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Comparison of Three Reference Methods for Testing Susceptibility of Staphylococci to Trimethoprim-Sulfamethoxazole▽

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Three reference MIC methods approved by the Clinical and Laboratory Standards Institute were compared by testing 567 staphylococci against trimethoprim-sulfamethoxazole. Category agreement ranged from 94.9% (broth macrodilution versus broth microdilution) to 98.6% (agar dilution versus broth microdilution). Twenty-seven strains resistant by broth macrodilution were susceptible by broth microdilution.

While it is commonly accepted that reference method results can vary by one dilution, some antimicrobial-organism combinations have even greater variability (4). Bacteriostatic antimicrobials such as trimethoprim-sulfamethoxazole (SXT) can demonstrate trailing end points when reference MIC testing is performed, especially with staphylococci (6). Trailing growth interpretation, even by trained technologists, can be subjective, and variations among different reference methods add to the complexity of end point determination. A study was performed to compare SXT MICs determined for staphylococci by three reference methods (agar dilution [AD], broth microdilution [BMI], and broth macrodilution [BMA]) approved by the Clinical and Laboratory Standards Institute (CLSI).

A total of 567 clinical *Staphylococcus* strains were tested; 472 from an internal stock collection were tested, and 95 were collected and tested externally at Barnes-Jewish Hospital, St. Louis, MO. This set comprised 333 *S. aureus* isolates and 234 strains of various other *Staphylococcus* species (Table 1).

Several batches of AD plates and BMA tubes were prepared internally. TREK Diagnostic Systems, Inc., Cleveland, OH, manufactured one lot of frozen BMI panels. Quality control was performed on each day of testing by following the guidelines outlined in CLSI documents (1, 2).

Fresh clinical isolates or isolates stored at −70°C were subcultured twice on Trypticase soy agar containing 5% sheep blood prior to testing. All isolates were incubated 18 to 24 h at 35°C in either ambient air or 5% CO_2. The isolates were suspended in aqueous 0.45% NaCl to achieve a turbidity equivalent to a 0.5 McFarland standard and then diluted appropriately and inoculated into the reference materials according to the CLSI guidelines. All inoculated reference materials were incubated for 16 to 20 h at 35°C in ambient air.

SXT was tested in serial twofold dilutions ranging from 0.5/9.5 to 8/152 μg/ml. Results from all three MIC reference methods were interpreted with respect to an end point of ≥80% reduction in growth compared to that in the positive control (1). BMI and BMA results were read using transmitted light; AD results were read using reflected light. All study participants were trained to read the tests in the same manner. The data were compiled and compared. The following CLSI breakpoints were used: MIC of ≤2/38 μg/ml, susceptible, and MIC of ≥4/76 μg/ml, resistant.

Twenty-three *S. aureus* strains for which differences in ref-

TABLE 1. Staphylococcal isolates tested

Species	No. of isolates
S. aureus	333
S. auricularis	3
S. capitis	3
S. chromogenes	3
S. cohnii subsp. *cohnii*	3
S. cohnii subsp. *urealyticus*	2
S. epidermidis	102
S. haemolyticus	43
S. hominis	10
S. hyicus	3
S. intermedius	3
S. kloosii	3
S. lentus	3
S. lugdunensis	3
S. saprophyticus	23
S. schleiferi	3
S. sciuri	3
S. simulans	6
S. warneri	4
S. xylosus	3
Coagulase-negative staphylococci, not otherwise specified	8

TABLE 2. Results from AD versus BMI[a]

BMI MIC (μg/ml)	No. of isolates with AD MIC (μg/ml) of:					Total
	≤0.5/9.5	1/19	2/38	4/76	≥8/152	
≤0.5/9.5	425	3				428
1/19	13	12				25
2/38		2	7	3		12
4/76			5	26	10	41
≥8/152				3	58	61
Total	438	17	12	32	68	567

[a] CLSI breakpoints are as follows: MIC ≤ 2/38 μg/ml, susceptible; MIC ≥ 4/76 μg/ml, resistant.

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TABLE 3. Results from BMA versus BMI[a]

BMI MIC (μg/ml)	No. of isolates with BMA MIC (μg/ml) of:					Total
	≤0.5/9.5	1/19	2/38	4/76	≥8/152	
≤0.5/9.5	410	8	1	1	6	426
1/19		10	3	4	8	25
2/38			4	2	6	12
4/76			2	27	12	41
≥8/152				1	60	61
Total	410	18	10	35	92	565

[a] CLSI breakpoints are as follows: MIC ≤ 2/38 μg/ml, susceptible; MIC ≥ 4/76 μg/ml, resistant.

erence MICs were greater than one doubling dilution were typed using the DiversiLab system (bioMérieux, Inc., Durham, NC). The strains were tested by following the manufacturer's procedure.

Tables 2 and 3 display the MIC results for comparing BMI to AD and BMA. Results for quality control organisms *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were within CLSI-published ranges for all reference testing (data not shown).

While BMI and AD had comparable results in identifying strains as susceptible or resistant (category agreement, 98.6% [559 of 567 isolates were assigned to the same categories]), BMA identified 27 strains as resistant that BMI identified as susceptible. This outcome caused the category agreement between BMI and BMA to drop below 95% (to 94.9% [536 of 565 isolates were assigned to the same categories]). While four errors were due to one-dilution discrepancies, the discrepancies for 25 strains were greater than one dilution (Table 4). The majority of these 25 strains were *S. aureus* ($n = 21$), with the results for one isolate each of *S. cohnii* subsp. *cohnii*, *S. hominis*, *S. saprophyticus*, and *S. warneri* also exhibiting discrepancies of greater than one dilution.

The isolates with discrepancies of greater than one dilution were chosen for further study. Testing of these strains by the three reference methods was repeated. The trend toward increased MICs from BMA compared to those from AD and BMI was reproduced with very few exceptions. Due to limited materials/resources, 10 of the *S. aureus* isolates with discrepant results were selected for reproducibility testing. This testing was performed by three laboratory technicians, and isolates were tested by the three methods for 3 days, resulting in nine results per strain for each method. The data demonstrated that the trend toward increased MICs from BMA compared to those from AD and BMI was reproducible (repeat/reproduc-

TABLE 4. List of strains for which category errors are found when BMI is used as the standard[a]

Species	Sample no.	BMI		AD		BMA	
		MIC (μg/ml)	Interpretation	MIC (μg/ml)	Interpretation	MIC (μg/ml)	Interpretation
S. aureus	7570	4/76	R	2/38	S, VME*	8/152	R
S. aureus	8753	2/38	S	1/19	S	8/152	R, ME
S. aureus	8816	2/38	S	2/38	S	8/152	R, ME
S. aureus	8865	4/76	R	2/38	S, VME*	8/152	R
S. aureus	12026	1/19	S	≤0.5/9.5	S	4/76	R, ME
S. aureus	12027	≤0.5/9.5	S	≤0.5/9.5	S	4/76	R, ME
S. aureus	12028	1/19	S	1/19	S	>8/152	R, ME
S. aureus	12029	1/19	S	≤0.5/9.5	S	>8/152	R, ME
S. aureus	12030	≤0.5/9.5	S	≤0.5/9.5	S	8/152	R, ME
S. aureus	12031	1/19	S	≤0.5/9.5	S	8/152	R, ME
S. aureus	12032	1/19	S	≤0.5/9.5	S	8/152	R, ME
S. aureus	12033	1/19	S	≤0.5/9.5	S	8/152	R, ME
S. aureus	12034	1/19	S	≤0.5/9.5	S	>8/152	R, ME
S. aureus	12039	1/19	S	≤0.5/9.5	S	>8/152	R, ME
S. aureus	12133	2/38	S	2/38	S	8/152	R, ME
S. aureus	12135	≤0.5/9.5	S	1/19	S	8/152	R, ME
S. aureus	12139	1/19	S	1/19	S	4/76	R, ME
S. aureus	12160	1/19	S	1/19	S	4/76	R, ME
S. aureus	12163	2/38	S	1/19	S	8/152	R, ME
S. aureus	12166	≤0.5/9.5	S	≤0.5/9.5	S	8/152	R, ME
S. aureus	12168	1/19	S	≤0.5/9.5	S	>8/152	R, ME
S. aureus	13305	≤0.5/9.5	S	1/19	S	8/152	R, ME
S. aureus	B111	1/19	S	≤0.5/9.5	S	4/76	R, ME
S. cohnii subsp. *cohnii*	9414	2/38	S	2/38	S	8/152	R, ME
S. epidermidis	7109	2/38	S	4/76	R, ME*	4/76	R, ME*
S. epidermidis	8568	2/38	S	4/76	R, ME*	2/38	S
S. epidermidis	12040	2/38	S	4/76	R, ME*	4/76	R, ME*
S. epidermidis	13204	4/76	R	2/38	S, VME*	8/152	R
S. epidermidis	13298	4/76	R	2/38	S, VME*	4/76	R
S. epidermidis	13299	4/76	R	2/38	S, VME*	2/38	S, VME*
S. hominis	12038	≤0.5/9.5	S	≤0.5/9.5	S	8/152	R, ME
S. hominis	13971	4/76	R	4/76	R	2/38	S, VME*
S. saprophyticus	12037	2/38	S	2/38	S	8/152	R, ME
S. warneri	12035	≤0.5/9.5	S	≤0.5/9.5	S	8/152	R, ME

[a] *, one-doubling-dilution category error; ME, major error; VME, very major error; S, susceptible; R, resistant.

ibility data not shown). Sterility testing was also performed; no contamination was found in the reference materials. The same 10 isolates were sent to the ICARE laboratory at Emory University's Rollins School of Public Health for BMI and molecular testing. ICARE confirmed that these isolates were identified as susceptible to SXT when tested by the BMI method (data not shown). Testing of these isolates for *dfrA*, which confers trimethoprim resistance, was also performed, with seven isolates yielding positive results while three were negative. These findings suggest that *dfrA* was not solely responsible for SXT resistance.

The discrepancy in results from the different methods may be explained by the observation that results from the BMA method are easier to read, since an 80% growth reduction standard can be made by mixing 2 ml of the positive control tube with 8 ml of uninoculated broth. Test samples can then be compared to the 80% growth reduction standard against a lined sheet of paper to evaluate turbidity differences more objectively. BMI is similar in that an 80% reduction control well can be made, but the BMI end point is still more subjective to read than the BMA end point. AD results are the most difficult to interpret, as it is not possible to make an 80% reduction control for comparison. The BMA method may, in theory, allow for resistance to be expressed differently than it is in other growth media, although no studies were done to investigate whether this was the case. It is impossible to conclude which method gives the correct MIC since all are approved reference methods for determining MICs. Clinical outcome data were not available for this study.

To investigate the possibility that the strains with discrepant results were genetically related, repetitive-sequence-based PCR using the DiversiLab system was performed. The DiversiLab system works by using an optimized repetitive-sequence-based PCR method, Agilent Lab-on-a-Chip electrophoresis and detection, and computerized electropherogram matching (3). The 23 *S. aureus* strains with discrepant results were separated into seven distinct genotypic groups. Of these groups, four were distinct from any of the USA PFGE types or the Iberian or Brazilian strains (5). One group containing three strains was similar to the Iberian clone, and one strain was similar to USA 100. It appeared that 13 (56.5%) of the 23 strains belonged to one related group, and 8 (61.5%) of those 13 strains came from one site. These 13 strains were indistinguishable from representatives of PFGE type USA 100 by the DiversiLab system. Thus, although there appeared to be a clone of highly related organisms within this test set, six unrelated genotypic groups also showed discrepant results.

Reference method variability is of greatest concern when the variation occurs around the breakpoint and no intermediate category exists, giving the appearance that an isolate is changing from susceptible to resistant or vice versa when in fact the true MIC for the isolate may place it somewhere in between. The data shown here indicate that laboratory personnel should be aware that even CLSI-approved reference methods can give different MICs for the same isolate, particularly for bacteriostatic antimicrobials such as SXT. In addition, care must be taken that trailing end points are read consistently among laboratory personnel in order to minimize variability. In many cases, the clinical relevance of the differences among MIC reference methods has not been determined.

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