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In Vitro Susceptibility of *Bacillus anthracis* to Antimicrobial Agents Including Carbapenems and Fosfomycin

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Summary. ***Bacillus anthracis*** **strains were tested for their in vitro susceptibilities to 25 antimicrobial agents, including carbapenems and fosfomycin. Ciprofloxacin, doxycycline, and amoxicillin were highly active against the *B. anthracis* strains, as reported earlier. Carbapenems as well as clarithromycin and clindamycin also showed considerable activity. Fosfomycin showed moderate levels of MIC against the *B. anthracis* strains, regardless of the presence of glucose-6-phosphate or blood. This glucose-6-phosphate-independent manner was also observed with the *B. cereus* strains. The data showed that, in addition to the drugs reported earlier, carbapenems are also active against *B. anthracis*, and that a fosfomycin-transport system in *B. anthracis* is distinct from the glucose-6-phosphate-inducible type in *Escherichia coli* or *Stapylococcus aureus*.**

Key words—*Bacillus anthracis*, *Bacillus cereus*, antimicrobial agents, carbapenems, fosfomycin, in vitro susceptibility.

INTRODUCTION

Bacillus anthracis, an endospore former, is the causative agent of anthrax. Anthrax is a zoonotic disease usually found in bovine, and direct human-to-human transmission is not known[1]. Human anthrax is caused by contact with contaminated animals or meats. Three types of anthrax are found in humans[1,2]. They include cutaneous (the most common form), pulmonary, and intestinal cases. Cutaneous anthrax is usually curable. Systemic infection mainly results from inhalation of the *B. anthracis* spore. This is followed by massive bacteremia, and results in severe anthrax toxin-mediated complications with shock and sudden death, with the mortality rate approaching 100%[1,2]. Athrax meningitis is rare but almost always fatal. In Japan, no human anthrax has been reported since 1995, and rare animal (bovine) anthrax since 1992[3].

In 2001, bioterrorism using anthrax spores as a biologic weapon[2] was leased in the United States with a total of 18 people infected (7 cutaneous cases and 11 inhalational cases), including a 7-month-old infant, and 5 deaths (all inhalational cases)[4]. Mailed letters have been identified as a vehicle of the *B. anthracis* spore[5].

Ciprofloxacin and doxycycline have been recommended for treatment of cutaneous as well as inhalational anthrax, and for prophylaxis for inhalational anthrax exposure for both adults and children[2,5]. In general, however, use of the two antimicrobial agents has not been recommended in Japan for children. Instead, fosfomycin has been recommended for treatment of children who experience (e.g.) enterohemorrhagic *Escherichia coli* (O157: H7) infection[6]. In vitro susceptibility data of *B. anthracis* to fosfomycin and carbapenems have not been reported.

In this study, we investigated the in vitro susceptibility of *B. anthracis* to 24 antimicrobial agents including fosfomycin and carbapenems, and the data were compared with that of *B. cereus* (an agent associated with food-poisoning).

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MATERIALS AND METHODS

Bacterial strains

B. anthracis strains (three strains) used in this study were from stock cultures in this laboratory. *B. cereus* strains (27 strains) were from patients or foods associated with food-poisoning in 2001. Enterohemorrhagic *E. coli* (serotype O157: H7) strains were derived from large outbreaks in Japan, 1996. The strains were stored at −80℃ in 3% skim milk (Difco Laboratories, Detroit. Mich. U.S.A) supplemented with 5% glucose (Difco).

Media and bacterial growth

For bacterial growth, we used L broth (Difco Laboratories, Detroit. Mich., U.S.A) as liquid media which was inoculated and incubated at 37℃ for 12–18 h with agitation. Nutrient agar (Eiken Chemical, Tokyo) and 5% sheep blood agar (Becton Dickinson, Tokyo) were used as solid media.

Antimicrobial agents

The antimicrobial agents were a gift from their manufacturers. They included: ampicillin (Meiji Seika, Tokyo), amoxicillin (Fujisawa Pharmaceutical Co., Tokyo) and benzylpenicillin (Banyu Pharmaceutical Co., Tokyo) for penicillins; cefazolin (Fujisawa Pharmaceutical Co.), cefotiam (Takeda Chemical Industries, Osaka), ceftriaxone (Nippon Roche, Tokyo), and cefozopran (Takeda Chemical Industries) for cefems; flomoxef (Shionogi & Co., Osaka) for Oxacephem; imipenem (Banyu Pharmaceutical Co.), panipenem (Sankyo Co., Tokyo), and meropenem (Sumitomo Pharmaceuticals Co., Osaka) for carbapenems; vancomycin (Shionogi) and Teicoplanin (Aventis Pharma Ltd., Tokyo) for glycopeptides; erythromycin (Shionogi), roxithromycin (Aventis Pharma), clarithromycin (Taisho Pharmaceutical Co., Tokyo), and azithromycin (Pfizer Pharmaceuticals Inc., Tokyo) for macrolides; clindamycin (Pharmacia, Tokyo); tetracycline (Wyeth Lederle Japan, Tokyo), doxycycline (Pfizer Pharmaceuticals), and minocycline (Wyeth Lederle Japan) for tetracyclines; norfloxacin (Daiichi Pharmaceutical Co., Tokyo) and ciprofloxacin (Bayer Yakuhin, Osaka) for quinolones; and fosfomycin (Meiji Seika). Furazolidone, 3-(5-nitrofurfurylideneamino)-2-oxazolidinone, was kindly provided by G. Balakrish Nair (National Institute of Cholera and Enteric Diseases, Culcutta, India).

Reagents

D-Glucose-6-phosphate disodium salt was purchased from Oriental Yeast Co. (Tokyo).

Susceptibility testing

Susceptibility testing of the bacterial strains was done by the agar dilution method with Mueller-Hinton agar according to standard procedures[7,8]. The final concentrations of antimicrobial agents were from 0.002 to 128 μg/ml. The test bacteria were grown for 12 to 18 h at 37℃ with agitation in L broth, and diluted to approximately 10^6 CFU/ml. Aliquots of the bacterial suspension (approximately 10^4 CFU of bacteria per spot) were inoculated on the surface of the antimicrobial agent-containing agar plates. Incubation was for 18 h at 35℃. The MIC was determined as previously described[7,8]. *E. coli* NIHJ JC-2 was used as a reference strain for quality control[8]. When the susceptibility to fosfomycin was tested, Mueller-Hinton agar supplemented with glucose-6-phosphate (at a concentration of 5 or 50 μg/ml) or with 10% (vol/vol) defibrinated horse blood (frozen and thawed) was also used, in addition to Mueller-Hinton agar alone[9]. The inoculated agar plates were also subjected to incubation in an anaerobic atmosphere.

RESULTS

In vitro susceptibility of B. anthracis

The MICs of the antimicrobial agents against the *B. anthracis* strains are summarized in Table 1. Among the antimicrobial agents tested, penicillins (ampicillin, amoxicillin, and benzylpenicillin), carbapenems (imipenem, panipenem, and meropenem), tetracyclines (tetracycline, doxycycline, and minocycline), and ciprofloxacin showed the greatest activity (MICs, $\leq$ 0.03 μg/ml). Clarithromycin and clindamycin also showed significant activity (MICs, $\leq$ 0.06 μg/ml). The *B. anthracis* strains were resistant to some of the broad-spectrum cephems, especially ceftriaxone and cefozopran (MICs, $\geq$ 2 μg/ml). Flomoxef (an oxacephem) showed more activity compared with ceftriaxone and cefozopran.

The MICs of fosfomycin were of a moderate level ($\geq$ 2 μg/ml, Table 1). The addition of glucose-6-phosphate or blood in the presence or absence of an anaerobic atmosphere showed no significant change in the MIC levels for fosfomycin.

Comparison with B. cereus

In many cases, the *B. anthracis* strains were more susceptible to the microbial agents than were the *B. cereus* strains (Table 1). The *B. cereus* strains were also highly susceptible to doxycycline (MICs, $\leq$ 0.06 μg/ml), but resistant to a wider range of β-lactam

Table 1. MICs of antimicrobial agents for the *B. antharcis* and *B. cereus* strains

Antimicrobial agents	MIC (μg/ml) against			
	B. anthracis (n=3)	*B. cereus* (n=27)		
	Range	Range	50%	90%
Penicillins				
Ampicillin	0.008-0.03	0.12-2	2	2
Amoxicillin	0.015	0.06-4	2	4
Benzylpenicillin	0.015-0.03	1-16	4	16
Cephems				
Cefazolin	0.06-0.12	0.25-32	8	32
Cefotiam	0.008-2	4-128	8	32
Ceftriaxone	4-32	8-64	32	64
Cefozopran	2-32	16-32	32	32
Oxacephem				
Flomoxef	0.008-0.5	0.5-4	1	1
Carbapenems				
Imipenem	0.015-0.03	0.03-1	0.06	0.06
Panipenem	0.008-0.015	0.015-0.5	0.03	0.06
Meropenem	0.03	0.03-0.5	0.06	0.12
Glycopeptides				
Vancomycin	0.5-1	0.5-1	0.5	1
Teicoplanin	0.008-0.25	0.12-0.25	0.125	0.25
Macrolides				
Erythromycin	0.06-0.5	0.06-0.12	0.06	1
Roxithromycin	0.008-0.25	0.12-0.5	0.25	0.25
Clarithromycin	0.03-0.06	0.06-0.12	0.06	0.12
Azithromycin	0.12-0.5	0.5-2	0.5	0.5
Clindamycin	0.008-0.06	0.06-0.12	0.125	0.125
Tetracyclines				
Tetracycline	0.008-0.015	0.12-2	0.25	0.5
Doxycycline	0.008	0.015-0.06	0.03	0.06
Minocycline	0.008	0.063-1	0.12	0.12
Quinolones				
Norfloxacin	0.008-0.25	0.12-0.5	0.25	0.5
Ciprofloxacin	0.008-0.03	0.06-0.12	0.12	0.25
Others				
Fosfomycin	2-16	1-32	4	16
Fosfomycin[a)]	2-16	1-32	4	32
Fosfomycin[b)]	2-16	1-32	8	32
Furazolidone	0.25-0.5	0.5-1	0.5	1

a) MICs determined in the presence of 5 μg/ml of glucose-6-phosphate.

b) MICs determined in the presence of 50 μg/ml of glucose-6-phosphate.

Table 2. MICs of fosfomycin in the presence or absence of glucose-6-phosphate

Strain	MIC (μg/ml) in the presence of:		
	none	glucose-6-phosphate	
		5 μg/ml	50 μg/ml
Enterohemorrhagic *E. coli* (O157: H7)			
S1	8	0.5	0.25
F31	128	0.5	0.5
B. anthracis			
PS1	4	4	4

antimicrobial agents than was the *B. anthracis*. In vitro susceptibility of the *B. cereus* strains to fosfomycin was also at a moderate level, and exhibited a glucose-6-phosphate-independent manner, similar to the *B. anthracis* (Table 1).

DISCUSSION

Bioterrorism using *B. anthracis* spores occurred in the United States in October, 2001[4,5]. The United States Government recommended the use of ciprofloxacin and doxycycline for treatment of inhalation anthrax and cutaneous anthrax[4].

Tetracyclines, including doxycycline, have side-effects of teeth discoloration and bone growth inhibition, and thus its use for treatment is generally not recommended in children of less than 8 years of age[10]. Fluoroquinolones, including ciprofloxacin, have central nervous toxicity[10]. Again, the use of the agents is not recommended for children, with the exception of norfloxacin in Japan.

Fosfomycin has a good safety record in children in Japan[6]. For instance, fosfomycin has been the first choice of antimicrobial agents for treatment of enterohemorrhagic *E. coli* infections in both adults and children[6]. Carbapenems have been an important agent for the treatment of sepsis due to (e.g.) *Pseudomonas aeruginosa*[11]. Furazolidone is often recommended for treatment of cholera[12]. In this study, we investigated the in vitro susceptibilities of *B. anthracis* to 25 antimicrobial agents, including carbapenems and fosfomycin.

Carbapenems (imipenem, panipenem, and meropenem) showed the greatest activity among the agents examined, even against cephem-resistant strains. The activity was comparable to those of penicillins (ampicillin, amoxicillin, and benzylpenicillin), tetracyclines (tetracycline, doxycycline, and minocycline), and ciprofloxacin.

Penicillins have been labeled for use in treating inhalational anthrax. However, since the *B. anthracis* isolated from the bioterrorism samples in the United States seemed to possess constitutive and inducible β-lactamases, treatment of systemic anthrax using penicillin alone is not recommended[5]. Indeed, all the *B. anthracis* strains used in this study were resistant to ceftriaxone (a 3rd-generation cephem) and cefozopran (a 4th-generation cephem), probably due to cephalosporinase (one of β-lactamases). Some of the strains also showed resistance to cefotiam (a 2nd-generation cephem) at a moderate level. Penicillin-resistant *B. anthracis* (producing penicillinase) has been reported[13]. Further studies investigating the in vitro activity against the penicillin-resistant *B. anthracis* are necessary for carbapenems.

The MICs of fosfomycin to *B. anthracis* were at a moderate level and comparable to those for *B. cereus*. Fosfomycin is transported into bacterial cells via (at least) two transport systems, the sn-glycerol 3-phosphate transport (GlpT) system and the hexose phosphate transport (UhpT) system[9]. The latter is inducible by glucose-6-phosphate. Therefore, MICs of fosfomycin can be lowered by adding glucose-6-phosphate to the medium[9], in (e.g.) *E. coli* and *Staphylococcus aureus*[9], as shown in Table 2. In contrast, the MICs of fosfomycin to *B. anthracis* and *B. cereus* were not affected by the addition of glucose-6-phosphate. There is a possibility that fosfomycin is transported mainly by the GlpT system in *B. anthracis* (and *B. cereus*).

Fosfomycin activity estimated in vitro is a function of the assay conditions[9], as reported above. Fosfomycin achieves a serum concentration of 166 μg/ml following intravenous injection at 50 mg/kg[14]. Further studies are necessary to evaluate the protective effect of fosfomycin against *B. anthracis* infection in vivo. Clarithromycin and clindamycin, which showed relatively good in vitro activity, may also be an alternative for treatment of a *B. anthracis* infection.

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