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Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods

Vicki A. Luna[1]*, Debra S. King[1], Jenny Gulledge[1], Andrew C. Cannons[1], Philip T. Amuso[2] and Jacqueline Cattani[1]

[1]Center for Biological Defense, College of Public Health, University of South Florida, 3602 Spectrum Boulevard, Tampa, FL 33612, USA; [2]Florida Department of Health, Bureau of Laboratories, 3602 Spectrum Boulevard, Tampa, FL 33612, USA

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***Objectives*: To examine susceptibilities of *Bacillus anthracis* and related species to 24 antimicrobials using and concurrently comparing two methods.**

***Methods*: Twenty-four antimicrobials were tested against 95 isolates of the *Bacillus cereus* group including 18 *B. anthracis*, 42 *B. cereus*, 5 *Bacillus mycoides*, 5 *Bacillus mycoides/pseudomycoides*, 6 *Bacillus pseudomycoides* and 19 *Bacillus thuringiensis* to determine their MICs, MIC ranges, MIC_{50}s and MIC_{90}s with Etest® and Sensititre® at 30 and 35°C for 18, 24 and 48 h.**

***Results*: Both methods yielded near-identical results at both temperatures for all antimicrobials except trimethoprim/sulfamethoxazole. Resistance to trimethoprim/sulfamethoxazole in 97% (92/95) was not always evident until tests were incubated for 48 h at 30°C. All *B. anthracis* isolates were susceptible to 22 antimicrobials and resistant to trimethoprim/sulfamethoxazole while three isolates were erythromycin-intermediate. Whereas the *B. thuringiensis* were resistant to the β-lactams, two *B. cereus*, one *B. mycoides*, five *B. pseudomycoides* and two *B. mycoides/pseudomycoides* were susceptible. Three *B. cereus* were solely clindamycin-resistant. Of the seven erythromycin-intermediate or -resistant *B. cereus*, three were resistant to clindamycin and one was resistant to clarithromycin and clindamycin. One *B. mycoides* was intermediately resistant to quinupristin/dalfopristin and meropenem and one was clindamycin-resistant. All *B. pseudomycoides* were clindamycin-resistant with one quinupristin/dalfopristin-resistant. Two *B. mycoides/pseudomycoides* were intermediately resistant to quinupristin/dalfopristin and clindamycin and a third was intermediately resistant to clindamycin alone. All isolates were susceptible to chloramphenicol, ciprofloxacin, gatifloxacin, gentamicin, levofloxacin, linezolid, moxifloxacin, rifampicin, streptomycin, tetracycline, tigecycline and vancomycin.**

***Conclusions*: This paper expands the list of therapeutic or prophylactic antimicrobials potentially effective against *B. cereus* group isolates using two testing methods that produced comparable results.**

Keywords: method comparison, minimum inhibition concentration, trimethoprim, β-lactams

Introduction

In the United States, human cases of anthrax are rare with the most recent cases occurring in 2001 and 2006.[1–3] European cases involved travellers who had contact with mammal carcasses.[4–6] *Bacillus cereus* causes gastrointestinal distress, necrotic enteritis, liver failure, bacteraemia, endocarditis, meningitis, pneumonia and skin lesions.[7–15] Long considered non-pathogenic and used extensively for insect pest control, *Bacillus thuringiensis* has been implicated in burn wound infections and food-poisoning.[16,17] Of the other *B. cereus* group members, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus*

*Corresponding author. Tel: +1-813-974-3873; Fax: +1-813-974-1479; E-mail: vluna@health.usf.edu

weihenstephanensis, only *B. mycoides* has been implicated in human infection (endophthalmitis).[18]

Although *Bacillus anthracis* has been reported as susceptible to a limited number of antimicrobials, resistance to penicillin, erythromycin and quinolones has been noted.[19–25] Therefore, it is conceivable that an isolate could be engineered to be resistant to one or more antimicrobials.[26–28] Because susceptibility testing of *B. anthracis* in the sentinel and reference laboratories is not recommended by the Centers for Disease Control and Prevention (CDC) who prefers to perform the testing at the national laboratory, the attending physician will treat a patient with a suspected *B. anthracis* infection empirically until a report is received from the CDC.[29] In contrast, *B. cereus*, typically resistant only to β-lactams, can be tested in the clinical laboratory.[30,31] Reports of resistance in *B. cereus* to erythromycin and tetracyclines in the United States and Europe predict the development of further resistance.[32–34] *B. thuringiensis* and *B. mycoides* can be difficult to distinguish from *B. cereus* before susceptibility tests are performed. *B. pseudomycoides* cannot be distinguished from *B. mycoides* without fatty acid methyl-ester analysis and its antibiogram is unknown.[35] Thus, all four species were included in the study.

Mohammed *et al.*[36] at the CDC reported that the Etest® by AB BIODISK (North America Inc., Piscataway, NJ, USA) could be used with *B. anthracis* and this implies that the method can be used for testing *B. cereus*. Turnbull *et al.*[25] also compared the Etest® to agar dilution tests for nine antimicrobials and extended this testing to include *B. cereus*, *B. thuringiensis* and *B. mycoides*. The dual purpose of this research was to (i) expand our knowledge of susceptibility patterns of *B. anthracis* and related *Bacillus* against a larger number of antimicrobials using the Etest® and (ii) to compare the Etest® method with the Sensititre® automated microbroth dilution method with selected antimicrobials.

Materials and methods

Bacteria and growth conditions

We examined 95 *Bacillus* isolates: 18 *B. anthracis*, 42 *B. cereus*, 5 *B. mycoides*, 5 *B. mycoides/pseudomycoides*, 6 *B. pseudomycoides* and 19 *B. thuringiensis*. The *B. anthracis* isolates were received from the Florida Department of Health, Bureau of Laboratories, Tampa, FL, USA (FDOH), the NIH Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Bethesda, MD, USA) or isolated from diverse environment and soil samples. The other *Bacillus* isolates in the study were received from FDOH, American Type Culture Collection (ATCC, Manassas, VA, USA) or Agriculture Research Service Culture Collection (ARSCC, Peoria, IL, USA), purchased as commercial pesticide products or isolated from various non-replicate environmental, marine and soil samples (one isolate per sample). Only one *B. anthracis* isolate was known to be from a human culture. Six of the *B. thuringiensis* isolates were potentially related.[37]

While all manipulations of *B. anthracis* isolates were performed in a class 2 biological safety cabinet, *B. anthracis* Pasteur isolates were stored and handled in a biosafety level 2 laboratory using biosafety level 3 (BSL3) practices and all other *B. anthracis* isolates were handled in a BSL3 laboratory. This arrangement followed the Institutional Bio-safety Committee requirements at USF. All safety protocols and requirements required by US federal regulation DHHS 42 CFR 73 were strictly met. Prior to susceptibility tests, the bacteria were grown on tryptic soy agar supplemented with 5% sheep red blood cells [blood agar (BA)] (Remel, Lenexa, KS, USA). All plates were incubated at 30 or 35°C (±2) overnight (~18 h) in ambient air before testing.

Susceptibility tests

All Etest® susceptibility tests were set up on Mueller–Hinton agar plates (Remel) and read following the manufacturer's directions. Plates were incubated at 30 or 35°C for 18, 24 and 48 h. Interpretation of the MIC values followed CLSI standards.[38–40] *Staphylococcus aureus* ATCC 29213 was included as a control.

The Sensititre® by TREK Diagnostic Systems (Cleveland, OH, USA) is an automatic system that uses a 96-well plate format with a panel of several antimicrobials that are precision dosed at appropriate dilutions and equates to the classical microbroth dilution method. The instrument detects growth as a fluorescent substrate is utilized by bacterial surface enzymes. The amount of detected fluorescence is proportional to bacterial growth. A data system interprets the MIC values following CLSI recommendations although manual interpretations can be performed with novel antimicrobials. Fail-safe features built into the database preclude interpreting tests read at inappropriate times, correctly interpret manually read tests and automatically flag unusual results. Susceptibility tests were performed following the manufacturer's instructions at both 30 and 35°C and read at 18, 24 and 48 h. *S. aureus* ATCC 29213 was included as a test control.

Of 24 antimicrobials examined, 16 were tested by both Etest® and Sensititre®: ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gatifloxacin, gentamicin, levofloxacin, moxifloxacin, oxacillin, penicillin, quinupristin/dalfopristin, rifampicin, trimethoprim/sulfamethoxazole, tetracycline and vancomycin. Streptomycin and clarithromycin were only tested by the microbroth dilution method because Etest® strips were not available. The remaining six antimicrobials were not available on the Sensititre® format and so were performed by Etest® only: amoxicillin, ceftriaxone, daptomycin, linezolid, meropenem and tigecycline.

In addition, isolates were grown on tryptic soy agar (Fisher Scientific, Suwanee, GA, USA) supplemented with 0.06 mg/L erythromycin (Sigma-Aldrich, St Louis, MO, USA) and retested against clindamycin. Tests for the β-lactams oxacillin and meropenem were repeated and read at 18, 24 and 48 h.

Results

The susceptibility tests with Etest® and the Sensititre® microbroth dilution methods, using either 30 or 35°C incubation temperatures, gave identical or near identical MICs (less than a 2-fold dilution difference) for all of the antimicrobials (except for trimethoprim/sulfamethoxazole) at 18, 24 and 48 h readings. This resulted in identical or near identical MIC_{50}s and MIC_{90}s for each antimicrobial. In general, with both methods, the endpoints for the 23 antimicrobials were easier to read at 24 h than 18 h, but we did not see any increase in MICs at the later time point. Even extending the time frame to 48 h did not produce higher MICs. The few times more growth was noted at 48 h with the Etest®, the new MIC values were less than or equal to a 2-fold difference with either temperature (data not shown) and were not considered a true increase in MIC. This agrees with other researchers.[25]

Because of the reports of trimethoprim/sulfamethoxazole resistance[41,42] and the molecular confirmation of trimethoprim resistance in *B. anthracis*,[43] the assumption was made that the other related *Bacillus* species were also resistant to the drug combination. Therefore, this antimicrobial was not originally planned to be tested. Yet because it is included in the pre-made microtitre plate by Sensititre®, the MICs were recorded. Initial testing at 35°C as recommended by CLSI did not show resistance to the trimethoprim/sulfamethoxazole combination in all of the *B. anthracis* isolates, the majority of *B. cereus* or *B. thuringiensis*. Retesting at 30°C, reading the tests at 24 and 48 h and also testing by Etest® proved that almost all of the isolates were indeed resistant to trimethoprim/sulfamethoxazole. The Sensititre® consistently gave resistant MICs for the *B. anthracis* at both time points after incubation at either 30 or 35°C while the Etest® produced resistant results for all *B. anthracis* isolates only at 30°C. For other *Bacillus* species, both methods yielded more numerous resistant MIC values at the lower temperature at 24 h, but the number of resistant isolates usually increased if the Etest® and 48 h incubation was employed.

When testing the other 23 antimicrobials, all (100%) of the *B. anthracis* isolates were susceptible to all of the tested therapeutics except erythromycin (Figure 1). Four *B. anthracis* were intermediately resistant to this antimicrobial: two reported only by Etest®, one reported only by Sensititre® and one by both methods (Table 1). This was due to the MIC values overlapping the susceptible breakpoint of 0.5 mg/L and was not considered a method discrepancy.

The majority of the *B. cereus* and all of the *B. thuringiensis* isolates were resistant to amoxicillin, ampicillin, ceftriaxone, penicillin and oxacillin while susceptible to the remaining antimicrobials (Figure 1). Resistance in *B. cereus* was uncommon as six (14%) isolates were resistant to meropenem, seven (17%) were intermediate or resistant (inducible and constitutive) to clindamycin and only a single (2%) isolate was resistant to both clarithromycin and erythromycin (Tables 1 and 2). Only one isolate of the seven that were intermediately resistant to erythromycin by Etest® (1 mg/L) was called susceptible by Sensititre® (0.5 mg/L). A single *B. thuringiensis* had intermediate resistance to clindamycin. The six potentially related *B. thuringiensis* isolates did not demonstrate identical MIC values for all of the antimicrobials, but since antibiograms can differ within a related population of strains, this did not rule out their possible relatedness.

One *B. mycoides*, five *B. pseudomycoides* and two *B. mycoides/pseudomycoides* isolates were susceptible to the β-lactams, whereas the other isolates of these species were resistant (Table 1). The tests were repeated and tests held for an additional 24 h but the MICs remained the same. Sporadic intermediate resistance was seen against meropenem, quinupristin/dalfopristin and clindamycin. All of the *B. pseudomycoides* were intermediate or resistant to clindamycin, whereas the *B. mycoides* and *B. mycoides/pseudomycoides* were generally susceptible.

Discussion

When 16 antimicrobials were tested by both Sensititre® and Etest®, the two techniques produced for 15 of the antimicrobials identical or near identical (less than a 2-fold dilution) results at either 30 or 35°C when read after 24 h of incubation. Attempts at discerning growth at <24 h of incubation rendered unreliable results no matter the method or the antimicrobial tested. This difficulty appeared to be due to the growth traits of the bacteria and not to the particular test method especially for *B. mycoides* and *B. pseudomycoides* isolates since they commonly produced their characteristic rhizoid-like branching. Although we were not able to fully compare the two methods with the other eight antimicrobials, we expect that both the Etest® and the microbroth dilution format by Sensititre® will be valid to use.

Like the Etest®, the Sensititre® protocols can be executed within a Bio-Safety Cabinet (BSC). The Sensititre® instrument has a modular design in which the inoculating unit is small

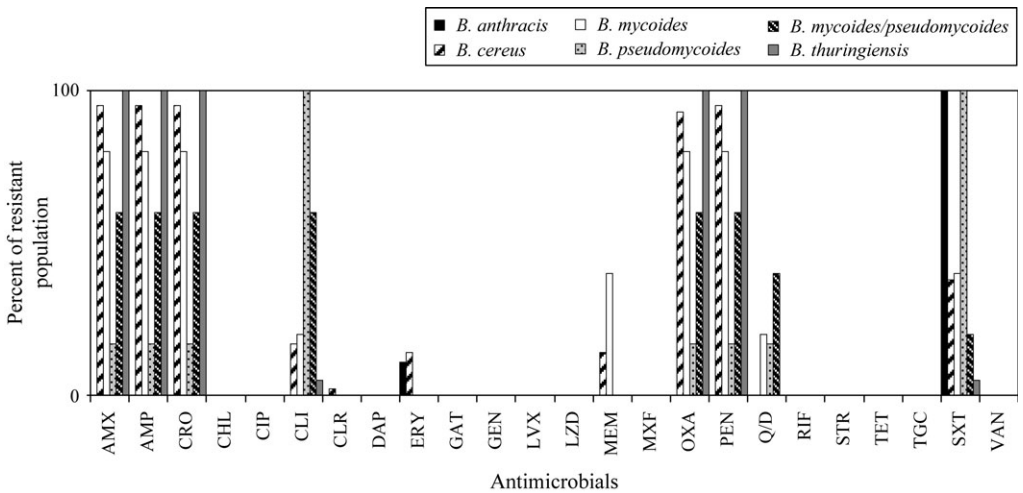

Figure 1. Percentage of *Bacillus* isolates showing intermediate and full resistance to the antimicrobials based upon the MIC results from both methods. AMX, amoxicillin; AMP, ampicillin; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CLR, clarithromycin; DAP, daptomycin; ERY, erythromycin; GAT, gatifloxacin; GEN, gentamicin; LVX, levofloxacin; LZD, linezolid; MEM, meropenem; MXF, moxifloxacin; OXA, oxacillin; PEN, penicillin; Q/D, quinupristin/dalfopristin; RIF, rifampicin; STR, streptomycin; TET, tetracycline; TGC, tigecycline; SXT, trimethoprim/sulfamethoxazole; VAN, vancomycin.

Table 1. Comparison of susceptibility[a] of 95 *Bacillus* isolates to antimicrobials[b] by Etest® and Sensititre® microbroth dilution

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L)			Breakpoints[c,d]		Interpretation *n* (%)		
				range	50%[h]	90%[h]	S (≤)	R (≥)	S	I	R
Ampicillin	*B. anthracis*	18	Etest	0.016–0.032	0.023	0.032	≤0.25	≥0.5	18 (100)		
			MBD	≤0.12	≤0.12	≤0.12					
	B. cereus	42	Etest	0.016–32	8	24			2 (5)		40 (95)
			MBD	≤0.12 to >8	8	>8					
	B. mycoides	5	Etest	0.64–48	4	48			1 (20)		4 (80)
			MBD	≤0.12 to >8	8	>8					
	B. pseudomycoides	6	Etest	0.094–8	0.094	8			5 (83)		1 (17)
			MBD	≤0.12 to 4	0.012	4					
	B. mycoides/pseudomycoides	5	Etest	≤0.016 to 32	0.5	32			2 (40)		3 (60)
			MBD	≤0.12 to >8	0.5	>8					
	B. thuringiensis	19	Etest	4–16	12	16					19 (100)
			MBD	4 to >8	8	>8					
	S. aureus 29213			0.4 (0.25–0.5)[i]							
Chloramphenicol	*B. anthracis*	18	Etest	0.75–4	4	4	≤8	≥32	18 (100)		
			MBD	≤2 to 4	4	4					
	B. cereus	42	Etest	1–4	2	3			42 (100)		
			MBD	≤2 to 4	≤2	4					
	B. mycoides	5	Etest	1–4	1	4			5 (100)		
			MBD	≤2 to 4	4	4					
	B. pseudomycoides	6	Etest	1–1.5	1	1.5			6 (100)		
			MBD	≤2	≤2	≤2					
	B. mycoides/pseudomycoides	5	Etest	0.75–2	1.5	2			5 (100)		
			MBD	≤2	≤2	≤2					
	B. thuringiensis	19	Etest	1–4	1.5	2			19 (100)		
			MBD	≤2	≤2	≤2					
	S. aureus 29213			6 (4–8)[i]							
Ciprofloxacin	*B. anthracis*	18	Etest	0.023–0.064	0.047	0.064	≤5[j]		18 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. cereus	42	Etest	0.047–0.38	0.125	0.25	≤1	≥4	42 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. mycoides	5	Etest	0.64–0.125	0.094	0.125			5 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. pseudomycoides	6	Etest	0.047–0.125	0.064	0.125			6 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. mycoides/pseudomycoides	5	Etest	0.047–0.094	0.064	0.094			5 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. thuringiensis	19	Etest	0.094–0.19	0.125	0.19			19 (100)		
			MBD	≤0.5	≤0.5	≤0.5					

Continued

Table 1. *Continued*

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L) range	MIC (mg/L) 50%[h]	MIC (mg/L) 90%[h]	Breakpoints[c,d] S (≤)	Breakpoints[c,d] R (≥)	Interpretation *n* (%) S	Interpretation *n* (%) I	Interpretation *n* (%) R
	S. aureus 29213			0.4 (0.25–0.5)[i]							
Clindamycin	*B. anthracis*	18	Etest	0.064–0.5	0.125	0.5	≤ 0.5	≥4	18 (100)		
			MBD	≤0.25 to 1	≤0.25	0.5					
	B. cereus	42	Etest	0.047–3	0.38	1			36 (85)	4 (10)	2 (5)
			MBD	≤0.25 to >2	0.5	1			35 (83)	5 (12)	2 (5)
	B. mycoides	5	Etest	0.25–1.5	0.25	1.5			4 (80)		1 (20)
			MBD	0.25–1	0.25	1					
	B. pseudomycoides	6	Etest	2–6	3	6				2 (33)	4 (67)
			MBD	>2	>2	>2					6 (100)
	B. mycoides/pseudomycoides	5	Etest	0.19–2	1	2			3 (60)	2 (40)	
			MBD	≤0.25 to 1	0.5	1			2 (40)	3 (60)	
	B. thuringiensis	19	Etest	0.125–0.5	0.38	0.38			18 (95)	1 (5)	
			MBD	≤0.25 to 1	≤0.25	0.5					
	S. aureus 29213			0.18 (0.12–0.25)[i]							
Erythromycin	*B. anthracis*	18	Etest	0.25–1	0.5	0.75	≤0.5	≥8	15 (83)	3 (17)	
			MBD	0.5–1	0.5	0.75			16 (89)	2 (11)	
	B. cereus	42	Etest	0.047–24	0.094	2			34 (81)	7 (17)	1 (2)
			MBD	≤ 0.12 to >4	≤0.12	1			35 (84)	6 (14)	1 (2)
	B. mycoides	5	Etest	0.016–0.094	0.047	0.094			5 (100)		
			MBD	≤0.12 to 0.25	≤ 0.12	0.25					
	B. pseudomycoides	6	Etest	0.064–0.19	0.064	0.19			6 (100)		
			MBD	≤0.12 to 0.25	≤0.12	0.25					
	B. mycoides/pseudomycoides	5	Etest	0.047–0.25	0.19	0.25			5 (100)		
			MBD	≤0.12 to 0.25	≤0.12	0.25					
	B. thuringiensis	19	Etest	0.047–0.125	0.064	0.094			19 (100)		
			MBD	≤0.12	≤0.12	≤0.12					
	S. aureus 29213			0.4 (0.25–0.5)[i]							
Gatifloxacin	*B. anthracis*	18	Etest	0.016–0.047	0.032	0.047	≤0.5	≥2	18 (100)		
			MBD	≤0.06	≤0.06	≤0.06					
	B. cereus	42	Etest	0.016–0.125	0.064	0.125			42 (100)		
			MBD	≤0.06 to 0.25	≤0.06	0.12					
	B. mycoides	5	Etest	0.023–0.064	0.032	0.064			5 (100)		
			MBD	≤0.06	≤0.06	≤0.06					
	B. pseudomycoides	6	Etest	0.023–0.047	0.032	0.047			6 (100)		
			MBD	≤0.06	≤0.06	≤0.06					
	B. mycoides/pseudomycoides	5	Etest	0.023–0.047	0.032	0.047			5 (100)		
			MBD	≤0.06	≤0.06	≤0.06					

Continued

Table 1. *Continued*

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L) range	MIC (mg/L) 50%[h]	MIC (mg/L) 90%[h]	Breakpoints[c,d] S (≤)	Breakpoints[c,d] R (≥)	Interpretation *n* (%) S	Interpretation *n* (%) I	Interpretation *n* (%) R
	B. thuringiensis	19	Etest	0.032–0.125	0.047	0.064			19 (100)		
			MBD	≤0.06–0.125	≤0.06	≤0.06					
	S. aureus 29213			0.04 (0.03–0.06)[i]							
Gentamicin	*B. anthracis*	18	Etest	0.032–0.19	0.064	0.125	≤4	≥8	18 (100)		
			MBD	≤2	≤2	≤2					
	B. cereus	42	Etest	0.016–1	0.38	0.75	≤4	≥16	42 (100)		
			MBD	≤2	≤2	≤2					
	B. mycoides	5	Etest	<0.016 to 0.25	0.19	0.25			5 (100)		
			MBD	≤2	≤2	≤2					
	B. pseudomycoides	6	Etest	0.094–0.38	0.125	0.38			6 (100)		
			MBD	≤2	≤2	≤2					
	B. mycoides/pseudomycoides	5	Etest	0.094–0.38	0.19	0.38			5 (100)		
			MBD	≤2	≤2	≤2					
	B. thuringiensis	19	Etest	0.19–1.5	0.25	0.38			19 (100)		
			MBD	≤2	≤2	≤2					
	S. aureus 29213			0.5 (0.125–1)[i]							
Levofloxacin	*B. anthracis*	18	Etest	0.064–0.125	0.06	0.125	≤1	≥4	18 (100)		
			MBD	0.06–0.12	0.06	0.125					
	B. cereus	42	Etest	0.064–0.25	0.125	0.25	≤2	≥8	42 (100)		
			MBD	≤0.03 to 0.25	0.06	0.25					
	B. mycoides	5	Etest	0.064–0.125	0.094	0.125			5 (100)		
			MBD	0.03–0.12	0.06	0.12					
	B. pseudomycoides	6	Etest	0.064–0.125	0.094	0.125			6 (100)		
			MBD	0.06–0.12	0.06	0.125					
	B. mycoides/pseudomycoides	5	Etest	0.032–0.125	0.094	0.125			5 (100)		
			MBD	0.06	0.06	0.06					
	B. thuringiensis	19	Etest	0.032–0.25	0.064	0.125			19 (100)		
			MBD	≤0.03 to 0.12	0.06	0.125					
	S. aureus 29213			0.12 (0.06–0.25)[i]							
Moxifloxacin	*B. anthracis*	18	Etest	0.016–0.094	0.032	0.047	≤0.5	≥2	18 (100)		
			MBD	≤0.25	≤0.25	≤0.25					
	B. cereus	42	Etest	0.023–0.19	0.064	0.19			42 (100)		
			MBD	≤0.25	≤0.25	≤0.25					
	B. mycoides	5	Etest	0.023–0.25	0.047	0.25			5 (100)		
			MBD	≤0.25	≤0.25	≤0.25					
	B. pseudomycoides	6	Etest	0.023–0.064	0.023	0.064			6 (100)		
			MBD	≤0.25	≤0.25	≤0.25					

Continued

Table 1. *Continued*

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L) range	MIC (mg/L) 50%[h]	MIC (mg/L) 90%[h]	Breakpoints[c,d] S (≤)	Breakpoints[c,d] R (≥)	Interpretation *n* (%) S	Interpretation *n* (%) I	Interpretation *n* (%) R
	B. mycoides/pseudomycoides	5	Etest	0.016–0.032	0.032	0.032			5 (100)		
			MBD	≤0.25	≤0.25	≤0.25					
	B. thuringiensis	19	Etest	0.032–0.125	0.047	0.094			19 (100)		
			MBD	≤0.25	≤0.25	≤0.25					
	S. aureus 29213			0.09 (0.06–0.125)[i]							
Oxacillin	*B. anthracis*	18	Etest	0.047–0.5	0.25	0.5	≤2	≥4	18 (100)		
			MBD	≤0.25	≤0.25	≤0.25					
	B. cereus	42	Etest	0.094 to >256	>256	>256			3 (7)	1 (2)	38 (91)
			MBD	≤0.25 to >2	>2	>2					
	B. mycoides	5	Etest	0.5 to >256	>256	>256			1 (20)		4 (80)
			MBD	≤0.25 to >2	>2	>2					
	B. pseudomycoides	6	Etest	0.25 to >256	0.25	>256			5 (83)		1 (17)
			MBD	≤0.25 to >2	≤0.25	>2					
	B. mycoides/pseudomycoides	5	Etest	0.094 to >256	3	>256			2 (40)		3 (60)
			MBD	≤0.25 to >2	>2	>2					
	B. thuringiensis	19	Etest	>256	>256	>256					19 (100)
			MBD	>2	>2	>2					
	S. aureus 29213			0.18 (0.125–0.25)[i]							
Penicillin	*B. anthracis*	18	Etest	0.008–0.032	0.016	0.032	≤0.12	≥0.25	18 (100)		
			MBD	≤0.03 to 0.06	≤0.03	0.06					
	B. cereus	42	Etest	0.16 to >256	24	>256			2 (5)		40 (95)
			MBD	≤0.03 to >8	>8	>8					
	B. mycoides	5	Etest	0.125 to >256	96	>256			1 (20)		4 (80)
			MBD	≤0.03 to >8	>8	>8					
	B. pseudomycoides	6	Etest	0.064–12	0.094	12			5 (83)		1 (17)
			MBD	≤0.03 to >8	≤0.03	>8					
	B. mycoides/pseudomycoides	5	Etest	0.016–24	1.5	24			2 (40)		3 (60)
			MBD	≤0.03 to >8	0.25	>8					
	B. thuringiensis	19	Etest	24–256	48	192					19 (100)
			MBD	>8	>8	>8					
	S. aureus 29213			0.4 (0.25–0.5)[i]							
Quinupristin/dalfopristin	*B. anthracis*	18	Etest	0.25–2	0.5	0.75	≤1	≥4	18 (100)		
			MBD	0.25–1	0.5	1					
	B. cereus	42	Etest	0.19–0.75	0.5	0.75			42 (100)		
			MBD	0.25–1	0.5	1					
	B. mycoides	5	Etest	0.75–2	1	2			4 (80)	1 (20)	
			MBD	1–2	1	2					

Continued

Table 1. *Continued*

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L)			Breakpoints[c,d]		Interpretation *n* (%)		
				range	50%[h]	90%[h]	S (≤)	R (≥)	S	I	R
	B. pseudomycoides	6	Etest	0.5–1	0.75	1			5 (83)	1 (17)	
			MBD	0.5–2	1	2					
	B. mycoides/pseudomycoides	5	Etest	0.5–2	1	2			3 (60)	2 (40)	
			MBD	0.25–2	1	2					
	B. thuringiensis	19	Etest	0.5–1	0.5	0.75			19 (100)		
			MBD	0.25–1	0.5	1					
	S. aureus 29213			0.3 (0.25–0.5)[i]							
Rifampicin	*B. anthracis*	18	Etest	0.064–0.5	0.19	0.25	≤1	≥4	18 (100)		
			MBD	≤0.5 to 1	≤0.5	1					
	B. cereus	42	Etest	0.002–1	0.038	1			42 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. mycoides	5	Etest	0.125–1	0.19	1			5 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. pseudomycoides	6	Etest	0.047–0.125	0.047	0.125			6 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. mycoides/pseudomycoides	5	Etest	0.064–0.5	0.094	0.5			5 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. thuringiensis	19	Etest	0.047–1	0.25	0.75			19 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	S. aureus 29213			0.4 (0.25–0.5)[i]							
Tetracycline	*B. anthracis*	18	Etest	0.016–0.047	0.023	0.032	≤1[j]		18 (100)		
			MBD	≤4	≤4	≤4					
	B. cereus	42	Etest	0.016–3	1.5	3	≤4	≥16	42 (100)		
			MBD	≤4	≤4	≤4					
	B. mycoides	5	Etest	0.032–1	0.5	1			5 (100)		
			MBD	≤4	≤4	≤4					
	B. pseudomycoides	6	Etest	0.125–0.5	0.19	0.5			6 (100)		
			MBD	≤4	≤4	≤4					
	B. mycoides/pseudomycoides	5	Etest	0.125–2	1.5	2			5 (100)		
			MBD	≤4	≤4	≤4					
	B. thuringiensis	19	Etest	0.25–2	1	1			19 (100)		
			MBD	≤4	≤4	≤4					
	S. aureus 29213			0.17(0.094–0.25)[i]							
Trimethoprim/ sulfamethoxazole[k]	*B. anthracis*	18	Etest	>32	>32	>32	≤2	≥8			18 (100)
			MBD	>4	>4	>4					
	B. cereus	42	Etest	0.25 to ≥32	>32	>32	≤2	≥4	11 (26)		31 (74)
			MBD	≤0.5 to >4	1	>4			26 (62)		16 (38)

Continued

Table 1. *Continued*

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L) range	MIC (mg/L) 50%[h]	MIC (mg/L) 90%[h]	Breakpoints[c,d] S (≤)	Breakpoints[c,d] R (≥)	Interpretation *n* (%) S	Interpretation *n* (%) I	Interpretation *n* (%) R
	B. mycoides	5	Etest	0.125 to >32	2	>32			3 (60)		2 (40)
			MBD	≤0.5 to >4	0.5	>4					
	B. pseudomycoides	6	Etest	≥32	≥32	≥32					6 (100)
			MBD	>4	>4	>4					
	B. mycoides/pseudomycoides	5	Etest	0.064 to >32	2	>32			4 (80)		1 (20)
			MBD	≤0.5 to >4	0.5	>4					
	B. thuringiensis	19	Etest	0.125–8	0.25	2			18 (95)		1 (5)
			MBD	≤0.5 to >4	≤0.5	≤0.5					
	S. aureus 29213			0.09 (0.06–0.125)[i]							
Vancomycin	*B. anthracis*	18	Etest	0.5–2	2	2	≤2	≥16	18 (100)		
			MBD	≤0.5 to 2	2	2					
	B. cereus	42	Etest	0.125–4	2	2	≤4	>4	42 (100)		
			MBD	≤0.5 to 2	1	2					
	B. mycoides	5	Etest	0.125–2	1.5	2			5 (100)		
			MBD	≤0.5 to 1	≤0.5	1					
	B. pseudomycoides	6	Etest	0.38–1.5	0.75	1.5			6 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. mycoides/pseudomycoides	5	Etest	0.5–1.5	1	1.5			5 (100)		
			MBD	≤0.5	≤0.5	≤0.5					
	B. thuringiensis	19	Etest	1–2	1.5	2			19 (100)		
			MBD	≤0.5 to 4	≤0.5	1					
	S. aureus 29213			1 (0.5–1.5)[i]							

[a]Susceptibility tests were performed by Etest® and/or by Sensititre® microbroth dilution and MICs read at 18, 24 and 48 h. All values noted are the 24 h results. The antimicrobials tested by Etest® alone were: amoxicillin, ceftriaxone, daptomycin, linezolid, meropenem and tigecycline. The antimicrobials tested solely by microbroth dilution were: clarithromycin and streptomycin that were included in the panel normally produced by the company.

[b]Antimicrobials that were tested by only one method are not listed in this table.

[c]The breakpoints (in mg/L) used for ciprofloxacin, penicillin and tetracycline were for *B. anthracis* while the breakpoints used for the other antimicrobials were for *Staphylococcus* spp. as recommended by the CDC and the CLSI guidelines M100-S16 (2006) and M7-A7 (2006).[38,40,44] For *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. mycoides/pseudomycoides* and *B. thuringiensis*, the breakpoints used followed the guidelines by CLSI (M45-P, 2005)[39] for the following antimicrobials: amoxicillin, ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, levofloxacin, penicillin, rifampicin, trimethoprim/sulfamethoxazole, tetracycline and vancomycin. For clarithromycin, daptomycin, gatifloxacin, linezolid, meropenem, moxifloxacin, oxacillin, quinupristin/dalfopristin and tigecycline, the breakpoints for *Staphylococcus* spp. were used. The manufacturer's literature and package insert gave the breakpoint for streptomycin.

[d]S, susceptible; I, intermediate; R, resistant.

[e]The table shows readings taken after 24 h of incubation at 30°C. All *B. anthracis* isolates were resistant at 24 h at 30 and 35°C when tested with Sensititre®. The *B. anthracis* isolates were resistant only at 30°C when tested with Etest® at 24 h, while at the higher temperature, some isolates appeared to be susceptible. For other species, tests performed and read at 24 h produced many false susceptible results at both 30 and 35°C. At 48 h all of the isolates of these other species were resistant to trimethoprim/sulfamethoxazole at 30°C.

[f]Five isolates were identified as *B. mycoides/pseudomycoides* until further testing.

[g]MBD denotes the microbroth dilution method by Sensititre®.

[h]MIC at which 50% or 90% of tested isolates are inhibited.

[i]The values given for *S. aureus* ATCC 29213 are the means and ranges of 12 repeat tests.

[j]CLSI approved standard M100-S16[38] states that no strains resistant to the tested drug were available for establishing standards. Only susceptible breakpoints established for these drugs.

[k]Trimethoprim/sulfamethoxazole was tested against *B. anthracis* because the antimicrobial is already on the Gram-positive panel offered by the manufacturer and unexpected susceptibility results were noted.

Table 2. Susceptibility[a] of 95 *Bacillus* isolates to antimicrobials[b] using one method

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L)			Breakpoints[c,d]		Interpretation *n* (%)		
				range	50%[h]	90%[h]	S (≤)	R (≥)	S	I	R
Amoxicillin	*B. anthracis*	18	Etest	0.016–0.047	0.016	0.032	≤0.25	≥0.5	18 (100)		
	B. cereus	42	Etest	0.016 to >256	16	>256			2 (5)		40 (95)
	B. mycoides	5	Etest	0.064 to >256	256	>256			1 (20)		4 (80)
	B. pseudomycoides	6	Etest	0.023–8	0.125	8			5 (83)		1 (17)
	B. mycoides/ pseudomycoides	5	Etest	0.023–12	1	12			2 (40)		3 (60)
	B. thuringiensis	19	Etest	4–256	24	96					19 (100)
	S. aureus 29213			0.4 (0.25–0.5)[i]							
Ceftriaxone	*B. anthracis*	18	Etest	0.38–32	12	24	≤8	≥64	18 (100)		
	B. cereus	42	Etest	8 to >256	128	>256			2 (5)		40 (95)
	B. mycoides	5	Etest	3 to >256	>256	>256			1 (20)		4 (80)
	B. pseudomycoides	6	Etest	8 to >256	8	>256			5 (83)		1 (17)
	B. mycoides/ pseudomycoides	5	Etest	1.5 to >256	16	>256			2 (40)		3 (60)
	B. thuringiensis	19	Etest	96 to >256	>256	>256					19 (100)
	S. aureus 29213			4 (1–8)[i]							
Clarithromycin	*B. anthracis*	18	MBD	≤0.12	≤0.12	≤0.12	≤2	≥8	18 (100)		
	B. cereus	42	MBD	≤0.12 to 4	≤0.12	0.25			41 (98)		1 (2)
	B. mycoides	5	MBD	≤0.12	≤0.12	≤0.12			5 (100)		
	B. pseudomycoides	6	MBD	≤0.12	≤0.12	≤0.12			6 (100)		
	B. mycoides/ pseudomycoides	5	MBD	≤0.12	≤0.12	≤0.12			5 (100)		
	B. thuringiensis	19	MBD	≤0.12	≤0.12	≤0.12			19 (100)		
	S. aureus 29213			0.18 (0.12–0.25)[i]							
Daptomycin	*B. anthracis*	18	Etest	0.38–4	1.5	4	≤1[j]		18 (100)		
	B. cereus	42	Etest	0.032–1.5	0.25	1			42 (100)		
	B. mycoides	5	Etest	0.016–1	0.25	1			5 (100)		
	B. pseudomycoides	6	Etest	0.023–0.25	0.032	0.25			6 (100)		
	B. mycoides/ pseudomycoides	5	Etest	0.032–1	0.064	1			5 (100)		
	B. thuringiensis	19	Etest	0.064–0.75	0.25	0.5			19 (100)		
	S. aureus 29213			0.5 (0.25–1)[i]							
Linezolid	*B. anthracis*	18	Etest	0.38–0.75	0.5	0.75	≤4[j]		18 (100)		
	B. cereus	42	Etest	0.125–0.5	0.25	0.38			42 (100)		
	B. mycoides	5	Etest	0.125–0.38	0.25	0.38			5 (100)		
	B. pseudomycoides	6	Etest	0.25–0.5	0.25	0.5			6 (100)		
	B. mycoides/ pseudomycoides	5	Etest	0.19–0.5	0.25	0.5			5 (100)		
	B. thuringiensis	19	Etest	0.19–0.25	0.19	0.25			19 (100)		
	S. aureus 29213			0.14 (0.06–0.25)[i]							
Meropenem	*B. anthracis*	18	Etest	0.008–0.047	0.032	0.047	≤4	≥16	18 (100)		
	B. cereus	42	Etest	0.012–32	0.094	32			36 (86)		6 (14)
	B. mycoides	5	Etest	0.032–12	0.064	12			3 (60)	2 (40)	
	B. pseudomycoides	6	Etest	0.016–2	0.032	2			6 (100)		
	B. mycoides/ pseudomycoides	5	Etest	0.003–0.125	0.032	0.125			5 (100)		
	B. thuringiensis	19	Etest	0.032–0.094	0.032	0.094			19 (100)		
	S. aureus 29213			0.08 (0.03–0.125)[i]							

Continued

Table 2. *Continued*

Antimicrobial[e]	Organism[f]	No.	Method[g]	MIC (mg/L) range	50%[h]	90%[h]	Breakpoints[c,d] S (≤)	R (≥)	Interpretation *n* (%) S	I	R
Tigecycline	*B. anthracis*	18	Etest	0.016–0.032	0.023	0.032	≤0.5[j]		18 (100)		
	B. cereus	42	Etest	0.023–0.125	0.032	0.094			42 (100)		
	B. mycoides	5	Etest	0.023–0.064	0.032	0.064			5 (100)		
	B. pseudomycoides	6	Etest	0.023–0.064	0.023	0.064			6 (100)		
	B. mycoides/ pseudomycoides	5	Etest	0.023–0.032	0.023	0.032			5 (100)		
	B. thuringiensis	19	Etest	0.032–0.047	0.032	0.032			19 (100)		
	S. aureus 29213			0.14 (0.06–0.25)[i]							

[a]Susceptibility tests were performed by Etest® or by Sensititre® microbroth dilution and MICs read at 18, 24 and 48 h. All values noted are the 24 h results. The antimicrobials tested by Etest® alone were: amoxicillin, ceftriaxone, daptomycin, linezolid, meropenem and tigecycline. The antimicrobials tested solely by microbroth dilution were: clarithromycin and streptomycin which were included in the panel normally produced by the company.
[b]Streptomycin results are not included in the table due to the fact that only one microbroth dilution was tested (1000 mg/L). All (100%) of the isolates were susceptible at this concentration.
[c]For *B. anthracis*, the breakpoints (in mg/L) for *Staphylococcus* spp. were used for all of the antimicrobials in the table as recommended by the CDC and the CLSI guidelines M100-S16 (2006) and M7-A7 (2006).[38,40,44] For *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. mycoides/pseudomycoides* and *B. thuringiensis*, the breakpoints used followed the guidelines by CLSI (M45-P, 2005)[39] for amoxicillin and ceftriaxone while for clarithromycin, daptomycin, linezolid, meropenem and tigecycline, the breakpoints for *Staphylococcus* spp. were again used. The manufacturer's literature and package insert gave the breakpoint for streptomycin.
[d]S, susceptible; I, intermediate; R, resistant.
[e]The table shows readings taken after 24 h of incubation at 30°C. All *B. anthracis* isolates were resistant at 24 h at 30 and 35°C when tested with Sensititre®. The *B. anthracis* isolates were resistant only at 30°C when tested with Etest® at 24 h, while at the higher temperature, some isolates appeared to be susceptible. For other species, tests performed and read at 24 h produced many false susceptible results at both 30 and 35°C.
[f]Five isolates were identified as *B. mycoides/pseudomycoides* until further testing.
[g]MBD denotes the microbroth dilution method by Sensititre®.
[h]MIC at which 50% or 90% of tested isolates are inhibited.
[i]The values given for *S. aureus* ATCC 29213 are the means and ranges of 12 repeat tests.
[j]CLSI approved standard M100-S16[38] states that no strains resistant to the tested drug were available for establishing standards. Only susceptible breakpoints established for these drugs.

enough to move into the BSC while the rest of the instrument can be placed permanently upon a workbench. A standard panel containing 19 antimicrobials is available although customized plates can be requested. The Sensititre® protocol is quick and easy to learn and perform (5–15 min per sample). The results are analysed and interpreted by the manufacturer's computer program. After review by personnel, all reports are automatically printed or sent to a main laboratory computer system. For the Etest®, normally only 5–6 Estrips® are placed onto a large media plate. Yet the choice of strips used can be customized on an as-needed basis and more antimicrobials can be added. More technical hands-on time is required with the Etest® in setting up tests, reading and interpreting the results. In addition, for branching *Bacillus* species, more expertise is needed to determine the growth line intersecting the MIC value on the Estrip®.

The results for penicillin, ciprofloxacin, gentamicin and vancomycin correlate with what other authors have described.[33,40] The fact that only 17% of the *B. anthracis* demonstrated any resistance to erythromycin, which is much lower than that found previously in one study,[25] may reflect the smaller number of *B. anthracis* examined in this work. The lack of resistance to tetracycline by any of the isolates was surprising since many came from environmental samples obtained from rural areas where farm animals may be routinely treated with tetracyclines.[33,34]

This paper demonstrates that most of the *Bacillus* isolates (no matter the species) were resistant to trimethoprim/sulfamethoxazole if the test was performed at 30°C and not at the CLSI recommended 35°C. Therefore, we suggest that this therapeutic combination not be tested nor reported for any isolates of the *B. cereus* group, especially if dealing with a *B. cereus* or *B. thuringiensis* causing a life-threatening infection or a suspected *B. anthracis*. It was interesting to note that all of the *B. anthracis* isolates were resistant to trimethoprim/sulfamethoxazole when tested in a broth at either 30 or 35°C, whereas most of the other *Bacillus* species did not reveal their resistance to the antimicrobial until after 48 h of incubation at 30°C. The lag in demonstrating resistance may be due to a low number of resistant individual organisms in a largely susceptible population that takes time to be observable in a well of broth or on an agar plate. Alternatively, there may be a more molecular-based reason for this difference. The actual mechanism for this false susceptibility is unknown, but one reason may be that the two enzyme targets of trimethoprim/sulfamethoxazole have different shapes at the higher temperature. This change in shape could allow the antimicrobials access to the enzymes and thus inhibit or slow their usual activity. The temperature/resistance differences could be useful in distinguishing *B. anthracis* from its relatives.

This is the first report of *B. mycoides* and *B. pseudomycoides* being susceptible to the β-lactams. The significance of this becomes important when their phenotypic characteristics are reviewed. Although some of the isolates produced enough haemolysin to give a β-haemolytic pattern, others did not. The latter were weakly haemolytic under the colony, mimicking a haemolysis pattern that has been seen in *B. anthracis*.[30] These latter isolates were non-motile and did not readily display the

characteristic spreading of these two species. Therefore, a sentinel laboratory would consider these cultures suspicious for *B. anthracis* and send them to a reference laboratory. If susceptibility tests were done before realizing that the organism was a potential *B. anthracis*, the penicillin susceptibility would give weight to the suspicion that they were indeed *B. anthracis*. In the meantime, the clinician would start prophylactic therapy. Our laboratory is currently exploring different characteristics of these isolates that might help to easily separate them from *B. anthracis* in the early stages of identification.

Under current guidelines in the United States, ciprofloxacin is the drug of choice for prophylaxis. For therapy, the CDC recommends a combination therapy of ciprofloxacin and at least one other efficacious antibiotic, while the CDC performs the MICs on a limited battery of antimicrobials: ciprofloxacin, clindamycin, erythromycin, penicillin, rifampicin, tetracycline and vancomycin.[3,44] And although *B. anthracis* can produce penicillinase and cephalosporinase, penicillin historically has been useful for treatment, especially when combined with streptomycin.[45] Because ciprofloxacin is the drug of choice for both prophylaxis and treatment, it is possible that in the event of a biothreat or accidental exposure to *B. anthracis*, the ciprofloxacin supply could be seriously depleted. To help ease the burden, the newer quinolones could be used in the place of ciprofloxacin with successful expectations since *B. anthracis* isolates were fully susceptible to all of the quinolones. The newer antimicrobials such as linezolid, daptomycin and tigecycline also offer newer choices for therapy against any of the *Bacillus* species (Table 2).

For the treatment of *B. cereus*, *B. thuringiensis* and other *Bacillus* infections, there is little advice found for treatment. *B. cereus* is usually susceptible to aminoglycosides, chloramphenicol, clindamycin, erythromycin, tetracycline and vancomycin.[30] Yet various *Bacillus* species in this study demonstrated small populations with some form of resistance to clindamycin and erythromycin. Therefore the wider choice of newer antimicrobials can be useful in treating an infection.

In conclusion, this paper has broadened the number of antimicrobials potentially useful against *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides* and *B. thuringiensis*. *In vitro* testing by Etest® and Sensititre® methods produced comparable results. Resistance in these species to trimethoprim/sulfamethoxazole was confirmed by using a lower testing temperature and longer incubation time during testing.

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Transparency declarations

None to declare.

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