were prepared in sterile water, rokitamycin (5.12 mg/mL) was prepared in ethanol and levofloxacin (1.28 mg/mL) in 0.1 M NaOH and water (50:50, v/v). 10 Working solutions of the drugs were obtained by diluting stocks with Mueller–Hinton broth.

**Determination of MIC and MBC**

Determination of MIC by means of microdilution broth method and MBC were carried out as follows: an adjusted inoculum of the tested organisms (10 μL) in the stationary phase of growth was added to 0.1 mL of Mueller–Hinton broth containing two-fold serial dilutions of a starting antibiotic solution, so that each well contained ∼1–5 × 10^5 cfu/mL. The inocula were verified by plating 0.1 mL of the microbial suspension after serial 10-fold dilutions (from 10^-1 to 10^-3) on to Columbia agar plates; when bacterial counts fell below this limit, MIC determinations were repeated. Results were observed after 18 h of incubation at 35°C, and MIC was defined as the lowest concentration able to inhibit visible growth. MBCs were determined by spotting 10 μL in duplicate from the wells showing no visible growth on Columbia blood agar plates and incubating them for 18–24 h. MBC was taken as the concentration at which a 99.9% reduction in cfu of the original inoculum occurred. As used by other authors, 11,12 the NCCLS staphylococcal breakpoints were used for determining susceptibility to levofloxacin (≤2 mg/L), gatifloxacin (≤2 mg/L), meropenem (≤4 mg/L) and penicillin (≤0.12 mg/L), whereas a breakpoint of ≤1 mg/L was used for rokitamycin.

**Time–kill experiments**

Each strain was grown overnight in Mueller–Hinton broth (37°C, aerobic atmosphere). Sterile drug solutions (0.1 mL) were added to 9.9 mL of the broth cultures (10^5–10^6 cfu/mL), to give final drug concentrations equivalent to 1 ×, 2 ×, 4 ×, 8 × and 16 × MIC. Antibiotic-free growth controls were also included. Tubes were incubated aerobically at 37°C under continuous agitation. Viable counts were carried out in duplicate 0, 3, 6, 9, 12 and 24 h after addition of antimicrobial agents, by spreading 0.1 mL on Columbia blood agar plates, after washing by centrifugation (1500g, 10 min at 4°C) to avoid antibiotic carry-over and serial 10-fold dilution in phosphate-buffered saline (pH 7.3). Colonies were counted after 24 h incubation in an aerobic atmosphere at 37°C.

The limit of count detection was 200 cfu/mL. The killing rate was determined by plotting the total number of viable cells (mean of cfu) as log_{10} cfu/mL against time. Bactericidal activity was defined as a 3 log_{10} decrease in cfu/mL (99.9% kill). Bacteriostatic activity was defined as <99.9% kill.

**Results**

Susceptibilities of *B. anthracis* isolates to the chosen antimicrobials are shown in Table 1.
Bactericidal activity of various agents against *B. anthracis*

Table 1. Antimicrobial susceptibility of the seven strains of *B. anthracis*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gatifloxacin MIC (mg/L)</th>
<th>Levofloxacin MIC (mg/L)</th>
<th>Meropenem MIC (mg/L)</th>
<th>Penicillin G MIC (mg/L)</th>
<th>Rokitamycin MIC (mg/L)</th>
<th>Gatifloxacin MBC (mg/L)</th>
<th>Levofloxacin MBC (mg/L)</th>
<th>Meropenem MBC (mg/L)</th>
<th>Penicillin G MBC (mg/L)</th>
<th>Rokitamycin MBC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.03</td>
<td>0.06</td>
<td>0.004</td>
<td>0.004</td>
<td>0.25</td>
<td>0.03</td>
<td>0.125</td>
<td>0.008</td>
<td>0.008</td>
<td>0.5</td>
</tr>
<tr>
<td>B5</td>
<td>0.06</td>
<td>0.125</td>
<td>0.008</td>
<td>0.008</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0.008</td>
<td>0.008</td>
<td>0.5</td>
</tr>
<tr>
<td>B9</td>
<td>0.03</td>
<td>0.06</td>
<td>0.008</td>
<td>0.008</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0.008</td>
<td>0.008</td>
<td>0.5</td>
</tr>
<tr>
<td>B15</td>
<td>0.03</td>
<td>0.06</td>
<td>0.004</td>
<td>0.016</td>
<td>0.25</td>
<td>0.03</td>
<td>0.06</td>
<td>0.008</td>
<td>0.016</td>
<td>0.25</td>
</tr>
<tr>
<td>B24</td>
<td>0.016</td>
<td>0.03</td>
<td>0.008</td>
<td>0.004</td>
<td>0.5</td>
<td>0.03</td>
<td>0.03</td>
<td>0.008</td>
<td>0.004</td>
<td>0.5</td>
</tr>
<tr>
<td>B30</td>
<td>0.016</td>
<td>0.06</td>
<td>0.008</td>
<td>0.004</td>
<td>0.5</td>
<td>0.03</td>
<td>0.06</td>
<td>0.008</td>
<td>0.004</td>
<td>0.5</td>
</tr>
<tr>
<td>B33</td>
<td>0.03</td>
<td>0.06</td>
<td>0.016</td>
<td>0.004</td>
<td>0.5</td>
<td>0.03</td>
<td>0.06</td>
<td>0.008</td>
<td>0.004</td>
<td>0.5</td>
</tr>
</tbody>
</table>

By considering the above-mentioned breakpoints, all the isolates were highly susceptible to the test antimicrobials. For gatifloxacin, MICs and MBCs ranged from 0.016 to 0.06 mg/L and MBCs corresponded to the MIC values for all the isolates. Levofloxacin showed an MIC range from 0.03 to 0.125 mg/L and MBCs were equal to MICs, with the exception of two strains, for which the MBCs of levofloxacin were twice the respective MICs. MICs and MBCs of meropenem and penicillin G were in the range 0.004–0.016 mg/L, with MBCs being equal to MICs for all the isolates but two, which had MBC values twice the MIC of meropenem. MICs of rokitamycin corresponded to MBCs and ranged from 0.125 to 0.5 mg/L.

Bactericidal activity, as defined by a $3 \log_{10}$ reduction in viable count, occurred after 12 h exposure to levofloxacin at $4 \times \text{MIC}$ (and above) and gatifloxacin or meropenem at $8 \times \text{MIC}$. Penicillin G (at $8 \times \text{MIC}$) or rokitamycin (at $4 \times \text{MIC}$ and above) was bactericidal only after 24 h incubation. A $2 \log_{10}$ reduction in viable count was observed after 6 h with levofloxacin at $2 \times \text{MIC}$ (and above) and gatifloxacin at $8 \times \text{MIC}$. Generally, little or no kill was observed after only 3 h incubation for all the antibiotics tested (Figure 1a–e).

**Discussion**

Although the knowledge of susceptibility of *B. anthracis* to antimicrobials represents an important issue for evaluating resistance, standardized methods of testing and interpretative criteria are not available. Papers evaluating *B. anthracis* susceptibility to different antibiotics have outlined the occurrence of resistance to penicillin and production of β-lactamases.\(^{11-13}\)

The isolates investigated here have probably never encountered the test antimicrobials because of their ‘clinical history’, with the probable exception of penicillin G. All the isolates were susceptible to the drugs tested, with MICs and MBCs within a narrow range. Other authors have investigated the MIC of penicillin, levofloxacin and gatifloxacin for *B. anthracis*.\(^{11,13}\) The MIC data presented here are lower than those reported by these authors for penicillin G, but they are within the reported ranges for gatifloxacin and levofloxacin.

This study showed that, although all the strains tested were susceptible to the chosen antimicrobials, the killing rate was different among the antibiotics under evaluation.

The data suggest that the fluoroquinolones tested could represent a valid choice for anthrax therapy. Levofloxacin was bactericidal, killing 99.9% of the initial inoculum within 12 h at concentrations $4 \times \text{MIC}$ and gatifloxacin required concentrations $8 \times \text{MIC}$ to be bactericidal within 12 h, although killing activity was also present after 24 h at lower concentrations. Although considered for many years the antibiotic of choice for therapy of all forms of anthrax,\(^1\) penicillin G bactericidal activity was the weakest among the antimicrobials tested, particularly at low concentrations, whereas a better bactericidal activity was shown by meropenem.

Rokitamycin showed a slow killing rate and furthermore was interesting in that similar levels of bactericidal activity occurred at $1 \times$, $2 \times$, $4 \times$ or $8 \times \text{MIC}$.

Data obtained in this study of the killing rate could contribute to the choice of candidate antibiotics as alternatives in anthrax therapy. In fact, in the case of inhalation of *B. anthracis* spores, vegetative cells are initially involved in the progress of infection and a rapid killing by antibiotics of the vegetative forms would avoid bacterial dissemination and toxin production. The fluoroquinolones were particularly bactericidal and their known potency against other non-dividing bacteria (which may also be true against *B. anthracis*) could also be important.

In conclusion, *in vitro* results indicate that all the drugs tested may represent valid alternatives in the treatment of *B. anthracis* infections, although with different killing rates, even if more data have to be collected for a future *in vivo* study.
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References


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