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Testing of *Streptococcus pneumoniae* for Resistance to Penicillin

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The increasing prevalence of penicillin-resistant *Streptococcus pneumoniae* requires antibiotic susceptibility tests that can be done with greater ease and reliability. We measured the MIC of penicillin for pneumococci by the tube macrodilution method with Mueller-Hinton broth (MHB), *Haemophilus* Test Medium (HTM), Todd-Hewitt broth with 0.5% yeast extract (THY), and MHB with 3% lysed horse blood (LHB). Eight (19%) and 6 (14%) of 42 pneumococcal isolates failed to generate turbid growth in MHB and HTM, respectively, whereas all pneumococcal isolates did so in THY and LHB. For those strains that replicated to turbidity, the mean MICs of penicillin were lower in MHB and HTM than in THY and LHB, with differences being significant ($P < 0.05$) for comparisons with LHB. Four isolates appeared to be penicillin susceptible in HTM but were actually moderately resistant in THY and LHB, and two isolates appeared to be moderately resistant but were resistant. A similar failure to detect resistance was seen with MHB. *S. pneumoniae* ATCC 49619, a moderately penicillin-resistant strain that has been proposed for quality control testing, gave variable results in MHB or THM and appeared to be susceptible to penicillin in some assays, whereas the MICs for *S. pneumoniae* ATCC 49619 in THY or LHB fell within a twofold dilution range, with geometric means of 0.16 and 0.18 μg/ml, respectively. Pneumococcal isolates thus may appear falsely susceptible to penicillin when tested in MHB or HTM. LHB remains the standard medium; however, because THY is an easily prepared clear medium that can be used in automated systems and appears to yield results similar to those obtained with LHB, THY deserves consideration for routine use.

Streptococcus pneumoniae, a leading cause of otitis media, pneumonia, and meningitis, remains a prominent cause of morbidity and mortality in people of all ages (3, 10). Since 1967 (4), penicillin-resistant pneumococci have been isolated worldwide, with especially high prevalences in Spain, Hungary, and South Africa (1). In the United States, the increasing incidence of disease caused by these organisms (5, 13) has made it increasingly important to be certain of the reliabilities of the techniques that measure the MIC of penicillin for *S. pneumoniae*.

The National Committee for Clinical Laboratory Standards recommends that 2 to 5% lysed horse blood (LHB) be added to Mueller-Hinton broth (MHB) for routine antibiotic susceptibility testing of pneumococci (11). Few laboratories, however, use MHB plus LHB because of the many extra steps required to obtain the reagents and prepare the medium. Manufacturers of automated systems recommend the use of Todd-Hewitt broth for assaying the MIC for *S. pneumoniae* (2). Unsupplemented, this medium fails to support the growth of about one-quarter of clinical isolates (9a). We have found, however, that when 0.5% yeast extract is added, the resulting medium, Todd-Hewitt broth plus yeast extract (THY), supports the growth of nearly all pneumococcal isolates, although CO_2 enrichment is occasionally required. In recognition of these problems, some investigators (6) have recently proposed the routine use of *Haemophilus* Test Medium (HTM), claiming that MIC results are comparable to those obtained in LHB. The present study was designed to identify an appropriate medium, standardize the inoculum (especially for fastidious strains), and identify conditions that enable reliable and reproducible MIC testing of *S. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains. Forty-two epidemiologically distinct isolates of *S. pneumoniae* were studied, including 29 isolates from the Veterans Affairs Medical Center, Houston, 10 from Texas Children's Hospital, and 3 from the American Type Culture Collection (ATCC 6308, ATCC 6304, and ATCC 49619). The clinical isolates were obtained between 1 November 1989 and 30 April 1992. Whereas isolates from the Veterans Affairs Medical Center, Houston, were selected entirely at random from a collection of stored isolates, those from Texas Children's Hospital were selected from strains that were previously shown (8) to be moderately resistant or resistant to penicillin. Reference strain ATCC 49619, a moderately resistant strain which has been proposed as a quality control test organism (6), was included in every study. The identification of each isolate as *S. pneumoniae* was verified after recovery from frozen aliquots by susceptibility to optochin and solubility in bile salts. The strains were serotyped in a standard agglutination assay by using appropriate antisera (Statens Seruminstitut, Copenhagen, Denmark). The serotypes of the *S. pneumoniae* strains were found to be 6 ($n = 7$), 19F ($n = 7$), 3 ($n = 6$), 23F ($n = 5$), 4 ($n = 2$), 8 ($n = 2$), and group 11 ($n = 2$). In addition, one strain each was found to have one of the following serotypes: 2, 7F, 11F, 12F, 14, 15, 35F, group 7, group 19, pool C, and nontypeable.

Growth media. MHB (Difco, Detroit, Mich.) was prepared

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as recommended by the manufacturer. THY was prepared by adding 5 g of yeast extract (Difco) per liter of Todd-Hewitt broth (Difco). LHB broth was prepared by adding 3% lysed horse blood to MHB as described in the guidelines of the National Committee for Clinical Laboratory Standards (11). HTM was made as described by Jorgensen et al. (7).

Oxacillin disk susceptibility screening. Five to 10 colonies from an overnight culture on Mueller-Hinton agar (MHA) containing 5% sheep blood (SB) (MHA-SB) were inoculated into 2.5 ml of THY. The broths were incubated to a visible turbidity (approximately 6 h) and then diluted to match a 0.5 barium sulfate turbidity standard before streaking onto MHA-SB. Oxacillin disks (1 μg; Becton Dickinson, Cockeysville, Md.) were then applied and the plates were incubated at 35°C under atmospheric conditions. For those strains that did not grow in room air, increased CO_2 was provided by incubation in a candle jar. Isolates that had clear zones of inhibition of ≥20 mm around the oxacillin disk were considered penicillin susceptible, whereas those with zones of <20 mm were considered penicillin resistant (12).

Standardization of bacterial inoculum. In order to determine the conditions that would reliably yield a predictable bacterial inoculum, growth in four liquid media under a variety of conditions was studied. In accord with the recommendations of the National Committee for Clinical Laboratory Standards, 5 to 10 colonies were selected from the surface of a Mueller-Hinton (MH)–SB plate and added to 2.5 ml of MHB, HTM, THY, or LHB. These suspensions were incubated under atmospheric conditions at 35°C. After 6- and 20-h incubations, aliquots were removed and serial 10-fold dilutions were made; 10 μl was streaked onto MH-SB for quantitation. If good growth was not obtained at 20 h, the experiment was repeated with incubation in a candle jar.

Antibiotic preparation. Penicillin G (Pfizer, New York, N.Y.) was dissolved in phosphate-buffered saline to 2,560 μg/ml and was stored in 1-ml aliquots at −20°C for ≤3 months.

MIC testing. The MICs of penicillin were determined in MHB, HTM, THY, or LHB by the broth tube macrodilution method and the broth tube microdilution method (11). A single lot of medium and antibiotic powder was used throughout the study. The bacterial inoculum was standardized to yield a final concentration of about 5×10^5 CFU/ml, as determined in preliminary studies with quantitative cultures (see Results). Twofold dilutions of penicillin G were tested at a range of decreasing concentrations from 8 to 0.004 μg/ml. All tubes were incubated at 35°C under atmospheric conditions for 20 h. MICs were read as the lowest concentration of antibiotic at which there was no visible growth. Isolates were designated as susceptible to penicillin if the penicillin MIC was <0.1 μg/ml, moderately resistant to penicillin if the MIC was ≥0.1 μg/ml but <2 μg/ml, and highly resistant to penicillin if the MIC was ≥2 μg/ml. Each isolate was studied by tube macrodilution on three separate occasions and by tube microdilution on two separate occasions.

Statistical methods. Student's *t* test was used to test significance in comparisons of the geometric mean MICs in the four media.

RESULTS

Susceptibility by oxacillin disk testing. A turbid broth culture is required to inoculate MH-SB agar plates for oxacillin disk susceptibility testing. Initial studies showed that about one-quarter of the strains of *S. pneumoniae* failed to grow reliably to turbidity in MHB or HTM during incubation for 6 h at 37°C under atmospheric conditions. Even at 20 h, turbid growth was not seen in MHB or HTM in 19 and 14% of the strains, respectively. In contrast, all isolates yielded rich growth after 6 h in THY or LHB. Accordingly, 42 isolates were inoculated into THY, incubated at 37°C under atmospheric conditions for 6 h, diluted to a 0.5 McFarland turbidity standard in THY, and spread onto MH-SB plates. A 1-μg oxacillin disk was placed onto the MH-SB plates, and the plates were then incubated at 37°C under atmospheric conditions. Six isolates (14%) failed to grow sufficiently under these conditions to allow detection of a zone of inhibition but did grow to confluence during incubation in a candle jar; these isolates are termed fastidious. Fifteen of 42 isolates (37%) had no inhibition zone, 3 (7.1%) had zones of 11 to 15 mm in diameter, 3 (5%) had zones of 16 to 19 mm in diameter, and 21 (51%) had clear zones of ≥20 mm in diameter around the 1-μg oxacillin disk.

TABLE 1. Growth of 22 nonfastidious strains of *S. pneumoniae* in four liquid media[a]

Medium and incubation time	Log_{10} CFU/ml		
	Geometric mean	SEM	Range
MHB			
6 h	7.12	1.68	<2.0–8.8
20 h	5.04	1.76	<2.0–9.6
HTM			
6 h	7.48	1.05	5.5–8.7
20 h	6.08	1.35	<2.0–7.6
THY			
6 h	8.00	0.48	7.5–8.7
20 h	3.32	2.31	<2.0–7.0
LHB			
6 h	8.32	0.45	7.0–9.0
20 h	7.63	0.33	7.1–8.3

[a] Growth was at 37°C under atmospheric conditions for the indicated times.

Further study of growth conditions. In order to study growth conditions that reliably yield a desired bacterial inoculum for MIC testing, 21 nonfastidious isolates were selected randomly; ATCC 46919 was studied each time that an assay was performed. Five colonies were inoculated to 2.5 ml of MHB, HTM, THY, and LHB and were incubated for 6 or 20 h at 37°C under atmospheric conditions. As shown in Table 1, for these 22 isolates, turbid growth with minimal variation in CFU was achieved with culture in THY or LHB after 6 h (mean log_{10} CFU/ml, 8.00 ± 0.48 and 8.32 ± 0.45, respectively) and in LHB after 20 h (mean CFU/ml, 7.63 ± 0.33); the CFU in THY had declined greatly by 20 h. Variation was substantially greater in HTM and MHB, indicating that a reliable inoculum would not be readily obtainable.

With five of the six fastidious isolates, it was possible to obtain rich cultures at 6 h in THY or LHB by using a sufficient bacterial inoculum to give an initially turbid suspension and by culturing under atmospheric conditions at 35°C (Table 2). The remaining isolate yielded turbid growth only after incubation in 5% CO_2. Good growth of these fastidious isolates was also achieved in MHB and HTM by use of a heavy inoculum and incubation in 5% CO_2. The CFU reached by the six fastidious isolates in LHB or THY

TABLE 2. Growth of six fastidious strains of *S. pneumoniae* in four liquid media[a]

Medium	Incubation:		Log_{10} CFU/ml		
	Atmosphere	Time (h)	Range	Mean	SEM
MHB	RA	6	6.2–7.6	6.85	0.44
	CO_2	6	6.8–7.7	7.56	0.61
	RA	20	<2.0–<2.0		
	CO_2	20	<2.0–6.1	3.0	2.29
HTM	RA	6	5.0–7.3	6.58	0.92
	CO_2	6	5.1–7.8	6.89	0.93
	RA	20	<2.0–7.3	5.43	2.43
	CO_2	20	6.1–7.6	6.46	0.73
THY	RA	6	6.9–8.2	7.66	0.59
	CO_2	6	6.4–8.3	7.61	0.85
	RA	20	<2.0–<2.0		
	CO_2	20	<2.0–5.2	1.69	1.71
LHB	RA	6	7.1–8.6	7.91	0.49
	CO_2	6	7.8–8.6	8.30	0.32
	RA	20	<2.0–6.1	3.30	2.57
	CO_2	20	<2.0–7.8	4.57	2.98

[a] Results are reported as log_{10} CFU per milliliter during growth in room air (RA) or under 10% CO_2. Means are for six isolates under CO_2 but only five isolates under room air, since one isolate failed to replicate.

after a 6-h incubation was slightly less than that for nonfastidious isolates, although these differences were not significant. By 20 h, the CFU had declined substantially in all four media, although the decline was the least in HTM. These results suggest that 20-h cultures do not yield a predictable inoculum for fastidious isolates in any of the media studied.

MIC results. The susceptibilities of all 42 isolates to penicillin were studied by the tube macrodilution method. We considered standardization of the bacterial inoculum to be essential if reliable results were to be obtained. On the basis of the results of the studies summarized in Table 1 and in order to have turbid cultures available at the start of each workday, we used a 20-h culture in LHB. If it had been desirable to test isolates at the end of the day, then 6-h cultures in THY or LHB would have been equally reliable.

S. pneumoniae ATCC 49619 was tested on eight occasions. Results were reproducible in LHB and THY, with no more than a twofold difference throughout; the mean MICs were nearly identical in these two media (Table 3) and were consistent with the earlier characterization of this organism as moderately resistant to penicillin. In contrast, results in MHB and HTM were unreliable. In MHB, this organism failed to grow in two assays, and in six other assays it grew, yielding an eightfold spread in the measured MIC. Most importantly, in MHB this moderately resistant strain gave the appearance of being susceptible (mean MIC, 0.02 ± 0.29 μg/ml [Table 3]). In HTM, the results were more consistent (mean MIC, 0.11 ± 0.16 μg/ml), but this strain still gave the appearance of being susceptible in two of eight assays.

TABLE 3. Reproducibility of repeat MIC testing of *S. pneumoniae* ATCC 49619[a]

Medium	Penicillin concn (μg/ml)		
	Geometric mean	SEM	Range
MHB	0.02	0.29	0.0075–0.03
HTM	0.11	0.16	0.06–0.25
THY	0.16	0.13	0.125–0.25
LHB	0.18	0.13	0.125–0.25

[a] Data are average results of eight separate studies with each medium.

TABLE 4. MICs for 42 *S. pneumoniae* isolates determined by macrodilution in four liquid media[a]

Medium	Penicillin concn (μg/ml)			
	Penicillin susceptible		Penicillin resistant	
	Geometric mean	SEM	Geometric mean	SEM
MHB	0.014[b]	0.23[b]	0.37	0.40
HTM	0.016[c]	0.20[c]	0.32	0.38
THY	0.021[c]	0.14[c]	0.71	0.29
LHB	0.033[b]	0.15[b]	0.89	0.31

[a] Isolates were stratified into the penicillin-susceptible or penicillin-resistant category by oxacillin disk testing in order to calculate the geometric mean.
[b] $P < 0.01$.
[c] $P < 0.05$.

Growth of the other isolates was also unreliable in MHB and HTM. As a result, 8 of 42 (19%) isolates failed to grow in MHB and 6 of 42 (14%) isolates failed to grow in HTM, rendering the MICs indeterminate. In contrast, all 42 isolates yielded readable MICs in THY or LHB, although one isolate failed to grow under atmospheric conditions and required 10% CO_2. In order to report the geometric means of the MICs, results were stratified for penicillin-susceptible and penicillin-resistant isolates, as determined by oxacillin disk screening, because, without such stratification, the data were not normally distributed (Table 4). In every instance, strains appeared to be more resistant (higher MIC) in LHB and THY, the two media that best supported the growth of *S. pneumoniae*, and appeared to be more susceptible (lower MIC) in MHB and HTM, the two media that were not as supportive of growth of *S. pneumoniae*. Statistically significant differences in MICs were observed by comparing the results in LHB with those in MHB ($P < 0.01$) and HTM ($P < 0.05$). Table 5 displays the substantial variation in MICs comparing the results obtained with MHB and HTM with those obtained with LHB. In contrast, the MICs obtained with THY varied by only one dilution for 41 isolates and by two dilutions for the remaining isolate (0.06 versus 0.015 μg/ml).

Category changes. Two isolates that appeared to be susceptible when tested in MHB were found to be moderately resistant on the basis of MIC results in LHB, and one strain that appeared to be moderately resistant when tested in MHB was resistant in LHB. In HTM, four isolates that appeared to be susceptible were actually found to be moderately resistant in LHB, and two isolates that appeared to be moderately resistant were actually resistant.

Tube macro- versus microdilution MICs. Results of MIC testing in LHB by the macrodilution and microdilution methods were compared for the 41 strains that replicated in this medium without CO_2 supplementation. The results for

TABLE 5. Comparison of MHB, HTM, THY, and LHB and comparison of tube microdilution and tube macrodilution methods

Medium or method	No. of strains within the indicated twofold difference versus results in LHB[a]									
	−6	−5	−4	−3	−2	−1	0	+1	+2	+3
MHB[b]	1	0	1	1	8	15	6	1	0	0
HTM[c]	0	1	0	3	5	19	7	0	0	0
THY[d]	0	0	0	0	1	14	24	2	0	0
Tube microdilution[e]	0	0	0	0	6	8	21	4	1	1

[a] The numbers −6 to +3 indicate more susceptible to more resistant by twofold dilutions.
[b] MICs in MHB versus those in LHB; eight strains did not grow in MHB.
[c] MICs in HTM versus those in LHB; six strains did not grow in HTM.
[d] MICs in THY versus those in LHB; all strains grew in both media.
[e] MICs in LHB by the tube microdilution method versus those in LHB by the tube macrodilution method.

eight strains differed by two or more dilutions (Table 5). Five MICs were categorized as susceptible by microdilution and intermediately resistant by macrodilution, and one strain, which differed by only one dilution, was determined to be highly resistant by microdilution but only intermediately resistant by macrodilution.

Oxacillin disk testing versus MIC testing. Results of penicillin susceptibility as determined by oxacillin disk testing were compared with those obtained by tube macrodilution testing in each of the four test media. For all strains (100%) with inhibition zone diameters of ≥20 mm around the oxacillin disk, MICs were <0.1 μg/ml in LHB or THY, and 90% of the strains with inhibition zone diameters of <20 mm were found to be intermediately resistant (MICs, ≥0.1 to <2.0 μg/ml) or resistant (MICs, ≥2.0 μg/ml) in these media. For all strains (100%) with inhibition zone diameters of ≥20 mm, MICs were <0.1 μg/ml in MHB and HTM, but 22 and 32% of isolates that were resistant both by oxacillin testing and by MIC testing in LHB and THY, respectively, would have been regarded as susceptible by MIC testing in MH and HTM.

DISCUSSION

Because pneumococcal strains resistant to penicillin have become more prevalent, accurate assessment of *S. pneumoniae* susceptibility has become a major concern of the microbiology laboratory. Fastidious growth and lack of specific recommendations for susceptibility testing, coupled with an historic lack of experience in testing the susceptibilities of pneumococci, present additional confusion in working with this species. To address these problems, we examined the effects of different media on growth of the bacterial inoculum as well as in the MIC testing of *S. pneumoniae*. Our results show that unsupplemented MHB and Todd-Hewitt broth (Todd-Hewitt broth is recommended for use in automated systems [2]) do not reliably support the growth of pneumococci, with nearly one-quarter of unselected strains failing to replicate. Although LHB has been recommended as the appropriate liquid medium for cultivation and testing of *S. pneumoniae*, this medium is not commercially available and it is extremely tedious to prepare LHB in the laboratory. Accordingly, HTM has been proposed as an alternative (6, 7). Even with *Haemophilus influenzae*, however, some degree of strain variation has been shown in HTM (9), and we found that 6 of 42 (14%) unselected pneumococcal strains also failed to grow. More disturbing was our observation that pneumococci appeared to be more susceptible in MHB and HTM than in THY or LHB; the differences translated into a category change for 17% of isolates that did grow in HTM. These results suggest that the antibacterial effect may appear to be artifically high in less supportive media. This effect could be compounded if the bacterial inoculum were inadequate, a potential source of variability that we eliminated by standardizing the inoculum in THY and LHB. The magnitude of this problem could probably be reduced by incubation in 5% CO_2, but many laboratories still use candle jars, not CO_2 incubators, and neither tubes nor microtiter plates are easily adaptable to incubation in candle jars. In contrast, the results of susceptibility testing in LHB and THY in room air were highly reliable and reproducible; the results were nearly identical, and no isolate was categorized differently as to susceptibility or resistance in these two media. When *S. pneumoniae* ATCC 49619, a strain that has been proposed for quality control testing, was studied in eight separate assays, the results obtained with THY or LHB were nearly identical, whereas substantial unreliability was observed with MHB and HTM. These results suggest that neither MHB nor HTM should be used to test the susceptibility of *S. pneumoniae* to penicillin by standard MIC testing methodologies.

In comparing micro- and macrodilution techniques, we found that the MICs appeared to be lower with microdilution, as was previously shown by Jorgensen et al. (6). This difference was consistently demonstrated on repeat testing and led to recategorization of penicillin susceptibility in several instances; no explanation is readily apparent.

Although at the time of this writing THY is also not commercially available, its preparation requires only the addition of yeast extract to a standard microbiological medium; thus, it would appear to be an attractive alternative to the labor-intensive LHB. A further advantage is that, because THY is a clear medium, it can be used in automated microtiter systems. Widespread adoption of this medium, however, will require confirmation of the results of the present study. Further study of the relevance of in vitro susceptibility testing in predicting the response to therapy is also indicated. In the meantime, demonstration of susceptibility by oxacillin disk testing appears to correlate well with MICs of <0.1 μg/ml, although some strains that appear to be resistant by oxacillin disk testing may not be.

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