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Evaluation of Accuracy and Reproducibility of E test for Susceptibility Testing of *Streptococcus pneumoniae* to Penicillin, Cefotaxime, and Ceftriaxone

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We evaluated the reproducibility with which technologists perform and interpret the E test (AB Biodisk, North America, Inc., Piscataway, N.J.) for determining the susceptibility of *Streptococcus pneumoniae* to penicillin, cefotaxime, and ceftriaxone. Four technologists prepared E test assays to test 124 isolates of *S. pneumoniae*. Each technologist then interpreted the results of the E test blinded to the interpretation of the other technologists. In addition, E test results were compared with the reference method of broth microdilution. Intraobserver and interobserver agreement were assessed by use of the kappa statistic. Interpretation of the E test and broth microdilution results showed substantial to excellent agreement, with kappa values ranging from 0.878 to 0.987. Compared with broth microdilution, no very major errors and only four major errors were made with the E test. Most minor errors with penicillin and ceftriaxone occurred for isolates with intermediate or high-level resistance, whereas for cefotaxime the minor errors were more evenly distributed between susceptible and intermediate resistance and between intermediate and high-level resistance. These results indicate that there is good agreement between technologists for the interpretation of the E test when testing the susceptibility of *S. pneumoniae* to penicillin, cefotaxime, and ceftriaxone and that the results of the E test agree with those of broth microdilution.

Isolates of *Streptococcus pneumoniae* that are resistant to penicillin and other antibiotics are being found with increasing frequency (15, 18). These isolates have been associated with serious infections. Traditionally, laboratories have screened clinically significant isolates of *S. pneumoniae* for penicillin resistance with a 1-μg oxacillin disk (19). Although this test is relatively sensitive, it cannot distinguish between isolates with intermediate resistance (MIC, 0.1 to 1.0 μg/ml) and those that are highly resistant (MIC of ≥2.0 μg/ml) to penicillin. Therefore, all *S. pneumoniae* isolates with an oxacillin disk zone of ≤19 mm must be further investigated to ascertain their degree of resistance (8). This is important because recommended therapy for an infection due to an isolate that is highly resistant to penicillin differs from that for an intermediately resistant strain (4, 10). Several reports have now demonstrated a correlation between increased MICs of penicillin and increased MICs of cephalosporins and other β-lactam antibiotics (1, 5, 20). This, together with the fact that treatment failures with the use of broad-spectrum cephalosporins for meningitis due to penicillin-resistant pneumococci have now been well documented, suggests that pneumococcal resistance to penicillin may predict an unfavorable response to other β-lactam antibiotics (6).

Broth microdilution is the current standard method for determining the MIC for *S. pneumoniae* (17). Commercially prepared broth microdilution plates are not widely available, and therefore, many laboratories refer these isolates to reference laboratories for further testing. Recently, the E test (AB Biodisk, North America, Inc., Piscataway, N.J.) has been investigated for its ability to accurately determine the susceptibility of *S. pneumoniae* to penicillin and other β-lactam antibiotics (9, 12, 14). These studies have shown the E test to be easier to use than and as accurate as broth microdilution for determining the susceptibility of *S. pneumoniae* to various antibiotics. However, the reproducibility of the E test has not previously been evaluated.

Therefore, in this study, we evaluated the ease of use, accuracy, and reproducibility of the E test for determining the susceptibility of 124 *S. pneumoniae* isolates to penicillin, cefotaxime, and ceftriaxone. The E test results were compared with those obtained with the broth microdilution method.

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MATERIALS AND METHODS

Test organisms. One hundred twenty-four clinical isolates of *S. pneumoniae* (representing each susceptibility category for penicillin) were selected from a collection of strains obtained from a cross-Canada study carried out in 1994. Isolates were stored frozen at −70°C in phosphate-buffered glycerol and subcultured at least twice onto Columbia sheep blood agar (PML Microbiologicals, Mississauga, Ontario, Canada) prior to testing.

Antimicrobial agents. The antimicrobial agents evaluated were penicillin, cefotaxime, and ceftriaxone (Sigma Chemical Co., St. Louis, Mo.). Stock solutions of the drugs were prepared according to the National Committee for Clinical Laboratory Standards (NCCLS) document M7-A3 (17). The following ranges of twofold dilutions were used for testing: penicillin, 0.015 to 8 μg/ml; cefotaxime and ceftriaxone, 0.015 to 4 μg/ml. The E test strips were kindly provided by Unipath, Nepean, Ontario, Canada.

Reference broth microdilution testing. Broth microdilution panels were prepared in-house according to NCCLS recommendations (17) with cation-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) with 5% lysed horse blood. Inocula were prepared by suspending colonies from an 18- to 24-h culture of the test organisms in 2 ml of Mueller-Hinton broth to the turbidity of a 0.5 McFarland standard. This suspension was further diluted in the inoculum tray with sterile distilled water to a final inoculum of 5×10^5 CFU/ml in each well of the test panel. The panels were incubated in ambient air at 35°C

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TABLE 1. Susceptibility results for 124 isolates of *S. pneumoniae* by broth microdilution and E test

Test[a]	Mean no. of isolates (range) for antibiotic:								
	Penicillin			Cefotaxime			Ceftriaxone		
	Susc[b]	Inter[b]	Res[b]	Susc	Inter	Res	Susc	Inter	Res
Broth microdilution	27[c] (27–28)	36[c] (33–39)	62[c] (58–64)	52[c] (52–54)	56[c] (54–58)	16[c] (13–19)	51[c] (50–52)	58 (54–62)	15[c] (12–20)
E test									
Set up by A	20.0[c] (18–21)	40.0[c] (39–41)	63.8 (62–66)	48.0[c] (48)	61.3[c] (55–66)	14.8 (10–21)	45.3[c] (44–46)	53.3 (46–59)	25.5[c] (19–33)
Set up by B	20.8[c] (20–21)	41.5[c] (39–44)	61.8 (59–64)	47.8[c] (47–49)	62.5[c] (58–69)	13.8 (6–19)	43.0[c] (41–45)	52.3 (43–61)	28.8[c] (18–40)
Set up by C	21.0[c] (21)	46.8[c] (45–50)	56.3[c] (53–58)	50.0[c] (49–51)	69.8[c] (67–72)	4.3[c] (2–7)	46.0[c] (45–48)	62.8 (56–67)	15.3 (9–23)
Set up by D	20.0[c] (20)	44.0[c] (43–46)	60.0 (58–61)	49.5[c] (49–50)	69.8[c] (68–71)	4.8[c] (3–7)	44.3[c] (44–46)	59.3 (54–65)	20.5[c] (14–26)

[a] Broth microdilution was set up by one technologist and read by four different technologists twice (eight readings). The E test was set up by four different technologists. Each technologist read his or her own set of E tests as well as those of the other three technologists.

[b] Susc, susceptible; Inter, intermediate; Res, resistant.

[c] $P < 0.05$, Student's *t* test for comparison of the mean number of isolates within a susceptibility category between broth microdilution and E test.

for 20 to 24 h prior to the determination of MICs. Ten organisms that failed to grow in ambient air were retested and incubated in 5% CO_2 at 35°C for 20 to 24 h.

E test. A cotton swab dipped into a 0.5-McFarland standard suspension of the test isolate was used to inoculate a 15-cm Mueller-Hinton agar plate containing 5% sheep blood (PML Microbiologicals). Three E test strips (penicillin, cefotaxime, and ceftriaxone) were then placed onto each plate. The plates were incubated at 35°C in 5% CO_2 for 20 to 24 h before being examined. The MIC for the isolate was determined as per the manufacturer's instructions. In brief, the MIC was the point at which the growth margin intersected the E test strip. Because the E test strips are marked in one-half log concentrations, it is possible to record MICs in increments smaller than the usual twofold increments used for broth microdilution. Therefore, for purposes of classifying organisms as susceptible, intermediately resistant, and highly resistant, MICs obtained with the E test were rounded to the next higher $\log_2$ dilution if the MIC fell between the standard twofold increments of the reference method (7, 12).

Evaluation criteria. The E test MIC results were compared with those of the reference method. The breakpoints were those recommended by the NCCLS (17); for penicillin, susceptible, ≤0.06 μg/ml; intermediately resistant, 0.1 to 1.0 μg/ml; resistant, ≥2.0 μg/ml; for cefotaxime and ceftriaxone, susceptible, ≤0.25 μg/ml; intermediately resistant, 0.5 to 1.0 μg/ml; resistant, ≥2.0 μg/ml. The results were characterized as follows: concordant (reference and E test agree), very major error (reference resistant, E test susceptible), major error (reference susceptible, E test resistant), and minor error (reference resistant or susceptible and E test intermediate, or reference intermediate and E test susceptible or resistant) (12).

Study design protocol. Broth microdilution testing of the *S. pneumoniae* isolates was prepared and interpreted by a technologist not involved in this study. These results were used as the reference MICs. Subsequently, to determine intra- and interobserver agreement for interpretation of broth microdilution MICs, a second set of broth microdilution panels was set up by one technologist and interpreted twice by each of the four study technologists, each of whom was blinded to the results of his or her first reading and to those of the other technologists. Each of the four study technologists prepared a set of E test susceptibility assays for all of the isolates with three different E test strips (penicillin, cefotaxime, and ceftriaxone). In turn, each technologist independently read his or her own set of E test assays as well as those prepared by the other three technologists. Each technologist was kept blinded to the interpretation of the other three technologists and to the reference MIC as determined by broth microdilution.

Quality control. Two control isolates of *S. pneumoniae* (ATCC 49619 [penicillin intermediately resistant] and ATCC 6303 [penicillin susceptible]) were included in each test batch.

Statistical analysis. The kappa statistic (3) was used to measure agreement about the susceptibility of the *S. pneumoniae* isolates as determined by broth microdilution and the E test. The kappa statistic ranges from 0 to 1, with 1 representing perfect agreement and 0 representing no more agreement than would be expected to occur on the basis of chance alone. In assessing the technologists' interpretation of broth microdilution and the E test, a weight of 1 was given for perfect agreement (classification of an isolate into the identical susceptibility category) and a weight of 0 was given for no agreement (classification of an isolate into different susceptibility categories). Calculated kappa values of ≤0.40 are considered to reflect fair to poor reproducibility or agreement, those of ≥0.41 and ≤0.80 are considered to reflect moderate to substantial agreement, and those of ≥0.81 reflect almost perfect agreement (11, 16).

Because broth microdilution is the reference method, intra- and interobserver agreement of broth microdilution was determined first to ensure that any comparison with the E test would not be biased by poor reproducibility of the reference method. Intraobserver agreement for broth microdilution was determined by comparing each technologist's first reading with her or his second reading of the same set of broth microdilution panels. Interobserver agreement for broth microdilution was determined by pairing each interpretation of a technologist with that of each of the other three technologists for the same set of broth microdilution panels. Because the E test is a relatively new test, agreement between different readers (interobserver) and different setups was assessed. Interobserver agreement was determined by pairing each interpretation of a technologist with that of each of the other three technologists for the same set of E tests. Agreement between different setups was determined by pairing each technologist's reading of one set of E tests with his or her own reading of the other three sets of E tests. All evaluations were done for each isolate of *S. pneumoniae* tested and for each antimicrobial agent tested. Mean kappa values and 95% confidence intervals were determined (2).

Student's *t* test was used to analyze statistical differences between the results obtained by broth microdilution and those obtained by the E test.

RESULTS

Table 1 shows the mean number of isolates that were susceptible, intermediately resistant, and highly resistant to the three antibiotics tested. The mean values for broth microdilution are based on four technologists reading the same set of broth microdilution panels twice (eight readings). For the E test, the mean values shown for each set are based on four readers reading the same set of E tests. The mean number of isolates that were categorized by the E tests as susceptible to each of the three antibiotics was significantly ($P < 0.05$) lower than the number categorized as susceptible by broth microdilution (Table 1). For penicillin and cefotaxime, there was a significant ($P < 0.05$) increase in the number of isolates classified as intermediately resistant by all four E test setups compared with broth microdilution. For ceftriaxone, three of the four E test setups showed a significant ($P < 0.05$) increase in the number of isolates classified as resistant compared with broth microdilution. For setups C and D, the E test detected a mean of four to five isolates highly resistant to cefotaxime, whereas broth microdilution detected a mean of 16 highly resistant isolates ($P < 0.05$).

Table 2 shows the mean kappa values and 95% confidence

TABLE 2. Intra- and interobserver agreement among four different technologists reading the same 124 broth microdilution susceptibility tests twice for each of three different antibiotics

Antibiotic	Mean kappa value (95% confidence interval) for:	
	Intraobserver agreement	Interobserver agreement
Penicillin	0.962 (0.924–0.999)	0.954 (0.944–0.963)
Cefotaxime	0.930 (0.874–0.986)	0.886 (0.870–0.901)
Ceftriaxone	0.900 (0.888–0.929)	0.888 (0.871–0.904)

TABLE 3. Agreement among four technologists reading four sets of 124 E tests (A)[a] and among four different sets of 124 E tests read by the same technologist (B)[b]

Antibiotic	Mean kappa value (95% confidence interval)	
	A	B
Penicillin	0.938 (0.927–0.948)	0.835 (0.818–0.852)
Cefotaxime	0.888 (0.863–0.913)	0.761 (0.732–0.791)
Ceftriaxone	0.829 (0.799–0.859)	0.745 (0.716–0.773)

[a] Kappa values were calculated for pairs of technologists (four technologists, six pairings) reading the same set of 124 E tests. Mean kappa values were then calculated by averaging the kappa values of all pairings.

[b] Kappa values were calculated by pairing each set of 124 E tests (four sets, six pairings) read by the same technologist. Mean kappa values were then calculated by averaging the kappa values of all pairings.

intervals for intraobserver agreement (each technologist reading the same test twice) and interobserver agreement (each technologist's reading of the same test compared with those of the other three technologists) for broth microdilution. The mean kappa values of >0.80 suggest almost perfect agreement for both intra- and interobserver agreement.

Table 3 shows the mean kappa values and 95% confidence intervals for interobserver agreement for different technologists reading the same set of E tests. Agreement between technologists was good regardless of which two technologists were compared and which antibiotic was tested. Agreement for the same technologist reading different sets of E tests (as set up by different technologists) was slightly lower, with kappa values in the moderate to substantial categories for cefotaxime and ceftriaxone (Table 3, column B).

Comparison of the E test with the reference broth microdilution (1,984 comparisons, four sets of E tests × four technologists × 124 isolates) revealed 353, 385, and 378 minor errors for penicillin, cefotaxime, and ceftriaxone respectively (Table 4). Only four major errors were observed for cefotaxime, all of which were for the same isolate. There were no very major errors. Most minor errors for penicillin and ceftriaxone were for classification between intermediately and highly resistant isolates. For cefotaxime, the minor errors were more evenly distributed between susceptible and intermediately resistant and between intermediately and highly resistant isolates.

TABLE 4. Agreement between the reference broth microdilution and E test susceptibility results for 124 *S. pneumoniae* isolates based on 1984 comparisons[a]

Antibiotic	Concordance	No. (%) of interpretive errors[b]				
		Minor[c]			Major	Very major
		S/I	I/R	Total		
Penicillin	1,631	57 (2.9)	293 (14.8)	350 (17.6)	0	0
Cefotaxime	1,595	157 (7.9)	228 (11.5)	385 (19.4)	4 (0.2)	0
Ceftriaxone	1,606	52 (2.6)	326 (16.4)	378 (19.1)	0	0

[a] Each of four technologists prepared a set of E tests for 124 *S. pneumoniae* isolates. In turn, each technologist interpreted her or his own set of E tests as well as those of the other three technologists.

[b] Interpretive errors are based on newly established ranges published in NCCLS document M7-A3 (17).

[c] S/I refers to errors in which one method reported an isolate to be susceptible and the other method reported that same isolate to be intermediately resistant. Similarly, I/R refers to errors in which one method reported an isolate to be intermediately resistant and the other method reported that same isolate to be highly resistant.

DISCUSSION

The increasing incidence seen in *S. pneumoniae* of resistance to penicillin and cephalosporins has produced a need for a testing method that is rapid, accurate, and easy to perform. Although the Kirby-Bauer disk diffusion method, utilizing a 1-μg oxacillin disk, is simple and easy to perform, it may not accurately predict penicillin resistance (19). Therefore, isolates with zones of inhibition of ≤19 mm should be retested with an alternate method. Broth microdilution testing has been recommended by the NCCLS (17), but the medium required for this method is tedious to prepare and is not widely available from commercial sources (7), making it difficult to implement in the routine clinical laboratory. The E test, which is as simple to perform as a disk diffusion test and yet provides the user with a MIC determination, has been shown to be an accurate method for determining the susceptibility of *S. pneumoniae* compared with broth microdilution (7, 9, 12). Although several studies have confirmed the accuracy of the E test, none has evaluated the reproducibility with which it is performed or interpreted by technologists.

For broth microdilution, intraobserver and interobserver agreement was excellent, with kappa values of >0.80, suggesting almost perfect agreement. This may reflect the technologists' greater familiarity with this technique but more likely represents the fact that the $\log_2$ dilutions used in broth microdilution testing allow for a clearer separation of isolates into the different susceptibility categories. Interobserver agreement for technologists reading the same set of E tests was also excellent, reflecting the high level of reproducibility with which different technologists interpret the same E tests. Agreement between different sets of E tests (reflecting possible variation in the technique used to prepare the E tests) was also good, with kappa values in the moderate-to-substantial and almost-perfect agreement categories. In this study, we did not evaluate the effect of each of the four different technologists setting up different panels of broth microdilution tests. Therefore, we could not fully evaluate whether there was variation in the technique used to prepare broth microdilution panels as was noted for the E test. However, agreement between the reference broth microdilution results (set up and interpreted by a non-study technologist) and each of the four study technologists' reading of the broth microdilution panels set up by one of the study technologists was excellent (results not shown), suggesting good reproducibility between different sets of microdilution panels when prepared by different technologists.

When the E test is compared with the reference broth microdilution, the results of our study, together with those of previous studies, confirm that virtually all errors between the two methods are minor (7, 9, 12). The percentage of minor errors reported in our study (ranging from 17.6% for penicillin to 19.4% for cefotaxime) is similar to that previously reported by others, who have shown rates as high as 24% (7, 12). These minor errors may reflect the fact that the antimicrobial agents present in the E test are applied as a concentration gradient rather than in doubling dilutions, making the separation between susceptible and intermediate categories and between intermediate and resistant categories less distinct. In our study, most minor errors for penicillin and ceftriaxone were made between the categories of intermediate and high-level resistance. This suggests that most isolates resistant to penicillin and ceftriaxone tend to cluster around the breakpoint between these two categories. The separation between susceptible and intermediate resistance is more distinct, resulting in fewer errors between these two categories. For cefotaxime, however, minor errors were more evenly distributed between susceptible

and intermediate categories and between intermediately and highly resistant categories, suggesting that the development of resistance to cefotaxime results in a more gradual increase in MICs from susceptible to highly resistant.

Some authors have suggested that differences in MICs within 1 $\log_2$ dilution when determined by E test and broth microdilution are acceptable because they result in minor errors only (7, 12). However, a difference of 1 $\log_2$ dilution in the MIC for an isolate at or near the breakpoint between susceptibility categories could change the reported susceptibility of that isolate and result in a change in its recommended therapy. As shown in Table 1, there were significant differences between the E test and broth microdilution in the number of isolates in most of the susceptibility categories, despite the fact that virtually all errors were minor. This potential for shifting of isolates across different susceptibility categories depending upon the testing method used (or even when the same method is repeated) may account for some of the recent reports of treatment failures with the use of high-dose penicillin or broad-spectrum cephalosporins to treat infections caused by isolates thought to be intermediately resistant when in fact they may have been highly resistant (6, 13).

Since the E test is a simple method for determining the susceptibility of *S. pneumoniae* to penicillin, cefotaxime, and ceftriaxone compared with the reference broth microdilution method, and because a high level of interobserver agreement has been demonstrated, we believe that the E test alone can be used in the routine clinical laboratory to reproducibly determine the susceptibility of these isolates. It is important to recognize the clustering of isolates near the breakpoint between the various susceptibility categories and that this may result in minor errors when the E test is compared with broth microdilution. This may, in turn, result in a shift in the reported susceptibility category of an isolate. However, the significance of these differences between the E test and broth microdilution testing for the categorization of susceptibility of clinical isolates requires further evaluation to determine which method best correlates with clinical response to therapy.

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