Multilaboratory Evaluation of Disk Diffusion Antimicrobial Susceptibility Testing of Neisseria meningitidis Isolates

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Received 27 December 2005/Returned for modification 16 February 2006/Accepted 6 March 2006

In 2005, the Clinical and Laboratory Standards Institute published MIC interpretive criteria for 13 antimicrobial agents used for either therapy or prophylaxis of Neisseria meningitidis infections. The MIC method includes the use of lysed horse blood-supplemented Mueller-Hinton broth with incubation in 5% CO₂ for 20 to 24 h. Since some clinical laboratories might prefer the option of disk diffusion testing for infrequently encountered isolates a multicenter collaborative study was conducted to evaluate the reproducibility of a disk diffusion method for testing isolates of N. meningitidis. Interpretive criteria were developed for 12 antimicrobial agents. Four laboratories tested a common collection of 50 meningococcal strains and then tested 25 unique isolates per laboratory. Isolates were tested using Mueller-Hinton sheep blood agar plates incubated for 20 to 24 h in 5% CO₂; they were also tested by the reference broth microdilution method in parallel. Pooling of the MIC and disk diffusion data from the common and unique isolates provided a sufficient sample size to develop susceptible, intermediate, and resistant zone diameters. Interpretive criteria using the error rate-bounded method for the following agents: chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin, and rifampin. Due to the lack of resistant strains at the present time, “susceptible only” interpretive criteria were proposed for cefotaxime, ceftriaxone, meropenem, azithromycin, and minocycline. The numbers of minor interpretive errors with penicillin and ampicillin disk tests were unacceptably high and precluded recommended testing of those agents by the disk method. However, amdinocillin, an agent that preferentially binds to the altered penicillin binding protein resistant for diminished penicillin susceptibility, has potential utility as a surrogate screening reagent for ampicillin resistance. A disk diffusion breakpoint was derived for nalidixic acid to serve as a surrogate marker for gyrA mutations associated with diminished fluoroquinolone susceptibility. Disk diffusion testing with meningococci can be performed in a reproducible manner with several antimicrobial agents and represents a practical and cost-effective option for testing sporadic clinical isolates or for surveillance purposes by resource-limited laboratories.

The Clinical and Laboratory Standards Institute (CLSI) published MIC interpretive criteria for 13 antimicrobial agents for Neisseria meningitidis for the first time in 2005 (11). The criteria apply to both the broth microdilution method using lysed horse blood-supplemented Mueller-Hinton broth and the agar dilution method using Mueller-Hinton agar supplemented with 5% sheep blood. Both broth and agar MIC tests must include incubation in 5% CO₂ to achieve consistent and accurate results (18). The 13 antimicrobial agents for which interpretive criteria are available include those used for both therapy and prophylaxis of invasive meningococcal infections. The goal for developing MIC breakpoints was to facilitate surveillance of emerging resistance in N. meningitidis strains and to assist in managing treatment of invasive disease and prophylaxis of case contacts. The interpretive criteria were developed based upon MIC distributions of wild-type strains, MICs of strains with genetically-characterized resistance mechanisms, limited published clinical data, and the use of pharmacokinetic and pharmacodynamic simulations, as outlined in CLSI publication M23-A2 (8). Prior to the development of these standard CLSI MIC methods and breakpoints, studies using different susceptibility testing approaches showed discordant estimates of the frequency of meningococcal strains with decreased susceptibility to penicillin in the United States. Diminished penicillin susceptibility has been shown previously to result from production of altered penicillin binding protein 2 (PBP2) (2, 24). Two studies conducted by the Centers for Disease Control and Prevention (CDC) using a reference broth microdilution method found only 3% of strains with elevated penicillin MICs of 0.12 μg/ml (17, 23). However, another U.S. surveillance study that employed a commercial MIC test method reported 30% of strains with elevated penicillin MICs of 0.09 to 0.25 μg/ml (22). Applying the newly approved CLSI breakpoints to 325 CDC Active Bacterial Core surveillance isolates recovered between 1997 and 2002 revealed that 13.2% of the isolates were not susceptible to penicillin (MIC ≥ 0.12 μg/ml) and 5.8% were not susceptible to ampicillin (MIC ≥ 0.25 μg/ml) and that 52.7% were resistant to sulfisoxazole (MIC ≥ 4 μg/ml), 55% to trimethoprim-sulfamethoxazole (MIC ≥ 0.25 μg/ml), and 1.5% to rifampin (MIC ≥ 1 μg/ml) (J. H. Jorgensen, S. A. Crawford, and N. E. Rosenstein, Abstr. 43rd Ann. Meet. Infect. Dis. Soc. Amer., abstr. 503, p. 130, 2005).

Clinical microbiology or public health laboratories may receive requests to perform susceptibility testing of isolates from an...
individual patient when the patient is not responding clinically, when a cluster of meningococcal cases occurs, or when an outbreak is recognized and appropriate prophylactic agents need to be identified to control the spread of infections. For example, the inability to recognize rifampin-resistant isolates early was responsible for the failure of prophylactic rifampin to prevent meningococcemia among close contacts of patients (16, 21). In such instances, laboratories have sometimes performed MIC determinations using a commercial gradient diffusion method (11, 20, 28). Previous studies of meningococcal susceptibility performed using the disk diffusion method have shown that standard content penicillin (10 U), ampicillin (10 μg), and oxacillin (1 μg) disks do not reliably discriminate between penicillin-susceptible and relatively penicillin-resistant strains (5, 6). However, there have been encouraging data regarding the use of low-content penicillin (2 U) and ampicillin (2 μg) disks to identify meningococci with decreased susceptibility to beta-lactam drugs (5, 6). Evaluation of the results of these previous studies, however, is complicated by the numerous differences in the testing methods, e.g., the different media used for MIC and disk diffusion tests, different inoculum densities and disk contents, and use of either ambient air or CO₂ atmosphere for incubation (1, 3, 5, 6, 19). This has resulted in some sharp differences of opinion regarding the utility of disk diffusion testing for N. meningitidis (3; J. Campos, Letter, J. Clin. Microbiol. 37:879–880, 1999).

Initial studies to develop a disk diffusion method and interpretable criteria for meningococci were conducted using a diverse collection of meningococcal strains in a single laboratory, the University of Texas Health Science Center, San Antonio (UTHSCSA). The preliminary studies suggested that reproducible disk diffusion results could be obtained for a variety of antimicrobial agents by use of Mueller-Hinton sheep blood agar with incubation for 20 to 24 h at 35°C in a 5% CO₂ atmosphere (J. H. Jorgensen, S. A. Crawford, and L. C. Fulcher, Abstr. 105th Gen. Meet. Amer. Soc. Microbiol., abstr. C-352, 2005). Those initial studies suggested the need to perform a multilaboratory study to assess the interlaboratory reproducibility of disk testing with meningococci and to derive potential disk diffusion breakpoints that might be accepted by the CLSI.

MATERIALS AND METHODS

Test isolates. A collection of 50 meningococcal strains was assembled using both CDC Active Bacterial Core surveillance isolates from the United States and selected antimicrobial resistant isolates from outside the United States. Specifically there were 38 isolates from at least 10 U.S. states, 6 from Australia, 3 from Spain, and 1 each from Bangladesh, Canada, and Saudi Arabia. These included 3 isolates of serogroup A, 14 isolates of serogroup B, 20 isolates of serogroup C, 2 isolates of serogroup W135, 1 isolate of serogroup X, 9 isolates of serogroup Y, and 1 group Z strain. There were 21 strains with documented resistance to penicillin and 2 chloramphenicol-resistant, 2 quinolone-resistant, 7 rifampin-resistant, 24 sulfonamide-resistant, and 2 tetracycline-resistant strains, as described earlier (13, 15, 16). This collection was tested independently and in a blinded fashion by each of the four participating laboratories. In addition, each laboratory tested 25 unique isolates of meningococci from its own culture collection.

Biosafety precautions. Since this study involved testing a large number of meningococcal isolates, and because laboratory-acquired infections have re-
TABLE 1. Interpretive category errors associated with application of the proposed breakpoints to the strains tested in this study

<table>
<thead>
<tr>
<th>Antimicrobial agent (disk content in μg unless otherwise noted)</th>
<th>% Error for 50 strains tested in four laboratories</th>
<th>% Error for 100 unique strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (10 U)</td>
<td>26  0.5  0  19  0  0</td>
<td></td>
</tr>
<tr>
<td>Ampicillin (10)</td>
<td>13.5  0  0  11  0  0</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime (30)</td>
<td>0  0.5  0  0  0  0</td>
<td></td>
</tr>
<tr>
<td>Meropenem (10 μg)</td>
<td>0  0  0  0  0  0</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol (30 μg)</td>
<td>1  0  0  2  0  0</td>
<td></td>
</tr>
<tr>
<td>Rifampin (5 μg)</td>
<td>0  0  0  0  0  0</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole (1.25-23.75 μg)</td>
<td>0  0  2  0  0  0</td>
<td></td>
</tr>
<tr>
<td>Minocycline (30 μg)</td>
<td>0  0  0  0  0  0</td>
<td></td>
</tr>
<tr>
<td>Azithromycin (15 μg)</td>
<td>0  0  0  0  0  1</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin (5 μg)</td>
<td>1  0  0  0  0  0</td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid (30 μg)</td>
<td>0  0  0  0  0  0</td>
<td></td>
</tr>
</tbody>
</table>

a m, minor interpretive error (i.e., intermediate category versus susceptible or resistant categories); M, major interpretive error, susceptible by the reference MIC method, resistant by the disk test; VM, very major interpretive error, resistant by the reference MIC method, susceptible by the disk test.

FIG. 2. (A) Combined penicillin MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Penicillin MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.
RESULTS

Testing of the 50 common isolates in the four laboratories resulted in very similar MIC and zone diameter determinations by each laboratory. There were no significant differences in zone diameters based upon the brand of Mueller-Hinton sheep blood agar or the brand of antimicrobial agent disks (data not depicted). However, zone diameters differed markedly when disk tests were incubated in candle extinction jars instead of a standard CO₂ incubator. Zones were much larger and growth was somewhat poorer in the candle jars (data not depicted).

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FIG. 3. (A) Combined ampicillin MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Ampicillin MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.

FIG. 4. (A) Penicillin MICs and amdinocillin disk zone diameters recorded with 102 selected strains tested in one laboratory (UTHSCSA). (B) Ampicillin MICs and amdinocillin disk zone diameters recorded with 102 selected strains tested in one laboratory (UTHSCSA). The vertical line represents the single proposed zone diameter breakpoint for screening purposes. Strains with known altered PBP2 are indicated in boxes. Strains that were not examined for PBP2 alterations are indicated by asterisks.
further). Figure 1 illustrates the similarity in MIC and zone diameter distributions with penicillin tested in the four laboratories. Similar results were obtained with the 50-strain collection with the remaining drugs in this study (data not depicted). Figure 2A depicts the same data included in Fig. 1, although all data points generated by the four laboratories are depicted on a single scattergram \((n = 200)\). Figure 2B illustrates the MICs and zone diameters that resulted from testing penicillin with the unique 25 isolates tested separately by the four laboratories; i.e., all 100 data points are presented on a

FIG. 5. (A) Combined cefotaxime MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Cefotaxime MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.

FIG. 6. (A) Combined ceftriaxone MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Ceftriaxone MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.
single scattergram. Best-fit interpretive criteria derived using the modified error rate-bounded method for penicillin are indicated as vertical lines in Fig. 2. Unfortunately, the proposed breakpoints resulted in a large number of minor errors (26%) with the zone diameter breakpoints selected (Table 1). Figure 3A depicts the combined test data from the four laboratories from tests of the challenge strains with ampicillin, while Fig. 3B indicates the pooled results of testing the unique isolates with ampicillin. While somewhat lower interpretive error rates were observed with ampicillin (as opposed to penicillin), error rates, especially minor errors, were still substantial (i.e., 11 to 13.5% and 19 to 26%; Table 1). Since the proposed interpretive criteria for both the penicillin and ampicillin disk tests showed unacceptably high minor error rates, a selected group of 102 strains of meningococci was tested using aminocillin disks in one laboratory (UTHSCSA) as a potential surrogate marker for decreased susceptibility to beta-lactam agents due to production of a modified PBP2. Figure 4 illustrates the potential utility of aminocillin as a disk screening test. The aminocillin disk results appear to differentiate effectively between ampicillin-susceptible isolates and those isolates with decreased susceptibility to ampicillin due to PBP2 alterations. (Fig. 4B). However, aminocillin zones correlated less well with penicillin MICs (Fig. 4A). There was only one

FIG. 7. (A) Combined meropenem MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Meropenem MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.

FIG. 8. (A) Combined chloramphenicol MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Chloramphenicol MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.
major error (0.9%) with the proposed amdinocillin breakpoint compared with ampicillin MIC results, although there were 13.7% minor errors compared with penicillin MICs.

Cefotaxime, ceftriaxone, and meropenem were highly active against all meningococci examined in this study. Figures 5A and B, 6A and B, and 7A and B, respectively, depict the MIC zone size correlations with these three drugs observed when testing the two organism collections in this study. For all three of these agents, a single “susceptible only” zone diameter breakpoint is indicated on each scattergram. Figures 8, 9, and 10 represent MIC zone diameter comparisons for chloramphenicol, rifampin, and trimethoprim-sulfamethoxazole, respectively. The availability of strains with resistance mechanisms affecting the activities of those agents allowed proposed interpretive criteria for susceptible, intermediate, and resistant categories with few interpretive errors (Table 1).

The lack of strains with acquired resistance mechanisms that affected minocycline and azithromycin precludes defining breakpoints other than the susceptibility breakpoint. Figures 11A and B, 12A and B indicate the single disk diffusion breakpoints proposed for those two agents. Last, only two strains were available for inclusion in this study that demonstrated reduced fluoroquinolone susceptibility. The high potency of ciprofloxacin against meningococci results in only modest elevations of MICs and concomitant reductions in zone diameters in strains that contain gyrA mutations, i.e., the principle target of ciprofloxacin in meningococci. However, the combined data set allows interpretive criteria to be established, as shown in Fig. 13. The nonfluorinated quinolone nalidixic acid appears to be a useful indicator of gyrA mutations in meningococcal testing. Figure 14 demonstrates that strains with reduced quinolone susceptibility can be readily separated from normal wild-type meningococci by use of a single-zone-diameter breakpoint.

Both the approved MIC interpretive criteria and the zone diameter interpretive criteria derived in this study are summarized in Table 2. The proposed breakpoints are associated with generally low numbers of category-interpretive errors, with the notable exceptions of those of penicillin and ampicillin (Table 1).

**DISCUSSION**

The primary goal of this study was to determine whether disk diffusion was a reliable method for assessing the susceptibility of *N. meningitidis* to a variety of therapeutic and prophylactic agents and, if so, to develop interpretive criteria for those antimicrobial agents. A previous study (Jorgensen et al., Abstr. 105th Gen. Meet. Amer. Soc. Microbiol.) demonstrated that disk diffusion zones can be

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**FIG. 9.** (A) Combined rifampin MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Rifampin MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.
measured reproducibly using the test conditions described in this study. In that single laboratory study, 94.9% zones measured by four separate observers agreed within 3 mm for the 14 antimicrobial agents studied. In the present study, all four laboratories achieved similar disk diffusion results when they tested a collection of 50 meningococcal strains that included a series of contemporary wild-type strains and strains exhibiting a variety of resistance mechanisms. There was very good agreement among both the MIC results and the zone diameters reported by the participating laboratories for the 12 antimicrobial agents included in this study. The favorable agreement among the laboratories and the availability of strains with resistance or decreased susceptibility to several of the antimicrobial agents has led to the proposed interpretive criteria that define standard susceptible, intermediate, or resistant categories or categories for

FIG. 10. (A) Combined trimethoprim-sulfamethoxazole MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. Trimethoprim-sulfamethoxazole disks are abbreviated as SXT on these graphs. (B) Trimethoprim-sulfamethoxazole MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.

FIG. 11. (A) Combined minocycline MICs and disk zone diameters recorded with the 50-strain collection by the four laboratories. (B) Minocycline MICs and disk zone diameters recorded with 100 unique meningococcal isolates contributed individually by the four laboratories. The vertical lines represent the proposed zone diameter breakpoints.
antimicrobial agents for which there currently is no recognized resistance (the “susceptible only” category).

For most of the antimicrobial agents included in this investigation, the interpretive error rates (very major, major, and minor) are low and are similar to those observed for other fastidious and nonfastidious bacterial isolates. This is encouraging given the large zone diameters produced by many of the drugs when meningococci are tested. It is disappointing, however, that there were an excessive number of minor interpretive errors when the standard content penicillin (10 U) and ampicillin (10 μg) disks were used in this study. An earlier study (Jorgensen et al., Abstr. 105th Gen. Meet. Amer. Soc. Microbiol.) was not able to achieve lower error rates by using lower-content penicillin (1 U) or ampicillin (2 μg) disks. Thus,
we explored a variety of other beta-lactam agents as an alternate means of identifying isolates with reduced susceptibility to penicillin and ampicillin. Other disks examined in one laboratory (UTHSCSA) included amoxicillin-clavulanic acid, cefoxitin, ticarcillin, and amdinocillin. Of those, only amdinocillin showed promise as a screening test for isolates with decreased penicillin and ampicillin susceptibility. The fact that amdinocillin specifically binds to PBP2 (numbered according to the E. coli numbering system) likely explains why meningococci with mosaic PBP2 (26) can be detected effectively with an amdinocillin disk test. The results of the amdinocillin disk test correlate better with elevated ampicillin MICs than with elevated penicillin MICs. An earlier study demonstrated a closer correlation between elevated ampicillin MICs and the presence of PBP2 alterations than the correlation of penicillin MICs with the altered drug target (18). Disk diffusion testing with amdinocillin may be a cost-effective approach to screening a large number of isolates that have reduced susceptibility to beta-lactams, particularly when the isolates are suspected to belong to a single or a few clonal groups (27).


<table>
<thead>
<tr>
<th>Antimicrobial agent (disk content in μg unless otherwise noted)</th>
<th>MIC (μg/ml)</th>
<th>Zone diam (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
<td>Intermediate</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Ampicillin (10)</td>
<td>≤0.12</td>
<td>0.25-1</td>
</tr>
<tr>
<td>Penicillin (10 U)</td>
<td>≤0.06</td>
<td>0.12-0.25</td>
</tr>
<tr>
<td>Amdinocillin* (10)</td>
<td>≤0.12</td>
<td>≥0.25</td>
</tr>
<tr>
<td>Cefotaxime (30)</td>
<td>≤0.12</td>
<td>≥0.25</td>
</tr>
<tr>
<td>Ceftiraxone (30)</td>
<td>≤0.12</td>
<td></td>
</tr>
<tr>
<td>Meropenem (10)</td>
<td>≤0.25</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol (30)</td>
<td>≤2</td>
<td>4</td>
</tr>
<tr>
<td>Rifampin (5)</td>
<td>≤0.5</td>
<td>1</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole (1.25-23.75)³</td>
<td>≤0.12-2.3</td>
<td>0.25-4.75</td>
</tr>
<tr>
<td>Minocycline (30)</td>
<td>≤2</td>
<td></td>
</tr>
<tr>
<td>Azithromycin (15)</td>
<td>≤2</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin (5)</td>
<td>≤0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Nalidixic acid (30)</td>
<td>≤4</td>
<td></td>
</tr>
</tbody>
</table>

* Amdinocillin disks can be used to screen for nonsusceptibility to ampicillin (MIC > 0.25 μg/ml).
³ Trimethoprim-sulfamethoxazole is the preferred disk for detection of sulfonamide resistance.
Chemother. 44:1116–2000) and have the potential for development of higher level fluoroquinolone resistance (25), screening with nalidixic acid by use of either MIC or disk could be a useful epidemiologic and diagnostic tool.

The disk diffusion method and interpretive criteria described herein provide a convenient method that can be used for epidemiologic surveys of emerging meningococcal resistance, or for clinical situations in which a physician needs confirmation that the drugs normally used for empirical therapy or prophylaxis of invasive meningococcal infections will likely be effective, or in resource-limited settings in which MIC determination methods are not readily available. It is important to follow the methodological details outlined above and in the CLSI document in order to obtain reproducible disk diffusion test results. Incubation of tests in candle extinction jars is not recommended as a means to achieve a suitable CO2 atmosphere. We do not recommend that disk testing be used to assess the activity of penicillin in cases of meningitis; in those situations, a MIC determination would be preferred.

The CLSI Antimicrobial Susceptibility Testing Subcommittee has reviewed the data presented in Table 2 and incorporated the disk diffusion breakpoints in CLSI publication M100-S16 (12), with the exception of the penicillin, ampicillin, and amoxicillin breakpoints. The CLSI concluded that the rates of minor errors with penicillin and ampicillin disk tests were too high for those tests to be recommended. The CLSI has not yet considered the possibility of using amoxicillin as a surrogate disk to screen for diminished penicillin and ampicillin susceptibility. The latest CLSI publication (12) now includes interpretive criteria for both MIC and disk diffusion testing of the drugs most often used for therapy and prophylaxis of meningococcal disease.

ACKNOWLEDGMENTS

This study was supported in part by grant RS1/CCR622402 from the Centers for Disease Control and Prevention. Most of the U.S. isolates in this study were collected as part of the Active Bacterial Core surveillance (ABCs) effort of the Emerging Infections Program of the CDC. The Meningitis and Special Pathogens Laboratory of the CDC facilitated access to those strains. Resistant non-U.S. isolates were generously provided by John Turnidge from Adelaide, Australia, and two chloramphenicol-resistant isolates and a strain with diminished quinolone susceptibility were kindly provided by Professor John Tap- sall from Randwick, NSW, Australia. Julio Vazquez (Spain) kindly provided a strain with diminished fluoroquinolone susceptibility. Robert Rennie (Canada) provided eight isolates with elevated penicillin MICs.

REFERENCES