

Antimicrobial Susceptibilities of Diverse *Bacillus anthracis* Isolates

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Received 21 March 2002/Returned for modification 29 July 2002/Accepted 5 September 2002

A test of 25 genetically diverse isolates of *Bacillus anthracis* was conducted to determine their susceptibility to seven clinically relevant antimicrobial agents. Etest strips (AB BIODISK, Solna, Sweden) were used to measure the MICs for the isolates. Using the National Committee for Clinical Laboratory Standards MIC breakpoints for staphylococci, three isolates were found to be resistant to penicillin and five were found to be resistant to cefuroxime. The penicillin-resistant isolates were negative for β -lactamase production. Continued surveillance of *B. anthracis* field isolates is recommended to monitor antimicrobial susceptibility.

The etiologic agent of anthrax, *Bacillus anthracis*, causes an acute disease, primarily of herbivores, that is transmissible to humans. Susceptible animals are primarily infected by the spores formed during the vegetative state of the bacteria. Spores may be found in soil contaminated by diseased animals or in diseased animal products, such as hair, wool, hides, and bones. The importance of treatment of the disease in humans has been underscored by the bioterrorism events of October 2001 in the United States. Ciprofloxacin was the antimicrobial agent of choice for prophylactic treatment after exposure to the spores of *B. anthracis* that were used in the bioterrorism events. Penicillin, traditionally the drug of choice for treatment, is still recommended in other parts of the world despite reports of penicillin resistance (2, 3). The Centers for Disease Control recommended ciprofloxacin, penicillin, and doxycycline for the treatment of human anthrax and for use as a prophylactic measure prior to and during the October event (1). In the past, streptomycin, penicillin, gentamicin, and chloramphenicol have also been recommended (7).

Other articles concerning *B. anthracis* and antimicrobial susceptibility have been written: two over 10 years ago and one within the past year. The first paper, published in 1990 by Lightfoot et al., determined the antimicrobial susceptibility of 70 isolates of *B. anthracis* to penicillin, amoxicillin, cefuroxime, gentamicin, streptomycin, erythromycin, tetracycline, chloramphenicol, and ciprofloxacin by agar dilution (6). Penicillin resistance and β -lactamase production were noted in two isolates. This paper was quickly followed in 1991 by a paper by Doganay and Aydin (2). They tested 22 *B. anthracis* isolates against 27 antimicrobial agents by agar dilution and reported that 19 isolates showed resistance to the five broad-spectrum cephalosporins tested. The isolates were shown to be sensitive to the other antimicrobials tested. These two papers were very basic in their approach and reporting. The latest paper, by Mohammed et al., tested 65 isolates with nine antimicrobial agents by using the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method (7). The results obtained from 50 of the isolates were compared to

those obtained with Etest strips. One isolate was penicillin resistant, and no statistically significant difference was found between the broth microdilution and Etest results.

The two earlier papers distinguished between sensitive and resistant with regard to the MIC noted for each isolate (2, 6). Unfortunately, no basis was given for the standard they used for these designations. The paper by Mohammed et al. used the NCCLS breakpoints for *Staphylococcus*, which we have also chosen to use to discriminate between sensitive (S), intermediate (I), and resistant (R) isolates. According to the NCCLS literature, studies of *Bacillus* spp. are not yet adequate to develop reproducible, definitive standards to interpret results (8). The infections caused by *Staphylococcus* spp. are very similar to the infections caused by *B. anthracis*. In addition, the distributions of the aforementioned antimicrobial data are in line with the *Staphylococcus* MIC breakpoints for the antimicrobials used in this study.

In this study, we examined 25 genetically diverse isolates of *B. anthracis* to determine their susceptibilities to seven clinically relevant antimicrobial agents. Etest strips (AB BIODISK, Solna, Sweden) were used to determine the MICs for the isolates.

MATERIALS AND METHODS

A total of 25 isolates diverse in time, space, and genotype were used in this study (Table 1). These 25 isolates are a representative subset of 89 distinct genotypes characterized by multiple-locus variable-number tandem repeat analysis (5).

MICs of the seven antimicrobial agents penicillin, cephalixin, cefaclor, tobramycin, doxycycline, ciprofloxacin, and cefuroxime were determined by using the Etest methodology described elsewhere (4). Tryptic soy agar (TSA) plates containing 5% sheep blood (Remel) were inoculated with a swab taken from a colony suspension equal to that of a 0.5 McFarland standard. One Etest strip was placed in each plate after 10 min. Quality control was assessed with *Staphylococcus aureus* ATCC 29213. The plates were incubated overnight at 37°C, and the plates were read at 16 and 24 h. This procedure was repeated three times for each isolate.

The β -lactamase activity of each isolate was determined with nitrocefin disks (BBL), which utilize a chromogenic cephalosporin. A drop of sterilized water was applied to the disk, and then a loop was used to remove a colony from the agar plate and it was applied to the disk. The inoculum was taken from an overnight TSA plate for each isolate. A positive reaction was denoted by the formation of a pink or red color. The reactions were held for 1 h to confirm negative results.

RESULTS

The MIC results are summarized in Table 2. Using the *Staphylococcus* MIC breakpoints, all isolates were susceptible to the antimicrobials, except for cefuroxime and penicillin.

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TABLE 1. *B. anthracis* isolates used by genotype and cluster

Geno- type	Cluster	Country	State/location	Source	Yr isolated
3	A1a	Canada	Unknown	Bovine	1974
10	A1a	United States	South Dakota	Bovine	1996
15	A1a	Poland	Krakow	Bovine	1962
20	A1a	Italy	Campania	Ovine	1994
23	A1b	Turkey	Yozgat	Human	1991
25	A1b	United States	Florida	Bovine	1937
28	A1b	Turkey	Sivas	Human	1984
29	A2	Pakistan	Unknown	Ovine	1978
30	A3a	Zambia	Mongu	Bovine	1992
34	A3a	South Korea	Kyungjoo	Human	1994
35	A3a	Namibia	Etosha Nat'l Park	Zebra	1993
38	A3a	Germany	Giessen	Porcine	1971
41	A3a	Turkey	Sivas	Human	1985
45	A3a	Argentina	Buenos Aires	Bovine	1980
51	A3a	United States	Maryland	Bovine	1939
55	A3a	Australia	Queensland	Bovine	1994
57	A3b	China	Changping	Bovine	Unknown
62	A3b	United States (Ames)	Texas	Bovine	1981
67	A3a	South Africa	Kruger National Park	Kudu	1993
68	A3d	United States	Ohio	Human	1968
69	A4	Pakistan	Unknown	Wool	1976
77	A4	United Kingdom (Vollum)	S. Oxfordshire	Bovine	1937
80	B1	France	Unknown	Bovine	1997
85	B2	Mozambique	Maputo	Porcine	1944
87	B2	South Africa	Kruger National Park	Kudu	1975

Only one isolate, GT 69, was sensitive. Five isolates (GT 23, 28, 34, 55, and 77) were resistant, and the remaining 19 isolates were categorized as intermediate to cefuroxime. The MIC at which 90% of the isolates tested are inhibited (MIC₉₀) was 32 µg/ml, which is considered the breakpoint for resistance. The MIC range for cefuroxime was wide: from 6 to 48 µg/ml.

Of the three recommended antimicrobials for the treatment of anthrax, ciprofloxacin and doxycycline exhibited potent activities against the *B. anthracis* isolates. Twenty-two of the isolates were susceptible to penicillin, and 3 (GT 20, 68, and 85) were classified as resistant. None of the isolates examined showed any β-lactamase activity. Ciprofloxacin, the drug of choice, had a MIC range of 0.032 to 0.38 µg/ml, with a MIC₉₀ of 0.094 µg/ml. This was the same as the MIC₅₀ and was the most active agent tested. All of the isolates were susceptible to doxycycline and the other antimicrobials: cephalixin, cefaclor, and tobramycin.

DISCUSSION

In this study, we have examined a spatially, temporally, and genetically diverse group of *B. anthracis* isolates for susceptibility to seven clinically relevant antimicrobials. This is the first report to use confirmed genetically diverse field isolates (5). Lightfoot et al. used 70 isolates, 33 of which were considered by the authors to be unrelated (6). Doganay and Aydin used 22 isolates collected from the same region in Turkey within a period of 7 years (2). The most recent paper, by Mohammed et al., used 50 historical and 15 recent isolates (7). The 50 historical isolates, collected between 1937 and 1997, were selected as representatives of temporally and spatially diverse strains. To-

gether, these studies have utilized 182 isolates to test antimicrobial susceptibilities. Unfortunately, the detailed isolate information from previous studies could not be obtained, and, therefore, no correlations or comparisons can be made genetically, spatially, or temporally.

Cefuroxime is a broad-spectrum cephalosporin and is reported to be effective against gram-positive bacteria. The older studies showed a high rate of resistance with cefuroxime, although the MIC₉₀s were lower in this study. The MIC₉₀ in the other studies was 64 µg/ml, and for this study, it was 32 µg/ml. This could be an artifact of the methodologies or could reflect a true difference in the isolates; however, in any case, the reason for this resistance is unknown, and the mechanism of resistance needs to be investigated further.

Penicillin and ciprofloxacin were also examined in all studies, and both agents showed good activity against the isolates of *B. anthracis*. Differences in the MIC₉₀s between the reports have been noted.

Penicillin resistance was reported in two of the other studies. Lightfoot et al. reported resistance (MIC > 0.25 µg/ml) in two isolates that originated from the same fatal case of infection, and Mohammed et al. reported three resistant isolates, including the same isolate used in the study by Lightfoot et al. (6, 7). Using the *Staphylococcus* breakpoints, our study demonstrated three resistant isolates. These isolates were tested for β-lactamase production and found to be nonproducers, as were all of the isolates. Since the highest concentration was within a doubling dilution of 0.25 µg/ml, it is possible that the *Staphylococcus* breakpoints are not appropriate for comparison in *Bacillus* species. Other explanations for this observation could be possible. Strains of *B. licheniformis* have been shown to produce large amounts of a β-lactamase and yet be sensitive to penicillin (9). In another study, a strain was shown to be highly susceptible to penicillin and still actively produce a β-lactamase (10). Penicillin resistance in *Bacillus* spp. is an area that needs more research than a cursory susceptibility panel every few years.

Ciprofloxacin, doxycycline, and the remaining antimicrobial agents, cefaclor, cephalixin, and tobramycin, showed that all isolates tested were highly susceptible. This was in agreement with the findings of other studies that tested the corresponding antimicrobial agent.

TABLE 2. MICs for and antimicrobial susceptibilities of 25 *B. anthracis* strains

Anti- microbial	MIC (µg/ml) ^a			Categorical interpretation						
	Range	50%	90%	No. of isolates			Staphylococcal breakpoint (µg/ml) ^b			
				S	I	R	S	I	R	
Cefaclor	0.125–0.75	0.38	1.65	25				≤8	16	≥32
Cefuroxime	6–48	21.33	32	1	19	5		≤8	16	≥32
Cephalixin	0.38–2	1.5	1.5	25				≤8	16	≥32
Ciprofloxacin	0.032–0.38	0.094	0.094	25				≤1	2	≥4
Doxycycline	0.094–0.38	0.23	0.34	25				≤4	8	≥16
Penicillin	<0.016–0.5	0.042	0.236	22		3		≤0.12		≥0.25
Tobramycin	0.25–1.5	0.75	0.97	25				≤4	8	≥16

^a 50 and 90%, MIC₅₀ and MIC₉₀, respectively.

^b NCCLS standard M100-S12.

Now that a reliable genotyping system is available, more genotypes should be tested against these and other antimicrobials to verify the trends noted here and in other studies. β -Lactamase production or nonproduction should not be considered an alternative to testing penicillin resistance. Hopefully, this study will contribute to the impetus for a more thorough study of β -lactamase production and *Bacillus* spp. Continued surveillance of *B. anthracis* field isolates is recommended to monitor antimicrobial susceptibility.

ACKNOWLEDGMENT

We express our gratitude to Eli Lilly for making this project possible.

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