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Comparison of Three Commercial MIC Systems, E test, Fastidious Antimicrobial Susceptibility Panel, and FOX Fastidious Panel, for Confirmation of Penicillin and Cephalosporin Resistance in *Streptococcus pneumoniae*

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The performances of three commercial broth microdilution MIC assays adapted for use with fastidious organisms—the E test (ET), Fastidious Antimicrobial Susceptibility panel (FAS), and FOX Fastidious panel (FOX)—were compared with a MIC using Mueller-Hinton broth with 5% lysed horse blood (MHLHB) to confirm penicillin and cephalosporin resistance in clinical isolates of *Streptococcus pneumoniae*. Of the isolates screened for penicillin resistance, 5 (12.8%) were categorized as susceptible, 16 (41.0%) were categorized as intermediate, and 18 (46.2%) were categorized as resistant by MHLHB. Only the isolates exhibiting intermediate-to-resistant MICs were included in the comparison. Agreement within ±1 $\log_2$ dilution was found in 91, 21, and 76% of the ET, FAS, and FOX MICs, respectively, compared with the MHLHB MIC. No very major or major discrepancies occurred with the ET or FOX; however, two very major interpretive errors occurred with the FAS. Agreement between the ET and MHLHB for cefotaxime, ceftriaxone, and cefuroxime was 88, 85, and 100%, respectively. Less than 50% of cephalosporin MICs categorized as >0.5 μg/ml by MHLHB were detected by FAS or FOX. Of the methods compared, the ET was the most reliable alternative for susceptibility testing of pneumococci.

Serious infections associated with penicillin-resistant strains of *Streptococcus pneumoniae* are being reported with increasing frequency (3, 4, 10). The laboratory assay most commonly performed to determine penicillin resistance in *S. pneumoniae* is the Kirby-Bauer disk diffusion procedure, which utilizes a 1-μg/ml oxacillin disk. Studies indicate that the oxacillin disk diffusion screen may not always accurately predict penicillin resistance (5, 14); therefore, isolates producing zones of inhibition of ≤19 mm (resistant) should be retested with an alternate method. Because *S. pneumoniae* often fails to grow adequately for testing in nonsupplemented media, laboratories are advised by the National Committee for Clinical Laboratory Standards (NCCLS) to perform a broth dilution MIC assay with cation-adjusted Mueller-Hinton broth (MH) supplemented with 3 to 5% lysed horse blood (MHLHB). Because of the difficulties often experienced in preparing the broth dilution MIC assay and lysed horse blood, many laboratories choose to purchase commercially prepared antimicrobial susceptibility testing systems designed to accommodate nutritionally fastidious organisms such as *S. pneumoniae*. In the present study, the performance of three commercial systems designed for use with fastidious isolates such as *S. pneumoniae*, the E test (ET) (AB Biodisk, North America, Inc., Culver City, Calif.), the Fastidious Antimicrobial Susceptibility panel (FAS) (Microtech Medical Systems, Aurora, Colo.), and the FOX Fastidious panel (FOX) (Micro Media Systems, Cleveland, Ohio), were evaluated for the determination of penicillin resistance in *S. pneumoniae*. In addition, the performances of the three systems for the accurate determination of cefuroxime, cefotaxime, and ceftriaxone MICs were also evaluated.

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MATERIALS AND METHODS

Bacterial isolates. Isolates were recovered from blood, cerebrospinal fluid, the middle ear, the eye, and tracheal aspirates. Among the isolates screened, 18 (46.2%) were resistant, 16 (41.0%) were intermediate, and 5 (12.8%) were susceptible to penicillin when tested with MHLHB. Only the isolates exhibiting penicillin MICs in the intermediate-to-resistant range were included in the comparison. *S. pneumoniae* ATCC 6305 (penicillin susceptible) and CDC-78-008104 (penicillin resistant) (REMEL, Inc., Lenexa, Kans.) were included as quality control strains for each assay. Clinical isolates were frozen at −70°C with porous beads in cryopreservative medium (Microbank; PRO-LAB Diagnostics, Austin, Tex.). One bead was removed from the vial and inoculated on Trypticase soy agar containing 5% sheep blood according to the manufacturer's recommendations. No differences in results were obtained when strains were tested either directly after isolation or after storage at −70°C. Isolates stored at −70°C

TABLE 1. Agreement between penicillin MICs obtained with *S. pneumoniae*[a] for ET, FAS, and FOX versus the reference method

Method	No. of isolates within the following $\log_2$ dilution increment(s) of reference MIC:							% Agreement (% minor interpretive errors)[b]
	<−2	−2	−1	0	+1	+2	>+2	
ET	0	2	8	21	2	1	0	91 (18)
FAS	12	15	6	1	0	0	0	21 (62)
FOX	0	7	16	10	0	1	0	76 (33)

[a] Only isolates with penicillin MICs within the intermediate-to-resistant range were included in the comparison.

[b] Determination of interpretive errors was based on newly established ranges published in NCCLS document M7-A3 (11). The majority of errors obtained with each panel type were minor interpretive errors; however, two very major errors were produced with the FAS.

TABLE 2. Agreement between cefuroxime, cefotaxime, and ceftriaxone MICs obtained with 34 isolates of penicillin-resistant *S. pneumoniae*[a] for ET versus reference method

Antimicrobial agent	No. of isolates within the following $\log_2$ dilution increment(s) of reference MIC:							% Agreement (% minor interpretive errors)[b]
	<−2	−2	−1	0	+1	+2	>+2	
Cefuroxime	0	0	9	20	5	0	0	100 (6)
Cefotaxime	1	2	15	13	2	1	0	88 (18)
Ceftriaxone[c]	0	5	12	12	5	0	0	85 (24)

[a] Only isolates with penicillin MICs within the intermediate-to-resistant range were included in the comparison.
[b] No very major or major errors occurred.
[c] The ceftriaxone ET currently has not been approved by the Food and Drug Administration for laboratory use.

were subcultured on sheep blood agar a minimum of three times prior to testing.

Antimicrobial agents, disks, and reagents. Antimicrobial agents evaluated included penicillin, cefuroxime, cefotaxime, and/or ceftriaxone. Standard antimicrobial powders were obtained from the U.S. Pharmacopeia Convention, Inc., Rockville, Md. Oxacillin (1 μg/ml) antimicrobial agent-impregnated disks were purchased from Becton Dickinson Microbiology Systems, Cockeysville, Md. Horse blood and microbiological media, unless otherwise specified, were obtained from REMEL, Inc. Broth MIC assays, disk diffusion assays, and antimicrobial susceptibility testing interpretive ranges were performed according to NCCLS documents M7-A3 (11) and M2-A5 (12) unless otherwise specified.

Reference method and ET. For both assays, an inoculum from a 24-h culture was suspended in 5 ml of MH and adjusted to match a 0.5 McFarland turbidometric standard. For the reference MIC, a 50-μl aliquot was added to each of the wells of a microtiter plate containing $\log_2$ dilutions of the antimicrobial agent in order to achieve a final concentration of 5×10^5 CFU/ml. Lysed horse blood was added to each well to achieve a final concentration of 5% (vol/vol). Plates were incubated at 35°C in ambient air for 20 to 24 h and read with a light box.

For the ET, a cotton swab dipped into the suspension was used to inoculate the entire surface of MH agar plate (15 by 150 mm) containing 5% sheep blood. After placement of the ET, the plate or plates were incubated at 35°C in ambient air or CO_2 for 18 to 24 h. The MIC was read at the point where the zone of inhibition intersected the antimicrobial agent concentration scale on the strip.

FAS and FOX. Assays were performed according to the individual manufacturer's recommendations. Wilkens-Chalgren and supplemented Trypticase-proteose peptone broth enriched with hematin were the growth media contained in the FAS and FOX, respectively. Both the FAS and FOX were stored at −20°C prior to testing. Bacterial suspensions prepared in sterile distilled water were inoculated into the respective panels to achieve a final concentration of between 3×10^5 and 5×10^5 CFU/ml. Assays were incubated in ambient air at 35°C for 18 to 24 h. MICs were determined according to established procedures.

Evaluation criteria. All results were compared with those from the reference method. Results were considered in agreement when MICs were within 1 $\log_2$ dilution increments by either method. Since the antimicrobial agents present in the ET are applied as a concentration gradient rather than in doubling dilutions, ET MICs that fell between the $\log_2$ dilution concentrations of the MHLHB were considered equivalent to the higher $\log_2$ dilution concentration. The determination of interpretive errors was based on the ranges for penicillin, cefuroxime, cefotaxime, and ceftriaxone recently published by the NCCLS in document M7-A3 (11). Discrepant results were categorized in the following manner: very major (false susceptibility), major (false resistance), or minor (susceptible or resistant versus an intermediate result).

RESULTS

Overall agreement. Antimicrobial susceptibility testing of penicillin, cefuroxime, cefotaxime, and/or ceftriaxone was performed only with isolates which produced intermediate-to-resistant penicillin MICs (Table 1). Of the penicillin MIC assays performed in the evaluation, 91% of the ET MICs were within 1 $\log_2$ dilution of the MIC obtained with the MHLHB, while only 21% of the FAS MICs and 76% of the FOX MICs were within this dilution range. The majority of interpretive errors obtained with each panel were minor errors; however, two very major errors were obtained with the FAS.

The percentage of agreement of the three methods compared with MHLHB when testing the cephalosporins is represented in Table 2. Of the 34 isolates evaluated, the percentages of agreement obtained with the ET were 100% for cefuroxime, 88% for cefotaxime, and 85% for ceftriaxone. The percentages of agreement between the FAS and FOX with MHLHB could not be determined because of the higher starting concentrations of the cephalosporins used in these panels (≤0.5 μg/ml), which do not reflect the recent changes in NCCLS cephalosporin interpretive ranges for *S. pneumoniae* (Table 3). Of the MICs of >0.5 μg/ml, only two cefuroxime and nine ceftriaxone MICs obtained with the FAS and two cefuroxime, eight cefotaxime, and eight ceftriaxone MICs obtained with the

TABLE 3. Agreement between cefuroxime, cefotaxime, and ceftriaxone MICs obtained with 34 isolates of penicillin-resistant *S. pneumoniae*[a] for FAS and FOX versus reference method

Method	Antimicrobial agent	No. of isolates within the following $\log_2$ dilution increment(s) of reference MIC:						
		<−2	−2	−1	Indeterminate[b]	+1	+2	>+2
FAS	Cefuroxime	8	9	2	15	0	0	0
	Ceftriaxone	3	8	9	14	0	0	0
FOX	Cefuroxime	12	5	2	15	0	0	0
	Cefotaxime	1	7	8	18	0	0	0
	Ceftriaxone	3	7	8	16	0	0	0

[a] Only isolates with penicillin MICs within the intermediate-to-resistant range were included in the comparison.
[b] Because of the higher starting concentration for both the FAS and FOX (≤0.5 μg/ml) versus MHLHB (0.03 μg/ml), MICs below this range could not be evaluated and were categorized as indeterminate. The numbers of $\log_2$ dilution differences between isolates producing MICs ≥0.5 μg/ml by FAS, FOX, and MHLHB are represented.

TABLE 4. Agreement between ET MICs obtained by incubation in ambient air versus CO_2 compared with the reference method for 55 *S. pneumoniae* isolates[a]

ET and incubation conditions	No. of isolates within the following log_2 dilution increment(s) of reference MIC:							% Agreement (% minor interpretive errors)[b]
	<2	−2	−1	0	+1	+2	>+2	
Penicillin								
Air	1	4	14	32	3	1	0	89 (18)
CO_2	2	3	17	28	5	0	0	91 (15)
Cefuroxime								
Air	2	2	18	28	5	0	0	93 (13)
CO_2	2	5	14	30	3	1	0	85 (16)
Cefotaxime[c]								
Air	6	4	26	16	3	0	0	82 (24)[d]
CO_2	0	3	21	20	10	1	0	93 (24)

[a] Isolates tested included study isolates and 21 additional isolates which were penicillin resistant by oxacillin disk diffusion screen; however, one isolate was susceptible when tested by the reference method. Only isolates tested with penicillin, cefotaxime, and cefuroxime which grew in both ambient air and CO_2 were compared.

[b] Determination of interpretive errors was based on newly established ranges published in NCCLS document M7-A3 (11). No very major or major interpretive errors occurred with penicillin and cefuroxime.

[c] Ceftriaxone was considered comparable to cefotaxime for this comparison and was, therefore, not tested.

[d] Three very major errors occurred with cefotaxime when incubation was in ambient air.

FOX were within 1 log_2 dilution of the MIC obtained with MHLHB (Table 3).

Comparison of the ET results obtained with penicillin, cefuroxime, and cefotaxime for selected isolates when incubated in CO_2 versus ambient air (Table 4) indicated that MICs did increase for assays incubated in CO_2, and the percentage of agreement with MHLHB was higher for penicillin and cefotaxime when incubated under these conditions.

DISCUSSION

The performance of any commercial assay must be carefully evaluated by the laboratory before the system is adopted for routine use. *S. pneumoniae* is a fastidious organism that requires supplemented media in order to produce acceptable growth for accurate antimicrobial susceptibility testing. While some isolates may grow sufficiently to produce an acceptable growth control in unsupplemented media such as those used in conventional commercial panels, the accuracies of the MICs compared with those of a reference method are often unacceptable. For example, the FAS produced acceptable results with all quality control strains tested and growth controls were considered adequate in all assays. The percentage of agreement with MHLHB, however, was considered unacceptable for all antimicrobial agents evaluated in the study. Quality control organisms previously recommended by the manufacturers for use with both FAS and FOX included American Type Culture Collection strains of *Escherichia coli* and *Staphylococcus aureus* as well as *Haemophilus influenzae*. The inability of the nonfastidious organisms to adequately challenge the potential of the broth formulation to support the growth of more fastidious organisms emphasizes the importance of using one or more strains of *S. pneumoniae* during quality control assessment. Recently, *S. pneumoniae* has been recommended for use by both manufacturers.

The FOX produced penicillin MICs within 1 log_2 dilution of those of the reference method for 26 of 34 isolates. These results are in contrast to those obtained in a previous evaluation of the FOX in which false susceptibility occurred with 11 of 12 *S. pneumoniae* isolates categorized as resistant by the oxacillin disk diffusion screen. Results were reported as 1 to 2 log_2 dilutions lower than those of both a broth macrodilution MIC assay supplemented with lysed horse blood and an agar dilution assay (2). In the present study, the majority of FOX MICs were also 1 to 2 log_2 dilutions lower than those obtained with MHLHB. A MIC that was 2 log_2 dilutions higher than that of MHLHB was obtained with one isolate when tested with both the FOX and ET. No differences in MHLHB MICs were obtained when rechecked, and these values were attributed to the poor growth of this isolate in MHLHB. The broth formulation used in the FOX is similar to *Haemophilus* test medium. *Haemophilus* test medium has been evaluated as a growth medium in MIC assays when testing *S. pneumoniae* (6, 9). Although the MICs determined in one study with *Haemophilus* test medium produced percentages of agreement of 100 to 96.2% for a battery of drugs including penicillin, cefaclor, and cefuroxime, the growth densities of the isolates were lower and the MICs were ≥1 log_2 dilution lower than those achieved with the reference method (6). Because NCCLS interpretive ranges for *S. pneumoniae* are established for isolates tested in MIC assays prepared with MHLHB, minor interpretive errors were attributed to the trend toward lower MICs obtained with *Haemophilus* test medium.

Our results, as well as data compiled from other studies, confirm the accuracy of the ET for susceptibility testing of *S. pneumoniae* (1, 7, 8, 13). Ngui-Yen et al. (13) evaluated the ET for determining the MICs for 208 gram-positive isolates, including 32 strains of *S. pneumoniae*. A percentage of agreement of 94% was obtained between the ET and the reference method (13). A recent multicenter evaluation of the ET for detection of penicillin and cephalosporin resistance among *S. pneumoniae* clinical isolates found that 136 of 147 (92.5%) isolates tested with the ET were in agreement with the reference method for penicillin and 96.6% were in agreement for cefotaxime (7). No very major or major interpretive errors occurred with either assay; however, minor errors of 9.5 and 5.4% were obtained with penicillin and cefotaxime ET MICs, respectively, and involved MICs categorized as intermediate by MHLHB and susceptible by ET.

Newly published NCCLS protocols recommend incubation of disk-diffusion tests for *S. pneumoniae* in CO_2 because of the poor viability of some isolates when incubated in ambient air (12). Jorgensen et al. (9) found increased agreement between the ET, when incubated in CO_2, and MHLHB. Percentages of agreement increased from 76% (air) to 91% (CO_2) for penicillin and 91% (air) to 100% (CO_2) for cefotaxime (9). In our evaluation, the percentages of agreement between MHLHB and penicillin and cefotaxime were also higher for the ET when incubated in CO_2, while cefuroxime ET MICs were comparable under both incubation conditions. More importantly, however, MICs were obtained for several isolates only after incubation in CO_2.

Of the assays evaluated, the ET was the most simple and accurate method to perform. The FOX was able to detect penicillin MICs in the intermediate-to-resistant ranges; however, adjustments in starting concentrations and/or recommendations concerning cephalosporin MIC interpretations are necessary in light of recent NCCLS changes in interpretive standards. The manufacturer of the FAS has announced the development of a newly formulated panel, which, when available, may afford laboratories with an additional choice in testing methods. The importance of laboratory validation of

the accuracy of any new antimicrobial susceptibility testing methodology, however, must again be stressed.

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