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Increased Rate of Isolation of Penicillin-Resistant *Streptococcus pneumoniae* in a Children's Hospital and In Vitro Susceptibilities to Antibiotics of Potential Therapeutic Use

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The isolation of *Streptococcus pneumoniae* with both high and intermediate resistance to penicillin has increased in our institution since 1989 to an average of 12.1% of all isolates. We determined the susceptibilities of 95 isolates (34 susceptible to penicillin, 42 intermediate in resistance to penicillin, and 19 resistant to penicillin) to 16 antimicrobial agents of potential use in the treatment of disease caused by *S. pneumoniae*. Susceptibility to penicillin was determined by broth macrodilution with Mueller-Hinton broth supplemented with 5% lysed horse blood. Isolates were classified as highly resistant when the MIC was ≥2.0 μg/ml, intermediate in resistance when the MIC was between 0.1 and 1.0 μg/ml, and susceptible when the MIC was <0.1 μg/ml. Fifteen of 19 isolates found to be highly resistant to penicillin were recovered from the middle ear of children. None of the isolates recovered from cerebrospinal fluid was highly resistant to penicillin. Fifteen of these isolates highly resistant to penicillin were found to be serogroup 6. Susceptibilities to other antibiotics were determined by the agar dilution method with Mueller-Hinton agar containing 5% lysed horse blood and an inoculum of 10^4 CFU per spot delivered by a replicator device. The MIC for 90% of isolates increased with increasing penicillin resistance for all antibiotics tested, except chloramphenicol, ciprofloxacin, rifampin, and vancomycin. Regardless of the classification of penicillin resistance, all isolates were classified as susceptible to cefotaxime, cefpirome, cefpodoxime, clarithromycin, imipenem, rifampin, and vancomycin on the basis of National Committee for Clinical Laboratory Standards interpretive guidelines. Interpretation of susceptibilities on the basis of currently available guidelines is difficult in that susceptibility guidelines applicable specifically to *S. pneumoniae* are not available.

Resistance to penicillin of *Streptococcus pneumoniae* has been reported sporadically since 1965 (1, 11). In 1978, investigators from South Africa reported high resistance (MIC, ≥2.0 μg/ml) to penicillin and to other antibiotics (9). The Centers for Disease Control recently reported that nationwide, the number of strains reported as resistant to penicillin rose to a peak of 8% in 1982, followed by an apparent decrease in the number of resistant strains isolated through 1987 (20). Of the 274 strains identified as having increased resistance to penicillin, 273 were intermediate in resistance and only 1 was highly resistant to penicillin (20). We reported the isolation of one highly resistant strain from the middle ear of a child with chronic otitis media at our institution in 1988 (3). Surveillance since 1988 has revealed an increased rate of isolation of *S. pneumoniae* with both intermediate and high resistance for the last 2 years. All but one strain with high resistance were recovered from middle ear cultures, and there was an association of prior treatment with amoxicillin-clavulanate in children from whom resistant strains were isolated (5). Because of our concern with the increase in the rate of isolation of *S. pneumoniae* resistant to penicillin and the need to identify therapeutic alternatives, we began a prospective study of the frequency of isolation of resistant *S. pneumoniae* and in vitro antibiotic susceptibility to a number of antibiotics. We also investigated whether the specificity of the oxacillin screen test for penicillin resistance could be improved by use of Mueller-Hinton (MH) agar supplemented with lysed horse blood (LHB), rather than with sheep blood, as is standard.

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MATERIALS AND METHODS

Bacterial strains. *S. pneumoniae* strains were isolated by the Clinical Microbiology Laboratory, Texas Children's Hospital, Houston, between January 1989 and December 1991. Additional strains received for quantitative susceptibility studies from other hospital laboratories also were included in the susceptibility survey. All strains were identified as *S. pneumoniae* by susceptibility to optochin and solubility in bile salts. Routine penicillin susceptibility screening with a 1-μg oxacillin disc on sheep blood-supplemented MH agar was performed, and a strain was suspected of being penicillin resistant when the zone surrounding the disc measured less than 20 mm following 24 h of incubation without CO_2 at 35°C (21).

Susceptibility studies. Penicillin susceptibility was confirmed with in-house-prepared MH broth supplemented with divalent cations and LHB to a final concentration of 3% (16). Doubling dilutions of standard penicillin G powder (Bristol-Myers Squibb Co., Evansville, Ind.) were used for the broth macrodilution method. The inoculum was a 1:1,000 dilution of overnight growth of the bacterium in MH broth with LHB, which consistently resulted in a final concentration of

1×10^5 to 3×10^5 CFU/ml, as confirmed by quantitative culturing. Isolates were designated susceptible when the MIC was <0.1 μg/ml, intermediate in resistance when the MIC was between 0.1 and 1 μg/ml, and highly resistant when the MIC was ≥2 μg/ml. Zone diameters surrounding 1-μg oxacillin discs were determined in two laboratories. On original isolation, the bacteria were tested by standard methods with 5% sheep blood agar plates, and at confirmation, they were tested with MH agar supplemented with 3% LHB under the conditions described above.

All culture media were subjected to quality control assessment as described by the National Committee for Clinical Laboratory Standards (NCCLS) (16). A strain of *S. pneumoniae* isolated previously (3) and confirmed to be highly resistant to penicillin by the Centers for Disease Control was maintained at −70°C and used as a control in each experiment.

The susceptibilities of the strains to amoxicillin-clavulanate (SmithKline Beecham, Inc., Philadelphia, Pa.), cefaclor, erythromycin, and vancomycin (Eli Lilly & Co., Indianapolis, Ind.), cefixime (Lederle Laboratories, Wayne, N.J.), cefotaxime and cefpirome (Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.), cefpodoxime and clindamycin (The Upjohn Co., Kalamazoo, Mich.), cefuroxime (Glaxo Pharmaceuticals, Research Triangle Park, N.C.), chloramphenicol (Parke-Davis, Morris Plains, N.J.), ciprofloxacin (Miles, Inc., West Haven, Conn.), clarithromycin (Abbott Laboratories, Abbott Park, Ill.), imipenem (Merck Sharp & Dohme, West Point, Pa.), rifampin (Marion Merrell Dow, Inc., Kansas City, Mo.), and sulfamethoxazole-trimethoprim (Roche Laboratories, Nutley, N.J.) were determined by the agar dilution method with MH agar supplemented with LHB (3% final concentration) (16). The inoculum was adjusted from the growth on an overnight blood agar plate so that the replicator prong delivered approximately 10^4 CFU per spot.

Serotyping. Isolates of *S. pneumoniae* were serotyped or serogrouped by the capsular swelling method with antisera from Statens Seruminstitut (Dako Inc., Carpinteria, Calif.) (14). Strains grown overnight on sheep blood agar were suspended in distilled water and reacted with omni and pooled antisera. Strains showing evidence of agglutination or capsular swelling on light microscopy were tested with the individual specific antisera contained in the reactive pool.

Statistics. The chi-square test was used to test the significance of proportions.

RESULTS

Prior to 1989, among suspected penicillin-resistant *S. pneumoniae* isolates received from all sources, quantitative tests confirmed 1 isolate intermediate in resistance to penicillin in 1985, 1986, and 1987. In 1988, one isolate intermediate in resistance and one isolate highly resistant to penicillin were documented. The numbers of confirmed penicillin-resistant isolates in both the intermediate- and the high-resistance categories increased to 22 of 172 (12.8%) pneumococcal isolates in 1989, 24 of 218 (11%) isolates in 1990, and 23 of 178 (12.9%) isolates in 1991 (Fig. 1).

All isolates from children seen at Texas Children's Hospital and suspected of being penicillin resistant on the basis of an oxacillin zone size of <20 mm in the 3-year period between January 1989 and December 1991 were evaluated by broth macrodilution for susceptibility to penicillin. Four of 127 isolates submitted for confirmation were duplicate isolates from other sites in the same patient (identical serotype and susceptibility) and were excluded from further study. Of the 123 isolates evaluated, 54 (43.9%) were classified as susceptible, 52 (42.3%) were classified as intermediate in resistance, and 17 (13.8%) were classified as highly resistant to penicillin. The distribution of isolates with specific MICs was as follows (MIC [in micrograms per milliliter], number of isolates): 0.002, 11; 0.004, 3; 0.008, 7; 0.016, 9; 0.030, 8; 0.060, 16; 0.125, 21; 0.25, 18; 0.5, 4; 1, 9; 2, 8; and 4, 9.

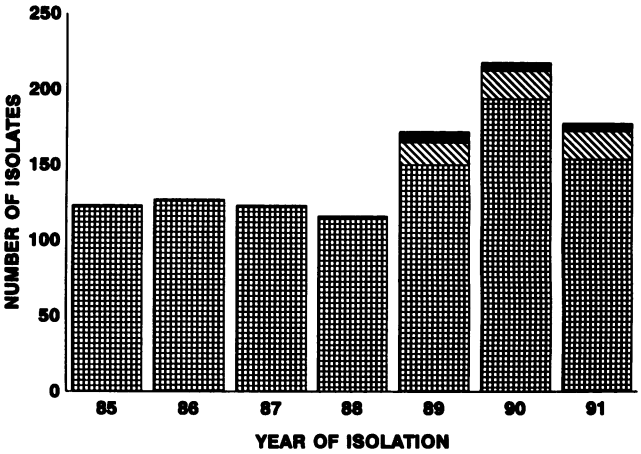

FIG. 1. Isolation of *S. pneumoniae* at Texas Children's Hospital between 1985 and 1991. Symbols: ■, susceptible to penicillin (MIC, ≤ 0.1 μg/ml); ▨, intermediate in resistance to penicillin (0.1 μg/ml ≤ MIC < 2 μg/ml); ■, highly resistant to penicillin (MIC, ≥ 2 μg/ml).

Of 101 isolates submitted with inhibition zone diameters specifically stated (all <20 mm), 62 had zone sizes of between 6 and 10 mm, 19 had zone sizes of between 11 and 15 mm, and 20 had zone sizes of between 16 and 19 mm (Fig. 2). Fourteen of 16 (88%) isolates highly resistant to penicillin showed no zone of inhibition around the oxacillin disc (6 mm = no zone of inhibition). Forty-one of 43 isolates with intermediate resistance to penicillin had zones of inhibition

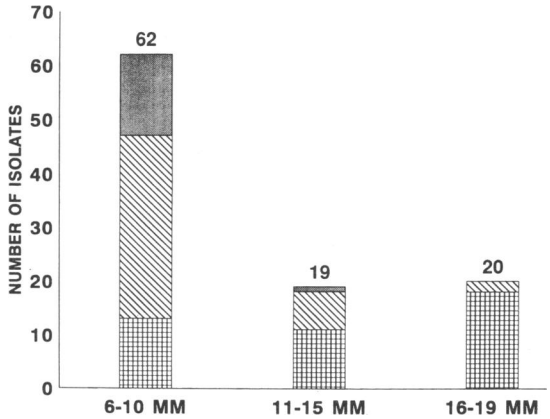

FIG. 2. Classification by quantitative susceptibility of isolates presumed to be resistant to penicillin by the oxacillin disc screen test. Symbols: ■, susceptible to penicillin (MIC, ≤ 0.1 μg/ml); ▨, intermediate in resistance to penicillin (0.1 μg/ml ≤ MIC < 2 μg/ml); ▩, highly resistant to penicillin (MIC, ≥ 2 μg/ml).

TABLE 1. Sources of isolation and susceptibilities to penicillin of *S. pneumoniae*[a]

Source	No. of isolates that were:		
	Susceptible	Intermediate in resistance	Highly resistant
Blood	26	14	0
Cerebrospinal fluid	3	4	0
Middle ear	16	23	15
Eye	3	3	0
Respiratory tract	8	10	3
Other	2	3	1

[a] 123 isolates from Texas Children's Hospital and 11 isolates from other hospitals or laboratories in Houston, Tex.

of ≤15 mm, and 2 had zones of inhibition of 16 to 19 mm. A false-positive result in the oxacillin screen was encountered for 24 of 81 (30%) isolates with a zone of inhibition of ≤15 mm. Eighteen of 42 (43%) isolates classified as susceptible to penicillin by the broth dilution method had oxacillin zone sizes of between 16 and 19 mm. There was little discrepancy between zone sizes obtained by the standard procedure (sheep blood-containing MH agar and reading in the general laboratory by different observers) and zone sizes measured on LHB-containing MH agar by a single observer in a research environment. Ten of 100 isolates for which oxacillin zone sizes were determined by both methods had zone sizes of ≥20 mm in the MH agar-LHB procedure, and all were classified as susceptible by quantitative dilution. Thus, the rate of false classification of resistance for the standard screening procedure was 42%, and that for the MH agar-LHB procedure was 32% (*P*, nonsignificant).

The sources of isolation and susceptibilities to penicillin are shown in Table 1. Fifteen of the 19 isolates highly resistant to penicillin were from the middle ear of children, as were 23 (40.4%) of the isolates with intermediate resistance to penicillin. None of the isolates from cerebrospinal fluid or blood demonstrated high resistance to penicillin. The serotype distribution of the *S. pneumoniae* isolates classified by penicillin resistance category is shown in Table 2. Fifteen of 19 (78.9%) isolates highly resistant to penicillin belonged to capsular type 6, while 21 of 57 (36.8%) isolates with intermediate resistance to penicillin and 9 of 58 (15.5%) isolates susceptible to penicillin were type 6 ($P = < 0.0001$).

A total of 95 isolates were selected for testing of susceptibility to potentially useful antibiotics other than penicillin. Eighty-four were cultured from children seen at Texas Children's Hospital, and 11 were submitted from other hospitals or laboratories in Houston, Tex. Isolates were selected to include available resistant isolates (both classifications) and a random group of susceptible isolates. It is recognized that all isolates were referred on the basis of an oxacillin zone of inhibition of <20 mm but were not selected for susceptibility studies on the basis of source of isolation or serotype. By standard broth macrodilution susceptibility testing, 34 were classified as susceptible, 42 were classified as intermediate in resistance, and 19 were classified as highly resistant to penicillin.

TABLE 2. Serotype distribution of penicillin-susceptible and -resistant isolates of *S. pneumoniae*[a]

Serotype	No. of isolates that were:		
	Susceptible	Intermediate in resistance	Highly resistant
4	1	0	0
6	9	21	15
9	1	0	0
11	1	0	0
14	13	22	1
19	26	7	1
23	6	7	1
Nontypeable	1	0	1

[a] 123 isolates from Texas Children's Hospital and 11 isolates from other hospitals or laboratories in Houston, Tex.

Table 3 shows the MICs for 50% of isolates (MIC_{50}s), MIC_{90}s, and percentages of isolates susceptible to each of the 16 antibiotics tested on the basis of the susceptibility breakpoints published by the NCCLS or by the manufacturer, in the case of antibiotics not yet evaluated. The MIC_{90} did not increase with increasing penicillin resistance for chloramphenicol, ciprofloxacin, rifampin, or vancomycin. Regardless of the classification of penicillin resistance, all isolates were susceptible to cefotaxime, cefpirome, cefpodoxime, clarithromycin, imipenem, rifampin, and vancomycin. If susceptibility to amoxicillin-clavulanate were interpreted on the basis of NCCLS guidelines for the antibiotic combination (MIC, ≤8 μg/ml), then 100% of the isolates would be classified as susceptible. If susceptibility were based on the amoxicillin component only and were interpreted on the basis of the guidelines for ampicillin, representative of the class containing amoxicillin (MIC, ≤0.125 μg/ml), then 85% of the isolates susceptible to penicillin, 48% of those intermediate in resistance to penicillin, and 0% of those resistant to penicillin would be found susceptible.

DISCUSSION

Despite reports of a decline in the rate of isolation of penicillin-resistant pneumococci in the United States as a whole, the rate of isolation of these bacteria appears to be increasing in our population of patients (12, 13, 15, 20). There are now reports of the failure of cefotaxime and ceftriaxone to adequately treat meningitis caused by *S. pneumoniae* showing intermediate resistance to penicillin (2, 19). One of these failures (2) is attributable to an isolate with atypical penicillin-binding proteins and cell wall peptides that the authors consider to cause the high resistance to cefotaxime and ceftriaxone (penicillin MIC, 0.3 μg/ml; cefotaxime and ceftriaxone MIC, 2.5 μg/ml) (4). Although the mechanism of resistance is different, erythromycin resistance has been found in association with penicillin resistance in adults in a study done at the Minneapolis Veterans Hospital (18). The selection of alternative antibiotics is hindered by the lack of clinical experience in treating systemic infections with antibiotics other than penicillin as well as by the lack of adequate susceptibility interpretation criteria. The NCCLS guidelines for susceptibility interpretation are based on experience with bacteria other than *S. pneumoniae* and may not apply specifically to *S. pneumoniae* in many cases. While distinct MIC interpretive standards exist for *Haemophilus influenzae* and *Neisseria gonorrhoeae*, *S. pneumoniae* susceptibility must be interpreted from data designated "for organisms other than *Haemophilus* and *Neisseria gonorrhoeae*" (15). As an example, Table 3 reports susceptibilities to amoxicillin-clavulanate on the basis of guidelines for "other organisms" (MIC, ≤8 μg/ml). Because clavulanate has no antimicrobial properties and β-lactamase is not the mechanism of resistance for pneumococci, interpretation of amoxicillin susceptibility on the basis

TABLE 3. Susceptibilities to selected antibiotics of *S. pneumoniae* isolates that were susceptible to penicillin, intermediate in resistance to penicillin, and highly resistant to penicillin

Antimicrobial agent	MIC breakpoint (μg/ml)	Susceptible to penicillin (M = 34)			Intermediate in resistance to penicillin (M = 42)			Highly resistant to penicillin (M = 19)		
		MIC_{50}	MIC_{90}	% Susceptible	MIC_{50}	MIC_{90}	% Susceptible	MIC_{50}	MIC_{90}	% Susceptible
Amoxicillin-clavulanate[a]	0.125[b]	0.06	0.25	85	0.25	2	48	2	4	0
Amoxicillin-clavulanate[a]	8[c]	0.06	0.25	100	0.25	2	100	2	4	100
Cefaclor	8	1	4	100	2	32	67	64	64	7
Cefixime	1	0.5	2	88	1	8	55	8	16	7
Cefotaxime	8	0.06	0.25	100	0.125	1	100	0.5	2	100
Cefpirome	8	0.015	0.25	100	0.125	0.25	100	0.5	1	100
Cefpodoxime	2	0.125	0.5	100	0.25	2	100	2	8	61
Cefuroxime	8	0.25	2	100	0.5	2	100	4	8	93
Chloramphenicol	8	2	2	96	1	2	100	2	2	100
Ciprofloxacin	1	1	2	87	1	2	89	1	2	82
Clarithromycin	2	0.008	0.03	100	0.015	2	100	1	2	100
Clindamycin	0.5	0.008	0.03	97	0.008	0.03	100	0.03	1	83
Erythromycin	0.5	0.015	0.5	94	0.03	2	69	1	4	21
Imipenem	4	0.008	0.03	100	0.03	0.25	100	0.5	1	100
Rifampin	1	0.06	0.125	100	0.06	0.125	100	0.06	0.125	100
Trimethoprim-sulfamethoxazole[d]	2	0.25/5	2/38	100	0.5/10	1/19	100	1/19	4/76	73
Vancomycin	4	0.25	1	100	0.25	2	100	0.25	0.5	100

[a] Ratio, 2:1; amoxicillin component reported.
[b] Interpretive standard for the ampicillin class (amoxicillin) in tests of "other streptococci."
[c] Interpretive standard for amoxicillin-clavulanate in tests of "other organisms."
[d] Ratio, 1:19.

of the breakpoint for ampicillin (MIC, ≤0.125 μg/ml), representative of the class containing amoxicillin, should be valid for interpretation of amoxicillin-clavulanate susceptibility in tests of *S. pneumoniae*. Isolates highly resistant to penicillin would be classified as susceptible by the former criterion and resistant by the latter interpretation. Penicillin resistance should be correlated with amoxicillin-ampicillin resistance, which is not the case if one interprets amoxicillin-clavulanate susceptibility on the basis of the guidelines presently in effect. The MICs for pneumococci are more appropriately interpreted on the basis of the ampicillin standards, and all interpretive standards for *S. pneumoniae* should perhaps be determined separately from those for other species.

A recently published multicenter evaluation of haemophilus test medium to define quality control limits addresses the problem of the lack of NCCLS guidelines for the interpretation of antibiotic susceptibility in tests of *S. pneumoniae* (10). Because the only specific NCCLS interpretive criterion for *S. pneumoniae* is for penicillin, these investigators chose to use the standards developed for *H. influenzae* to interpret results of tests with cefotaxime and ceftriaxone. Use of the interpretive criteria for *H. influenzae* would in many cases lower the susceptibility breakpoints for many antibiotics, compared with the values used for "rapidly growing bacteria other than *Haemophilus*," but would still fall short of an ideal solution. For cefotaxime, a susceptibility breakpoint lowered from 8 to 2 μg/ml would still lead to the interpretation that all of our isolates were susceptible to cefotaxime, including the isolates highly resistant to penicillin. While our study shows that there is a clear difference between susceptible isolates and isolates intermediate in resistance or resistant in vitro, a clinical correlation of this difference remains undetermined. Our experience indicates that cefotaxime and ceftriaxone may be adequate therapy for systemic infections caused by isolates intermediate in resistance to penicillin (22), while others have reported therapeutic failures with these agents (2, 19). On the other hand, use of the *H. influenzae* interpretive standards for amoxicillin alone and in combination with clavulanate would lower the combination criterion one dilution, to 4 μg/ml, but raise the criterion for amoxicillin alone to 1.0 μg/ml from 0.125 μg/ml under the guidelines for streptococci. It is clear from this study as well as from the study by Jorgensen et al. (10) that it is essential that specific interpretive guidelines for *S. pneumoniae* be developed to address what is becoming an increasing problem with penicillin resistance in this species.

Our results indicate that the oxacillin disc screen test for penicillin susceptibility has a false-positivity rate of about 42%. Use of LHB-containing agar lowered the false-positive screen results to 32%, but this improvement probably does not justify the additional effort in preparing the LHB supplement. Other investigators have reported false-positivity rates for the oxacillin disc screen test of 5.9% under experimental conditions (8, 21) and 39% in a clinical survey (23). On the contrary, we have not found isolates with a zone size of ≥20 mm to be resistant to penicillin (data not presented). As Fig. 2 shows, confidence in the prediction of resistance to penicillin is associated with decreasing zone size in the oxacillin disc screen test. As others have reported (21), we could not use the oxacillin zone size to separate isolates highly resistant to penicillin from isolates intermediate in resistance to penicillin, although only one isolate classified as highly resistant to penicillin was found to have any zone of inhibition surrounding the oxacillin disc. Because penicillin therapy may be preferable to alternate antibiotic therapy, confirmation of isolates with zone sizes of ≤20 mm should continue to be performed in an effort to define true resistance. Likewise, confirmation, preferably by an accurate quantitative method, is indicated for systemic infections and

for patients with an inappropriate response to adequate therapy.

It is of interest that there was a significant association of serogroup 6 with intermediate and high penicillin resistance in this study. Gray et al. found that over one-half of cases of septicemia and meningitis were caused by *S. pneumoniae* belonging to serogroup 6, while only 10% of isolates from otitis media were found to be serogroup 6 (6, 7). While serogroup 6 is a predominant isolate in children, it has not been consistently associated with resistance in the United States. It is possible that this phenomenon may be a geographical one, although an outbreak of disease caused by *S. pneumoniae* serotype 14 intermediate in resistance to penicillin but multiply resistant to other antibiotics has been reported in Houston, Tex. (17).

Recognition of intermediate and high penicillin resistance in the laboratory is necessary, as is the development of appropriate guidelines for the interpretation of MIC data specifically for *S. pneumoniae*. Additionally, high erythromycin resistance has also been found in association with penicillin resistance (18), and this fact will most probably require increased testing for antibiotic resistance in isolates of this once homogeneously susceptible species. While the clinical implications of penicillin resistance in *S. pneumoniae* from specific sites of infection remain to be determined, it is clear that some infections caused by these isolates may not respond adequately to seemingly appropriate therapeutic regimens and that additional clinical correlation is needed.

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