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Selection of *Bacillus anthracis* isolates resistant to antibiotics

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***Objective*: Long-term therapy for anthrax might induce antimicrobial resistance in *Bacillus anthracis*. The aim of the present study was to investigate the potential of 18 different antibiotics to select resistant isolates of *B. anthracis*, (ST-1 and Sterne strains).**

***Methods*: Resistant isolates were selected by serial passages on brain heart infusion agar containing increasing concentrations of antibiotics (from the MIC upwards).**

***Results*: The MICs of ciprofloxacin, ofloxacin and levofloxacin increased from 0.125–0.25 to 8 mg/L, that of moxifloxacin increased from 0.03–0.06 to 8 mg/L, in both strains, and the MIC of garenoxacin increased from 0.015 to 0.5 mg/L for the ST-1 strain and from 0.03 to 8 mg/L for the Sterne strain. The MICs of tetracycline and minocycline increased from 0.125 to 2–8 mg/L and 0.06 to 1 mg/L, respectively. The MIC of vancomycin increased from 2.5 to 20 mg/L for the ST-1 strain and from 5 to 20 mg/L for the Sterne strain. Linezolid exhibited an MIC increase from 2 to 4 mg/L for both strains. The MIC of quinupristin/dalfopristin increased from 0.125 to 64–128 mg/L. Erythromycin demonstrated an MIC increase from 1 to 128 mg/L, that of clarithromycin increased from 0.125 to 8–64 mg/L and that of telithromycin increased from 0.06–0.125 to 1–4 mg/L. The clindamycin MIC increased from 0.125–0.25 to 8 mg/L. Penicillin G and amoxicillin MICs increased from <1 mg/L to 128–512 mg/L. Isolates made resistant to one fluoroquinolone exhibited cross-resistance to the other quinolones except the ST-1 mutant strain which remained susceptible to garenoxacin. Cross-resistance to fluoroquinolones did not correlate with resistance to other antibiotics.**

***Conclusion*: The ease with which *B. anthracis* can be made resistant *in vitro* suggests that close monitoring of patients treated for anthrax is mandatory.**

Keywords: anthrax, quinolones, macrolides, cross-resistance

Introduction

Anthrax has been considered a potential biological weapon for at least 60 years. Prevention of anthrax infection relies mainly on series of vaccinations and prolonged antibiotic treatment. Owing to limited global availability of the anthrax vaccine, most treatment strategies utilize antibiotics. In the recent bioterror attack in the USA in 2001, antibiotic prophylaxis was administered to ~32 000 individuals suspected to have been exposed to anthrax.[1] A bioterror attack, which results in inhalational anthrax, if aimed at a large non-selected population such as shopping mall visitors, stadium spectators, rail station users, etc., will result in the exposure of a large number of individuals, and their environment, to massive doses of antibiotics.

The drugs of choice for post-exposure prophylaxis include: penicillin G, amoxicillin, doxycycline, and ciprofloxacin or ofloxacin given for 60 days or more.[2,3]

The emergence of antibiotic resistance is a global phenomenon that is on the increase and is partially related to extensive antibiotic usage.[4] Long-term antibiotic therapy, as would be administered for anthrax, might induce antimicrobial resistance in *Bacillus anthracis* by the selection of resistant mutants.

Although natural resistance of *B. anthracis* to antibiotics has been documented only rarely, *in vitro* studies have shown that *B. anthracis* can develop resistance to ciprofloxacin, doxycycline and β-lactam antibiotics.[5–7] The mechanism of resistance of *B. anthracis* has not been fully explored; however, strains that are fluoroquinolone resistant owing to development of mutations

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in *gyrA*, *parC* and *gyrB* have been recently described by Price *et al.*[7] Our group has also isolated a fluoroquinolone-resistant mutant in a different *B. anthracis* strain, with a different mutation to that described by Price *et al.*[7] β-Lactam-resistant strains have been attributed to the derepression of cephalosporinase.[8] Doxycycline resistance was conferred on *B. anthracis* by transfection with a pBC16 plasmid carrying a tetracycline resistant gene, *tet*.[7]

The aim of this study was to determine *in vitro* whether *B. anthracis* could develop resistance to antibacterial agents belonging to various classes, in particular those used for treatment of anthrax.

Materials and methods

Antibacterial agents

The antibiotics tested in this study were: ofloxacin and levofloxacin (gifts from Aventis, Netanya Israel, and Aventis, Paris, France, respectively), ciprofloxacin and moxifloxacin (a gift from Bayer Leverkusen, Germany), garenoxacin (a gift from Bristol-Myers Squibb, Waterloo, Belgium), minocycline (Dexxon, Haifa, Israel), tetracycline (Sigma, Rehovot, Israel), penicillin G (Rafa Laboratories, Jerusalem, Israel), amoxicillin (GSK, Petach-Tikva, Israel), ceftriaxone (Roche, Tel-Aviv, Israel), vancomycin (Eli Lilly, Italy), erythromycin (Sigma, Rehovot, Israel), clarithromycin [Abbott (Promedico, Petach-Tiqva, Israel)], telithromycin and quinupristin/dalfopristin (a gift from Aventis, Paris, France), clindamycin and linezolid [a gift from Pharmacia (Agis), Bnei-Braq, Israel and Pharmacia, Kalamazoo, MI, USA] and rifampicin (Sigma) and Penicillin G, minocycline, vancomycin, erythromycin, rifampicin, clindamycin, linezolid, ceftriaxone, garenoxacin and quinopristin/-dalfopristin were each received as a dry laboratory powder and were dissolved in phosphate-buffered saline (PBS) (pH 7.2). Amoxicillin was dissolved in distilled water. Clarithromycin was dissolved in analytical acetone, and telithromycin and tetracycline were dissolved initially in two drops of acetic acid and ethanol (100%), respectively, and subsequently diluted in distilled water to the required concentration (at the final concentrations used these solvents had no demonstrable antibacterial activity). Antibiotic solutions were sterilized through 0.45 μM pore-size filters (Millipore S. A., Paris, France). Ofloxacin, levofloxacin, moxifloxacin and ciprofloxacin were obtained as injectable solutions.

Bacterial strains and growth conditions

Bacteria used in this study were two strains of *B. anthracis*, the Sterne veterinary vaccine strain (a gift from the Colorado Serum Institute, Denver, CO, USA) and the Russian strain (ST-1) purchased from a veterinary supply shop in Moscow, Russia. Neither strain is a human pathogen, as both lack a plasmid necessary to produce the capsule of the vegetative form. Bacterial spores were stored in sterile 30% glycerol in PBS, and were spread on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI, USA) and incubated overnight at 37°C to obtain single colonies (vegetative form). A single colony was inoculated into 10 mL of BHI broth and incubated overnight at 37°C. The grown bacteria were used in the experiments.

Determination of MICs

MICs were determined by the microdilution technique according to the NCCLS criteria for *Staphylococcus aureus*.[9,10]

The antibacterial agents to be tested were prepared as concentrated stock solutions in distilled water. Two-fold dilutions were used in a concentration range from 0.015 to 1024 mg/L (for vancomycin and ceftriaxone the concentration range was 0.625–160 mg/L) diluted in 100 μL of BHI broth and poured into wells of flat-bottomed microtitre plates (Nunc 96-well flat-bottomed microtitre plates; Nunc Inc., Roskilde, Denmark). A 10 μL volume, which contained 10^5 cfu/mL of *B. anthracis* ST-1 or Sterne strains, was then added. Following incubation of the plates for 18 h at 37°C in ambient air, the MICs were determined. The MICs were recorded as the lowest concentration that completely inhibited visible growth of the bacteria.[10]

Induction of resistance

Spores were induced to transform into their vegetative form by streaking a spore suspension onto a BHI agar plate and incubating for 18 h at 37°C. Induction of resistance was performed by transferring 10 colonies $\sim 2 \times 10^7$ cfu ($\sim 2 \times 10^6$ cfu/colony), using sterile tooth-picks from the original BHI agar plate, to a plate containing the initial MIC of each antibiotic, followed by incubation for 24 h at 37°C in ambient air, and thereafter to a plate containing the next two-fold higher concentration (diluted from the stock antibiotic solution). The same procedure was repeated for the 18 serial passages. All experiments were performed in triplicate.

Results

Induction of resistance

The results in Table 1 depict the induction of resistance to 18 antibacterial agents for the two strains of *B. anthracis*, ST-1 and Sterne.

Table 1. Induction of antibiotic resistance in *B. anthracis* ST-1 and Sterne strains

	MIC (mg/L)			
	ST-1 strain		Sterne strain	
Antibacterial agent	passage 0	passage 18	passage 0	passage 18
Ofloxacin	0.25	8	0.25	8
Ciprofloxacin	0.125	8	0.125	8
Moxifloxacin	0.03	8	0.06	8
Levofloxacin	0.125	8	0.125	8
Garenoxacin	0.015	0.5	0.03	8
Erythromycin	1.0	128	1.0	128
Telithromycin	0.06	1.0	0.125	4
Clarithromycin	0.125	8	0.125	64
Penicillin G	0.125	128	0.5	128
Amoxicillin	0.03	512	0.25	256
Ceftriaxone	20	80	20	80
Vancomycin	2.5	20	5	20
Tetracycline	0.125	2	0.125	8
Minocycline	0.06	1	0.06	1
Linezolid	2	4	2	4
Clindamycin	0.125	8	0.25	8
Quinupristin/dalfopristin	0.125	64	0.25	128
Rifampicin	0.25	256	0.25	256

Table 2. Cross-resistance of resistant ST-1 and Sterne isolates of *B. anthracis* to fluoroquinolones (expressed as MIC in mg/L)

MIC for passaged strain (passage 18) (mg/L)	Ofloxacin	Ciprofloxacin	Moxifloxacin	Levofloxacin	Garenoxacin	Tetracycline	Amoxicillin
B. anthracis ST-1							
ofloxacin (8)	–	2	4	4	0.25	0.125	0.06
ciprofloxacin (8)	2	–	1	4	0.125	0.06	0.06
moxifloxacin (8)	0.5	0.5	–	2	0.25	0.125	0.06
levofloxacin (8)	1	1	2	–	0.25	0.06	0.06
garenoxacin (0.25)	0.25	1	0.5	0.25	–	0.03	0.03
tetracycline (2)	0.125	0.125	1	0.125	0.125	–	0.5
amoxicillin (512)	0.03	0.03	0.06	0.03	0.06	0.03	–
B. anthracis Sterne							
ofloxacin (8)	–	4	4	8	8	0.125	0.06
ciprofloxacin (8)	4	–	4	8	8	0.06	0.06
moxifloxacin (8)	1	2	–	2	8	0.06	0.03
levofloxacin (8)	2	2	4	–	8	0.125	0.06
garenoxacin (8)	0.125	0.5	8	0.125	–	0.06	0.03
tetracycline (8)	0.125	0.125	0.125	0.25	0.06	–	0.125
amoxicillin (256)	0.125	0.125	0.125	0.25	0.125	0.125	–

The MICs of the fluoroquinolones (ofloxacin, ciprofloxacin, moxifloxacin, levofloxacin and garenoxacin) increased after 18 passages, five to eight double-dilution steps (32- to 256-fold) except for garenoxacin, which, despite an increase in five double-dilution steps (32-fold increase), remained in the susceptible range for the ST-1 strain. The MICs of the β-lactams increased by two double-dilution steps for ceftriaxone to 14 double-dilution steps (>16 000-fold increase) for amoxicillin. Vancomycin MICs increased by two to three double-dilutions (four- to eight-fold). The MICs of tetracycline and minocycline increased by three to five double-dilutions (16- to 64-fold). For the macrolides (erythromycin and clarithromycin) and for the ketolide (telithromycin), the MICs increased by four to nine double-dilutions (16- to 512-fold). Despite a four double-dilutions increase (16-fold) in the MIC of telithromycin for the ST-1 isolate, it remained in the susceptible range. Clindamycin MIC increased by five to six double-dilutions (32- to 64-fold). The MIC of quinupristin/dalfopristin increased by nine double-dilutions (512-fold), that of rifampicin by 10 double-dilutions (1024-fold) and that of linezolid increased by a single double-dilution, but remained in the susceptible range.

Cross-resistance of antibiotics

Organisms that became resistant to one fluoroquinolone were also resistant to the other fluoroquinolones, i.e. they were cross-resistant. The MICs of the heterologous quinolones were two- to 16-fold lower than the MIC of the homologous agent. Susceptibility to garenoxacin in strain ST-1 was an exception, since this strain showed no cross-resistance to other fluoroquinolones (Table 2). In the Sterne strain (Table 2), ofloxacin- and ciprofloxacin-resistant isolates exhibited similar MICs of levofloxacin. No cross-resistance was observed in fluoroquinolone-resistant isolates, against amoxicillin and tetracycline (Table 2).

In the ST-1 strain made resistant to telithromycin, only erythromycin showed cross-resistance, while the MICs of clindamycin, quinupristin/dalfopristin and clarithromycin remained unchanged (Table 3). In the telithromycin-resistant Sterne isolate, only the clindamycin MIC was unchanged, while those of the macrolides increased (Table 3). For both the ST-1 and Sterne quinupristin/dalfopristin-resistant isolates, the MICs of clindamycin and the macrolides increased significantly (Table 3).

Table 3. Cross-resistance of resistant ST-1 and Sterne isolates of *B. anthracis* to macrolides, ketolide, clindamycin and quinupristin/dalfopristin (expressed as MIC in mg/L)

MIC for passaged strain (passage 18) (mg/L)	Telithromycin	Quinupristin/ dalfopristin	Erythromycin	Clarithromycin	Clindamycin
B. anthracis ST-1					
telithromycin (1)	–	0.125	4	0.125	0.06
quinupristin/dalfopristin (64)	16	–	64	16	8
erythromycin (128)	8	4	–	4	4
clarithromycin (8)	0.5	0.5	32	–	0.5
clindamycin (8)	1	0.25	0.5	0.5	–
B. anthracis Sterne					
telithromycin (4)	–	1	4	2	0.125
quinupristin/dalfopristin (128)	16	–	64	16	8
erythromycin (128)	16	16	–	32	4
clarithromycin (64)	16	8	32	–	1
clindamycin (8)	1	0.25	0.5	0.125	–

Erythromycin-resistant ST-1 and Sterne isolates exhibited high MICs of clarithromycin, telithromycin, clindamycin and quinupristin/dalfopristin (Table 3), while clarithromycin-resistant isolates had lesser increases in the MICs of the other agents. The results of experiments performed in triplicate were consistent.

Discussion

The results of this study show that *in vitro* increases in MIC for *B. anthracis* occur with various antibacterials belonging to different classes. Resistance within an antibiotic class may vary among the different class members.

With the fluoroquinolones, during the first eight to 10 passages no increases in MICs were observed. From then on, resistance was induced rapidly (data not shown), and at the eighteenth passage the MICs increased up to 8 mg/L. In other experiments, which utilized 30 similar passages, the MICs of these fluoroquinolones increased to 16–64 mg/L (data not shown). The lowest increase (from 0.015 to 0.5 mg/L) in the MIC of garenoxacin was observed in the ST-1 strain. Similar results have been reported in previous studies.[5,11,12] Price *et al.*[7] demonstrated in a ΔANR *B. anthracis* strain (plasmid-cured Aims strain) a stepwise increase in the MIC of ciprofloxacin from 0.06 to 64 mg/L, following repeated passages in increasing ciprofloxacin concentrations. This increase in MIC was associated with mutations appearing first in the *gyrA* quinolone resistance-determining region (QRDR). Second mutants appeared when the MIC reached 8–16 mg/L in the *parC* QRDR, and third mutants appeared when the MIC reached 64 mg/L in the *gyrA* or *gyrB* QRDR. In addition, mutants with an MIC ≥32 mg/L had evidence of the ciprofloxacin efflux mechanism. In the present study, in which different *B. anthracis* strains were used, similar resistance levels were obtained. In unpublished work, a mutation in the *gyrA* was discovered by our group at a position different from that described by Price *et al.*,[7] indicating the universality of this resistance selection phenomenon.

Fluoroquinolone-resistant isolates of both ST-1 and Sterne strains demonstrated, as expected, cross-resistance with the other fluoroquinolones. Resistance to macrolides, the ketolide telithromycin and to quinupristin/dalfopristin was also inducible in both *B. anthracis* strains. The number of passages required for induction of resistance varied between a single passage for quinupristin/dalfopristin, telithromycin and erythromycin, to nine passages for clindamycin (data not shown). Thus the development of resistance occurred earlier with this group of agents, except clindamycin, than with the fluoroquinolones. These results also are in accordance with previous observations.[5,11,12] Cross-resistance among the agents of this group of antibiotics, which possess a similar mechanism of action, was also demonstrable. Telithromycin was the weakest resistance inducer, particularly with the ST-1 strain, while quinupristin/dalfopristin was the strongest resistance inducer in the two strains.

Resistance to the β-lactam agents was intrinsic in both strains and was observed from the first passage onwards. The increment in the MIC was as high as >16 000 for amoxicillin in the ST-1 strain after the eighteenth passage, and ranged between 250- and 1000-fold for penicillin G and amoxicillin for the other isolates. Remarkably, the original ceftriaxone MIC was in the non-susceptible range (20 mg/L), but the increase in the MIC was only four-fold following 18 passages (data not shown). Penicillin-resistant natural *B. anthracis* strains have been reported in 2–16% of historical and recent US strains, but also in strains collected from animals in South Africa,[4,9] and exposure to β-lactams has also been reported to induce penicillin resistance in *B. anthracis*.[9,13,14] The mechanism underlying β-lactam resistance is due to the presence of two β-lactamases, *bla1* and *bla2*, with *bla1* being a penicillinase and conferring high-level resistance to ampicillin, amoxicillin and penicillin G, while *bla2* is a cephalosporinase conferring low-level resistance to ceftriaxone, cefazolin, cefoxitin and cefotetan.[8]

Rifampicin-resistant isolates were induced after a single passage, stressing the threat for development of resistance should this agent be used to eradicate the rapidly phagocytosed *B. anthracis* in the pulmonary compartment. In a previous observation, a spontaneous rate of rifampicin resistance was calculated to be 1.57×10^{-9}. In UV-irradiated *B. anthracis*, spontaneous rifampicin-resistant mutants and attenuated *B. anthracis* mutations conferring rifampicin resistance were similar to those observed in *Escherichia coli* and *Mycobacterium tuberculosis*. In addition, new mutations at different sites (positions 450 and 468) considered to be rifampicin-binding sites also observed.[15] The rifampicin-resistant isolates of this study have not yet been analysed, but we suspect that the resistance mechanisms might be similar.

Tetracycline is one of the agents of choice for the treatment of post-exposure prophylaxis of inhalational anthrax. Doubling of the original MIC occurred after three to five passages, reaching an MIC of 8 mg/L on passage 18 for the Sterne strain and an MIC of 2 mg/L for the ST-1 strain (data not shown). Thus no 'real' resistance was observed for this class of agents, as the breakpoint according to the NCCLS is >16 mg/L (for *S. aureus*).[10] Increases in the MICs, although less pronounced, were also observed with minocycline in the two strains. Previous investigators have increased the MIC of doxycycline in the Sterne strain from 0.025 to 0.1 mg/L following 14–21 passages, the MIC still being in the susceptible range for this agent as well.[5] Nevertheless, *B. anthracis* strains that carried a tetracycline-resistant plasmid did not respond *in vivo* to tetracycline therapy.[6] These results may suggest that this class of agents might be the preferred agent(s) compared with the fluoroquinolones and β-lactams, as long as the *B. anthracis* infecting strain does not carry a tetracycline resistance plasmid.

The overall results of this study demonstrate the potential for development of resistance in *B. anthracis* to antibiotics recommended for prophylaxis and treatment of anthrax.

Since treatment of anthrax is rather prolonged, continuous surveillance of susceptibility of *B. anthracis*, even if the original isolates are antibiotic susceptible, needs to be incorporated in public health measures of anthrax outbreak.

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References

1. Centers for Disease Control and Prevention. (2001). Update: Investigation of bioterrorism-related anthrax and adverse events from

antimicrobial prophylaxis. *MMWR Morbidity and Mortality Weekly Report* **50**, 973–6.

2. Centers for Disease Control and Prevention. (2001). Update: Interim recommendations for antimicrobial prophylaxis for children and breastfeeding mothers and treatment of children with anthrax. *MMWR Morbidity and Mortality Weekly Report* **50**, 1014–6.

3. Inglesby, T. V., O'Toole, T., Henderson, D. A. *et al.* (2002). Anthrax as a biological weapon, 2002: updated recommendations for management. *Journal of the American Medical Association* **287**, 2236–52.

4. Levy, S. B. (1998). Multidrug resistance: a sign of the times. *New England Journal of Medicine* **338**, 1376–8.

5. Brook, I., Elliott, T. B., Pryor, H. I. *et al.* (2001). *In vitro* resistance of *Bacillus anthracis* Sterne to doxycycline, macrolides and quinolones. *International Journal of Antimicrobial Agents* **18**, 559–62.

6. Pomerantsev, A. P., Shishkova, N. A. & Marinin, L. I. (1992). Comparison of therapeutic effects of antibiotics of the tetracycline group in the treatment of anthrax caused by a strain inheriting *tet*-gene of plasmid pBC16. *Antibiotiki i Khimioterapiia* **37**, 31–4.

7. Price, L. B., Volger, A., Pearson, T. *et al.* (2003). *In vitro* selection and characterization of *Bacillus anthracis* mutants with high-level resistance to ciprofloxacin. *Antimicrobial Agents and Chemotherapy* **47**, 2362–5.

8. Chen, Y., Succi, J., Tenover, F. C. *et al.* (2003). β-Lactamase genes of the penicillin-susceptible *Bacillus anthracis* Sterne strain. *Journal of Bacteriology* **185**, 823–30.

9. Mohammed, M. J., Marston, C. K., Popovic, T. *et al.* (2002). Antimicrobial susceptibility testing of *Bacillus anthracis*: comparison of results obtained by using the National Committee for Clinical Laboratory Standards broth microdilution reference and Etest agar gradient diffusion methods. *Journal of Clinical Microbiology* **40**, 1902–7.

10. National Committee for Clinical Laboratory Standards (1997). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Fourth Edition: Approved Standard M7-A4*. NCCLS, Wayne, PA, USA.

11. Choe, C. H., Bouhauala, S., Brook, I. *et al.* (2000). *In vitro* development of resistance to ofloxacin and doxycycline in *Bacillus anthracis*. *Antimicrobial Agents and Chemotherapy* **44**, 1766.

12. Bryskier, A. (2002). *Bacillus anthracis* and antibacterial agents. *Clinical Microbiology and Infectious Diseases* **8**, 467–78.

13. Cavallo, J. D., Ramisse, F., Girardet, M. *et al.* (2002). Antibiotic susceptibilities of 96 isolates of *Bacillus anthracis* isolated in France between 1994 and 2000. *Antimicrobial Agents and Chemotherapy* **46**, 2307–9.

14. Òdendaal, M. W., Pieterson, P. M., de Vos, V. *et al.* (1991). The antibiotic sensitivity patterns of *Bacillus anthracis* isolated from the Kruger National Park. *Onderstepoort Journal of Veterinary Research* **58**, 17–9.

15. Volger, A. J., Busch, J. D., Percy-Fine, S. *et al.* (2002). Molecular analysis of rifampicin resistance in *Bacillus anthracis* and *Bacillus cereus*. *Antimicrobial Agents and Chemotherapy* **46**, 511–3.