JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2000, p. 3341–3348 Vol. 38, No. 9
0095-1137/00/$04.00+0

Use of the National Committee for Clinical Laboratory Standards Guidelines for Disk Diffusion Susceptibility Testing in New York State Laboratories

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Received 6 March 2000/Returned for modification 12 May 2000/Accepted 22 June 2000

Accurate antimicrobial susceptibility testing is vital for patient care and surveillance of emerging antimicrobial resistance. The National Committee for Clinical Laboratory Standards (NCCLS) outlines generally agreed upon guidelines for reliable and reproducible results. In January 1997 we surveyed 320 laboratories participating in the New York State Clinical Evaluation Program for General Bacteriology proficiency testing. Our survey addressed compliance with NCCLS susceptibility testing guidelines for bacterial species designated a problem (*Staphylococcus aureus* and *Enterococcus* species) or fastidious (*Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*) organism. Specifically, we assessed compliance with guidelines for inoculum preparation, medium choice, number of disks per plate, and incubation conditions for disk diffusion tests. We also included length of incubation for *S. aureus* and *Enterococcus* species. We found overall compliance with the five characteristics listed above in 80 of 153 responding laboratories (50.6%) for *S. aureus* and 72 of 151 (47.7%) laboratories for *Enterococcus* species. The most common problem was an incubation time shortened to less than 24 h. Overall compliance with the first four characteristics was reported by 92 of 221 (41.6%) laboratories for *S. pneumoniae*, 49 of 163 (30.1%) laboratories for *H. influenzae*, and 11 of 77 (14.3%) laboratories for *N. gonorrhoeae*. Laboratories varied from NCCLS guidelines by placing an excess number of disks per plate. Laboratories also reported using alternative media for *Enterococcus* species, *N. gonorrhoeae*, and *H. influenzae*. This study demonstrates a need for education among clinical laboratories to increase compliance with NCCLS guidelines.

With antibiotic resistance increasing among many pathogens, accurate antimicrobial susceptibility testing results are vital for both patient care and public health surveillance. In order to monitor drug resistance, clinicians and public health officials depend on accurate performance and appropriate reporting of the results of these susceptibility tests. Disk diffusion (Kirby-Bauer) (3) is one of the most commonly used antimicrobial susceptibility testing (AST) methods among diagnostic laboratories (22). This method is a well-established procedure for which there are accepted standards including those endorsed by the National Committee for Clinical Laboratory Standards (NCCLS) (26). Methods and interpretive criteria for the disk diffusion methodology are published by NCCLS in *Performance Standards for Antimicrobial Disk Susceptibility Tests* and are revised and updated as needed. These documents include specific guidelines (i.e., medium recommendations, changes in inoculum preparation, incubation conditions, and interpretive criteria) to be followed for organisms designated problem or fastidious organisms by NCCLS (26, 27).

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The New York State (NYS) Clinical Laboratory Evaluation Program's Section of Bacteriology is responsible for ensuring the quality of AST and other clinical bacteriologic testing in laboratories throughout the state (~90%) as well as out-of-state laboratories testing specimens originating from NYS patients (~10%). Permitted laboratories are required to meet minimum standards and successfully participate in the NYS Department of Health's proficiency testing program.

Information regarding the appropriateness of the disk diffusion methods used in clinical laboratories is difficult to obtain. Most studies focus on a particular organism or a single step in a method. This study was designed to examine practices in NYS permitted laboratories with regard to disk diffusion testing. The primary objective was to evaluate the level of compliance with NCCLS guidelines for the disk diffusion methodology when testing two organisms designated problem pathogens (*Staphylococcus aureus* and *Enterococcus* species) and three organisms designated fastidious pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*) by NCCLS. We also examined the interpretation and follow-up of screening tests for penicillin resistance in *S. pneumoniae* and vancomycin resistance in *Enterococcus* species among NYS permitted laboratories.

MATERIALS AND METHODS

Questionnaire. In January 1997, questionnaires were sent to 320 laboratories participating in NYS's proficiency testing program for general bacteriology as part of a routine proficiency testing event. Laboratories in this comprehensive subcategory are approved to perform antimicrobial susceptibility testing with all organisms. The purpose of this survey was to draw attention to the modified methods required for AST of problem and fastidious organisms and to iden-

TABLE 1. Key to analysis of compliance with 1993 or 1997 NCCLS guidelines[a] for disk diffusion susceptibility testing

Characteristic	*S. aureus*	*Enterococcus* species	*S. pneumoniae*	*H. influenzae*	*N. gonorrhoeae*
Age of inoculum (h)	16–24	16–24	16–18[b]	20–24	16–24
Method of inoculum preparation	Direct	Direct or growth	Direct	Direct	Direct
Test medium[c]	MH (no supplement)	MH (no supplement)	MH + sheep blood	HTM	GC + 1% growth supplement
No. of disks of the indicated diam (mm) per plate	4 or 5 per 100; 9 or 12 per 150	4 or 5 per 100; 9 or 12 per 150	4 per 100; 9 per 150	4 per 100; 9 per 150	4 per 100; 9 per 150
Incubation conditions	35°C, room air	35°C, room air	35°C, 5% CO_2	35°C, 5% CO_2	35°C, 5% CO_2
Incubation time (h)	16–18; 24 for oxacillin	16–18; 24 for vancomycin	20–24	16–18	20–24
Table in NCCLS guidelines used[d]	Table 2	Table 2	Table 2C	Table 2A	Table 2B
Quality control strain(s)	ATCC 25923 (*S. aureus*)	ATCC 25923 (*S. aureus*)	ATCC 49619 (*S. pneumoniae*)	ATCC 49766 and ATCC 49247 (*H. influenzae*)	ATCC 49226 (*N. gonorrhoeae*)

[a] The methods presented here represent guidelines from 1993 (27) or 1997 (26). Laboratories must consult currently available guidelines from NCCLS to ensure that the methods used are in compliance with currently accepted guidelines.

[b] NCCLS guidelines in 1997 (26) recommend 16 to 18 h; NCCLS guidelines in 1993 (27) recommend 20 to 24 h.

[c] Abbreviations used for test media include the following: MH, Mueller-Hinton agar; MH + sheep blood, Mueller-Hinton agar containing 5% sheep blood; HTM, Haemophilus test medium; GC agar + 1% growth supplement, GC agar base containing 1% defined growth supplement.

[d] The tables were considered in compliance if they were from either the M2-A5 (27) or M2-A6 (26) series. Tables from M2-A4 (28) were considered outdated.

tify topics in which specific continuing education might be beneficial to participating laboratories. The cover letter clearly stated that responses would not affect the laboratories' proficiency testing score. The questionnaire focused on five organisms for which NCCLS guidelines recommend modified methods for AST: *S. aureus*, *Enterococcus* species, *S. pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*.

The questionnaire requested responses for 11 aspects of AST methods for each organism: (i) indications for testing, (ii) method used, (iii) maximum number of disks placed on a 100- or 150-mm plate, (iv) preparation of inoculum, (v) age of inoculum, (vi) type of medium used, (vii) incubation atmosphere and temperature, (viii) incubation time, (ix) NCCLS interpretive chart used to determine breakpoint, (x) American Type Culture Collection (ATCC) quality control strain used, and (xi) antimicrobial agents tested. Essentially all questions were presented as multiple-choice questions and covered methods described by both recent and older NCCLS documents (26, 27) as well as nonstandard methods. Separate choices were listed for disk diffusion and MIC testing when appropriate. Participants were asked to choose all responses applicable to their laboratory. Participants were also asked to indicate policies used by their laboratory to follow up on oxacillin disk zone sizes of ≤19 mm for *S. pneumoniae* and vancomycin disk zone sizes of >14 and <17 mm for *Enterococcus* species (intermediate results).

Data entry and analysis. The responses on the questionnaires were entered into a database created with Filemaker Pro, version 3.0, software (Claris Corporation, Santa Clara, Calif.) and were independently verified. Responses for questions relevant to disk diffusion were tabulated with Excel, version 4.0, software (Microsoft Corporation, Redmond, Wash.). For each question and organism, results were tabulated only for participants responding to the specific question resulting in a different total number (*n*) of responses for each question and each organism analyzed. We did not include missing responses in the analysis of the survey results.

Interpretation. For each organism, compliance was determined as the percentage of respondents whose practices agreed with 1993 (27) or 1997 (26) NCCLS guidelines for each step of the disk diffusion test. Either set of guidelines was considered acceptable, as the 1997 guidelines were released just prior to mailing of the survey. An outline of the responses that we considered in compliance with NCCLS guidelines is shown in Table 1. For temperature of incubation for *S. aureus* we included choices of 30 to 35°C in room air, 35°C in room air, and 30 to 35°C in 5% CO_2. The 1993 (27) and 1997 (26) NCCLS guidelines suggest 30 to 35°C in room air; however, because of the overlap between the choices, we accepted both 30 to 35°C in room air and 35°C in room air as being in compliance with NCCLS guidelines. For length of incubation, the response choices in our survey included 16 to 18, 20 to 24, and 24 h. Due to the overlap between the choices of 20 to 24 and 24 h, we considered both answers as being in compliance for the survey. We defined overall compliance as compliance with five key steps (inoculum preparation, choice of medium, number of disks per plate, incubation conditions, and length of incubation) for *S. aureus* and *Enterococcus* species or as compliance with the first four steps for the fastidious organisms (*S. pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*).

For questions regarding follow-up of an oxacillin zone size of ≤19 mm for *S. pneumoniae*, responses were grouped as follows: (i) the report is withheld and the result is confirmed by an MIC determination or by a reference laboratory, (ii) the isolate is reported as resistant and the result is then confirmed by an MIC determination or by a reference laboratory, (iii) either the isolate is reported as resistant or the oxacillin disk test is repeated with no other follow-up, and (iv) other. For questions regarding the follow-up for *Enterococcus* species with vancomycin disk zone sizes in the intermediate range (>14 and <17 mm), responses were grouped into three categories: (i) the report is withheld and the result is confirmed by an MIC determination or by a reference laboratory, (ii) the isolate is reported as intermediately susceptible and the result is then confirmed by an MIC determination or by a reference laboratory, or (iii) the isolate is reported as intermediate without confirmation of the result.

RESULTS

Overview. Two hundred eighty-eight of 320 laboratories (90%) returned a completed survey. The disk diffusion method was used by 58.0, 58.7, 93.0, 68.6, and 37.4% of the laboratories responding to the question regarding the method for testing of *S. aureus*, *Enterococcus* species, *S. pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*, respectively (Table 2).

Overall compliance with either the 1993 (27) or 1997 (26) NCCLS guidelines for the disk diffusion method (Table 3) was better for problem organisms (*S. aureus* and *Enterococcus* species) than for the fastidious organisms (*S. pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*). Laboratory deviation from NCCLS guidelines was most evident in areas where the guidelines for problem or fastidious organisms differed from the standard AST protocol.

S. aureus. Of the 166 laboratories that reported use of disk diffusion testing for *S. aureus*, 85.0% reported testing oxacillin disks rather than methicillin (10.2%) or nafcillin (0.6%) disks. Table 3 shows that many laboratories (50.6%) reported that their protocol was in compliance with NCCLS guidelines for five key steps (preparation of inoculum, choice of medium, number of disks per plate, incubation conditions, and length of incubation) of the disk diffusion procedure. The two most common areas of noncompliance were the use of the growth method to prepare the inoculum rather than the direct suspension method recommended by NCCLS and the failure to incubate the test for a full 24 h to accurately detect methicillin or oxacillin resistance (Table 3). Most (89.0%) laboratories complied with NCCLS protocols by using the strain (ATCC

TABLE 2. Laboratories reporting methods used for antimicrobial susceptibility testing[a]

Method	No. (%) of laboratories				
	S. aureus	*Enterococcus* species	*S. pneumoniae*	*H. influenzae*	*N. gonorrhoeae*
Laboratories answering method question	286	283	270	264	257
Disk diffusion	166 (58.0)	166 (58.7)	251 (93.0)	181 (68.6)	96 (37.4)
Beta-lactamase	44 (15.4)	57 (20.1)	8 (3.0)	163 (61.7)	202 (78.6)
Both disk diffusion and beta-lactamase	26 (9.1)	35 (12.4)	6 (2.2)	85 (32.2)	43 (16.7)
Not tested	0 (0.0)	2 (0.7)	15 (5.6)	21 (8.0)	31 (12.1)

[a] Data are shown here only for methods related to disk diffusion testing reported by 288 laboratories. Methods related to MIC testing are not included.

25923) recommended for quality control testing of the disk diffusion procedure, although some (18.9%) laboratories reported using the strain (ATCC 29213) recommended for MIC testing (Table 3).

***Enterococcus* species.** Table 3 shows that 47.7% of the laboratories reported compliance with the five key steps evaluated (preparation of inoculum, choice of medium, number of disks per plate, incubation conditions, and length of incubation). The most common deviation was the use of media other than unsupplemented Mueller-Hinton agar (Table 3). Laboratories often reported using sheep blood-supplemented Mueller-Hinton agar (31.6%) or Trypticase soy agar (2.3%). Laboratories (16.2%) also reported using a strain for quality control (ATCC 51299) that is not designated for quality control of disk diffusion testing (Table 3).

Another survey question dealt with each laboratory's protocol for follow-up of an intermediate disk diffusion testing result (defined by NCCLS as diameters of >14 and <17 mm) when testing *Enterococcus* species against vancomycin. Of 152 laboratories (data not shown) responding, 46.1% determine the MIC, perform a vancomycin agar screen, or send the isolate to a reference laboratory but do not report it as intermediate (according to NCCLS guidelines); 37.5% do not perform follow-up testing with an MIC determination or agar screen and do not send the isolate to a reference laboratory (which is not in compliance with NCCLS guidelines), and 16.5% follow-up with an MIC determination or an agar screen or send the isolate to a reference laboratory but report it as intermediate (which is partially in compliance with NCCLS guidelines). For laboratories reporting performance of follow-up in compliance with NCCLS guidelines, 35% send the isolate to a reference laboratory rather than performing in-house MIC testing. Ninety-three percent of laboratories reported testing *Enterococcus* species for resistance to vancomycin by either the disk diffusion method or MIC testing (data not shown).

S. pneumoniae. Overall, 41.6% of respondents (Table 3) reported following NCCLS guidelines for the four key steps analyzed for this organism (preparation of inoculum, choice of medium, number of disks per plate, and incubation conditions). Common departures from the NCCLS guidelines (Table 3) included placement of too many disks for the size of plate chosen (40.8%) and incubation in room air rather than 5% CO_2 (22.6%). NCCLS provides quality control zone diameter limits for only one strain of *S. pneumoniae*, ATCC 49619; however, only 68.6% of laboratories reported using this strain for quality control (Table 3). We found that 70.9% of laboratories reported that they interpreted zone sizes for *S. pneumoniae* using the table specifically designated for *S. pneumoniae* (Table 3). The 1993 NCCLS guidelines (27) differed from the 1997 NCCLS guidelines (26) by changing the age of inoculum from 18 to 20 h to 16 to 18 h. If the results of the survey are analyzed by the guidelines reportedly in use in the participants' laboratories, then 37.9% of laboratories were in compliance; if either answer was considered in compliance, then 52.4% of the laboratories were in compliance.

For *S. pneumoniae*, 86.0% of laboratories reported assessing resistance to penicillin either by testing for oxacillin resistance by the disk diffusion method or by testing for penicillin resistance by determination of the MIC (data not shown). When penicillin susceptibility in *S. pneumoniae* is tested by the determination of the disk diffusion method, the recommended procedure for zone sizes of ≤19 mm with an oxacillin disk is to determine an MIC and/or send the isolate to a reference laboratory. The organism is not to be reported as resistant until the result is confirmed by further testing, as reported by 42.8% of the 244 laboratories that tested *S. pneumoniae* by the disk diffusion method (data not shown). Our survey found that 31.7% of the laboratories either report the isolate as resistant or retest the isolate by the disk diffusion method without further follow-up, while 23.9% either determine an MIC in-house or send the isolate to a reference laboratory but report the isolate as resistant (partially in compliance with NCCLS guidelines) and 1.6% gave other responses (data not shown).

H. influenzae. Overall compliance with the four key steps (preparation of inoculum, choice of medium, number of disks per plate, and incubation conditions) was 30.1% (Table 3). Although compliance with NCCLS guidelines was evident as 65.2% chose Haemophilus test medium, others chose either chocolate or Mueller-Hinton agar. Many (53.6%) reported placing no more than four disks per 100-mm plate or nine disks per 150-mm plate, and 87.6% correctly chose to incubate their plates in CO_2 (Table 3). Similar to the results for *S. pneumoniae*, only 68.4% of laboratories reported using the table specifically designated for interpretation of the results for *H. influenzae* (Table 3).

N. gonorrhoeae. Only 96 of 257 (37.4%) laboratories reported performing disk diffusion testing with this organism. Many (61.9%) use only beta-lactamase tests, which do not detect chromosomally mediated resistance or resistance to other classes of antimicrobial agents. Table 3 shows that 14.3% of the laboratories reporting disk diffusion susceptibility testing for *N. gonorrhoeae* were in compliance with NCCLS guidelines for four key steps (preparation of inoculum, choice of medium, number of disks per plate, and incubation conditions). Many laboratories used chocolate agar (44.6%) or Mueller-Hinton chocolate agar (19.6%) rather than the recommended GC agar containing 1% growth supplement (29.3%). Many (57.8%) reported using no more than the recommended number of disks per plate, and only 69.7% of laboratories used strain ATCC 49226 for antimicrobial susceptibility testing of *N. gonorrhoeae* (Table 3). Also of concern, as shown in Table 3, only 58.2% of laboratories reported choosing the specific table for interpretation of the zone sizes obtained when testing *N. gonorrhoeae*.

TABLE 3. Responses and summary data for five key questions regarding specific protocols used by participating laboratories

Question and specific response[a]	No. (*n*) or % of laboratories reporting[b]				
	S. aureus	*Enterococcus* species	*S. pneumoniae*	*H. influenzae*	*N. gonorrhoeae*
No. of disks per plate of indicated diam	*n* = 164	*n* = 166	*n* = 233	*n* = 179	*n* = 90
4 per 100 mm	**8.5**	**16.9**	**29.2**	**21.8**	**43.3**
9 per 150 mm	**9.8**	**19.3**	**25.8**	**29.6**	**13.3**
12 per 150 mm	**75.0**	**46.4**	28.8	25.1	23.3
5 per 100 mm	**0.6**	**5.4**	3.0	7.3	10.0
6 per 100 mm	3.0	7.2	7.3	11.7	6.7
Combinations in compliance	**3.0**	**4.8**	**4.3**	**2.2**	**1.1**
Other responses	*0.0*	*0.0*	*1.7*	*2.2*	*2.2*
Total in compliance	**97.0**	**92.8**	**59.2**	**53.6**	**57.8**
Preparation of inoculum	*n* = 159	*n* = 164	*n* = 242	*n* = 169	*n* = 83
Direct suspension	**76.1**	**70.7**	**88.0**	**87.0**	**80.7**
Growth method	18.9	**23.2**	9.1	11.2	16.9
Both direct and growth	*5.0*	**6.1**	*2.9*	*1.8*	*2.4*
Total in compliance	**76.1**	**100.0**	**88.0**	**87.0**	**80.7**
Age (h) of inoculum	*n* = 82	*n* = 69	*n* = 124	*n* = 76	*n* = 37
16–18	**0.0**	0.0	**21.8**	26.3	**21.6**
20–24	**0.0**	**18.8**	**29.8**	**65.8**	**62.2**
18–24	**57.3**	**30.4**	1.6	0.0	0.0
18–20	0.0	0.0	34.7	0.0	0.0
16–24	**29.3**	**36.2**			
24		0.0	0.0	**2.6**	**5.4**
>24	1.2	0.0	0.8	0.0	2.7
Combinations in compliance	**3.7**	**10.1**	**0.8**		**5.4**
Other responses	*8.5*	*4.3*	*10.5*	*5.3*	*2.7*
Total in compliance	**90.2**	**95.7**	**37.9**[c]	**68.4**	**94.6**
Test medium[d]	*n* = 165	*n* = 171	*n* = 250	*n* = 181	*n* = 92
HTM	0.0	0.0	0.4	**65.2**	0.0
Chocolate	0.0	0.0	0.0	21.0	44.6
MH + Chocolate	0.0	0.0	1.2	8.3	19.6
MH + 5% sheep blood	0.0	31.6	**88.8**	0.0	0.0
MH	**90.3**	**62.6**	1.2	0.6	0.0
TSA	0.6	2.3	3.2		
GC agar + 1% growth	0.0		0.0		**29.3**
Sheep blood agar			2.8		
MH + 2% NaCl	0.0		0.0		
Combinations in compliance	**4.2**		**0.0**	**0.0**	**0.0**
Other responses	*4.8*	*3.5*	*2.4*	*5.0*	*6.5*
Total in compliance	**94.5**	**62.6**	**88.8**	**65.2**	**29.3**
Incubation conditions	*n* = 165	*n* = 167	*n* = 248	*n* = 177	*n* = 92
35°C, 5% CO_2	5.5	10.2	**74.2**	**87.6**	**94.6**
30 to 35°C, 5% CO_2	0.0				
35°C, room air	**79.4**	**89.2**	22.6	11.9	5.4
30 to 35°C, room air	**14.5**				
Other responses	*0.6*	*0.6*	*3.2*	*0.6*	*0.0*
Total in compliance	**93.9**	**89.2**	**74.2**	**87.6**	**94.6**
Incubation time (h)	*n* = 161	*n* = 169	*n* = 247	*n* = 176	*n* = 95
16–18	23.6	17.2	21.9	**43.8**	22.1
20–24	**30.4**	**37.3**	**58.3**	44.3	**56.8**
24	**41.0**	**39.6**	**17.4**	9.1	**16.8**
48	0.6				2.1
16–20	0.0	0.0	0.0	0.0	0.0
18–20	0.0	0.0	0.0	0.0	0.0
Combinations in compliance	**4.3**	5.9	**0.8**	**0.0**	**1.1**
Other responses	*0.0*	*0.0*	*1.6*	*0.0*	*1.1*
Total in compliance	**75.8**	**82.8**	**76.5**	**43.8**	**74.7**
NCCLS guideline used	*n* = 156	*n* = 161	*n* = 230	*n* = 174	*n* = 91
M2-A5 Table 2	**66.7**	**65.8**	13.5	14.9	24.2
M2-A5 Table 2A	0.0	0.0	0.0	**53.4**	0.0
M2-A5 Table 2B	0.0	0.0	0.0	0.0	**44.0**
M2-A5 Table 2C	0.0	0.0	**55.7**	0.0	1.1

Continued on following page

TABLE 3—*Continued*

Question and specific response[a]	No. (*n*) or % of laboratories reporting[b]				
	S. aureus	*Enterococcus* species	*S. pneumoniae*	*H. influenzae*	*N. gonorrhoeae*
M2-A6 Table 2	**19.2**	**20.5**	0.0	0.0	0.0
M2-A6 Table 2A	0.0	0.0	0.0	**14.9**	0.0
M2-A6 Table 2B	0.0	0.0	0.0	0.0	**14.3**
M2-A6 Table 2C	0.0	0.0	**15.2**	0.0	0.0
Other responses	*14.1*	*13.7*	*15.7*	*16.7*	*16.5*
Total in compliance	**85.9**	**86.3**	**70.9**	**68.4**	**58.2**
Quality control strain[e]	*n* = 164	*n* = 154	*n* = 226	*n* = 171	*n* = 89
ATCC 49619	0.0	0.6	**68.6**	2.3	3.4
ATCC 29212	0.0	**77.9**	0.0	0.6	0.0
ATCC 29212[f]		**9.7**			
ATCC 29213	18.9	0.6	0.9	1.2	1.1
ATCC 25922	**0.6**	**1.3**	8.4	0.6	0.0
ATCC 25923	**89.0**	**20.8**	8.0	1.2	0.0
ATCC 27853	**0.6**	**1.3**	0.4	0.6	
ATCC 33186	0.0	0.0	0.0	0.0	0.0
ATCC 35218	**0.0**	0.0	0.0	0.0	
ATCC 49766	0.0	0.0	0.9	**2.3**	0.0
ATCC 49247	0.0	0.0	0.0	**49.1**	10.1
ATCC 10211	0.0	0.0	0.0	9.9	1.1
ATCC 49226	0.0	0.0	0.0	0.0	**69.7**
ATCC 51299	0.0	16.2	0.0	0.0	
Other *S. pneumoniae*			20.8		
Other *H. influenzae*				8.8	
Other *N. gonorrhoeae*					14.6
Other *S. aureus*	3.7				
Other *Enterococcus* sp.		1.9			
Both ATCC 49247 and ATCC 49766				**29.2**	
Total in compliance	**89.0**	**93.5**	**68.6**	**80.7**	**69.7**
Overall compliance with four steps	*n* = 158	*n* = 154	*n* = 221	*n* = 163	*n* = 77
Total in compliance	**65.2**	**58.4**	**41.6**	**30.1**	**14.3**
Overall compliance with five steps	*n* = 153	*n* = 151			
Total in compliance	**50.6**	**47.7**			

[a] Some laboratories reported multiple answers. In general these multiple responses were tabulated as separate responses and were considered in compliance if all responses were in compliance or are shown as other responses either if they contained combinations of responses in compliance and not in compliance with NCCLS guidelines or if none of the responses were in compliance.

[b] The percentages of laboratories responding with an answer considered in compliance with NCCLS guidelines are shown in boldface. Responses in italics were considered unevaluable as insufficient information was provided by the reporting laboratories.

[c] Guidelines for age of inoculum of *S. pneumoniae* changed from 18 to 20 h in 1993 (27) to 16 to 18 h in 1997 (26). The percentage of laboratories shown here represents only those laboratories reporting the age of inoculum correlating with the guidelines used by the laboratory. If all laboratories reporting an age of inoculum of either 16 to 18 h or 18 to 20 h are considered in compliance, then 52.4% of laboratories are in compliance.

[d] Abbreviations used for test media: HTM, Haemophilus test medium; MH, Mueller-Hinton agar; TSA, Trypticase soy agar; GC agar + 1% growth, GC agar base containing 1% defined growth supplement.

[e] Many laboratories reported using multiple strains for quality control; laboratories were considered to be in compliance with NCCLS guidelines if they used a minimum of the recommended strain. For *S. aureus* we received 185 responses from 164 laboratories, for *Enterococcus* sp. we received 201 responses from 154 laboratories, for *S. pneumoniae* we received 244 responses from 226 laboratories, for *H. influenzae* we received 181 responses from 171 laboratories, and for *N. gonorrhoeae* we received 89 responses from 89 laboratories.

[f] *Enterococcus faecalis* ATCC 29212 is used for quality control of high-content aminoglycoside disks used for the susceptibility testing of *Enterococcus* species. SXT, sulfamethoxazole. It is also recommended for monitoring M-H agar for unacceptable levels of inhibitors when testing trimethoprim or sulfonamides.

DISCUSSION

This survey was designed to assess laboratory compliance with NCCLS guidelines for AST by the disk diffusion method for problem and fastidious organisms and to determine areas where further education was needed. The results presented here are based on the results specifically reported by NYS permitted laboratories; however, it is likely that a similar lack of compliance occurs in clinical laboratories in other locales as well. This is supported by the fact that the specific results found in this study are similar to those previously reported by the American College of Pathologists and other investigators (17–20).

S. aureus. Temperature and length of incubation are critically important in the detection of methicillin-resistant *S. aureus* (4, 6), and minor changes in these protocols may have a significant impact on susceptibility testing results. We found that 50.6% of our laboratories were in compliance with all five steps, which is comparable to other similar studies (20, 29). Some (18.9%) of the reporting laboratories chose to prepare their inocula by the growth method rather than the recommended direct suspension method. This is comparable to the rate reported by Pfaller et al. (29). In addition, laboratories also reported incubation times of less than the critical 24 h required for optimum detection of methicillin (4, 21, 25) and vancomycin (34) resistance. Pfaller et al. (29) also reported that laboratories did not incubate plates for the recommended full 24 h.

***Enterococcus* species.** Because multiple mechanisms of resistance in enterococci exist, it may be necessary to use more than one antimicrobial susceptibility testing method for accurate analysis (33). Overall, the laboratories in this study adhered to most of the steps in the procedure for *Enterococcus* sp. except that 31.6% of laboratories selected Mueller-Hinton agar supplemented with sheep blood as the test medium. Mueller-Hinton agar containing 5% sheep blood was considered acceptable in 1979 and 1983, but NCCLS subsequently recommended Mueller-Hinton agar without supplements when studies demonstrated that the blood in the agar interacted with aminoglycoside disks to yield false-positive results for susceptibility (16). The continued use of this medium by some laboratories raises concerns that outdated practices still occur in clinical laboratories.

Several surveys (1, 7, 30, 35) have established that laboratories may have difficulty detecting enterococci with phenotypes of intermediate- and low-level vancomycin resistance. These studies have shown problems with both disk diffusion and automated methods as well as ample interlaboratory variation in accuracy and consistency in detecting accurate vancomycin susceptibility for isolates with low- or intermediate-level resistance (1, 7, 30, 35). Although NCCLS has determined that the disk diffusion method is an acceptable choice for ascertainment of enterococcal resistance to vancomycin, current recommendations (26, 27) indicate that an incubation time of at least 24 h is critical to reliable identification of vancomycin-resistant enterococci. In addition, for isolates that yield an intermediate level of susceptibility to vancomycin as determined by the disk diffusion method, an MIC procedure must be performed to verify the level of susceptibility or resistance. Over 60% of NYS permitted laboratories appropriately follow up an intermediate result by applying an MIC technique, as advocated by NCCLS.

***S. pneumoniae*.** The progressive increase in the United States of pneumococci resistant to penicillin (see reference 12 for a review) underscores a need for accurate susceptibility testing so that therapeutic and epidemiologic decisions are appropriate. The overall rate of compliance with the four key steps in the disk diffusion technique for AST of *S. pneumoniae* was 41.6%. A similar survey of testing for pneumococcal antibiotic resistance in Tennessee found that only 27% of hospitals properly tested isolates according to NCCLS guidelines (8). Although penicillin resistance was less common in 1987, Brummitt et al. (5) found a lower percentage (23%) of 111 laboratories surveyed statewide in Minnesota performed the recommended test with a 1-μg oxacillin disk, whereas 80.1% of the laboratories in this study performed the test.

Isolates of *S. pneumoniae* with zone sizes for oxacillin in the nonsusceptible range (≤19 mm) may be resistant, intermediate, or susceptible when they are further tested by an MIC method for susceptibility to penicillin (10, 23, 32). It is therefore critical that laboratories perform confirmatory tests with isolates with zone sizes of ≤19 mm. In 1997, Doern et al. (10) reported that 31.7% of clinical isolates of *S. pneumoniae* had zone sizes of ≤19 mm. Moreover, nearly two-thirds of the participants reported performing appropriate follow-up for a nonsusceptible oxacillin disk test result (10), whereas 42.8% of the laboratories in our study reported that they did so. Heffernan et al. (15) found that the proportion of laboratories conforming to NCCLS guidelines for penicillin susceptibility testing of *S. pneumoniae* increased from 22% in 1993 to 69% in 1995. As our study includes essentially all the laboratories surveyed by Heffernan et al., it is not surprising that we obtained similar results.

***H. influenzae*.** There have been numerous revisions of NCCLS guidelines with respect to application of the disk diffusion method for *H. influenzae* (see reference 9 for a review). Recent NCCLS guidelines recommend that inoculum suspensions be made from colony growth 20 to 24 h old because of the importance of an accurate inoculum concentration (26, 27). Care must be taken in preparing the inoculum of this organism for disk diffusion testing because it may demonstrate an "inoculum effect" in that inaccurate zone size determinations result if the number of CFU exceeds that recommended. Overall compliance with NCCLS guidelines for the four key steps noted was 30.1% for this organism, which is similar to that reported by Scriver et al. (31). One-quarter of laboratories described preparation of the inoculum from a source with 16 to 18 h of growth, which is younger than recommended. The majority of respondents (87.0%) to this survey correctly chose the direct colony method to prepare the inoculum for this organism.

Overall, the results of this study of *H. influenzae* susceptibility testing protocols were comparable to those of Doern and Jones (11). Some laboratories, including 34.9% of respondents in the current study and 37% of those in the study of Doern and Jones (11), chose a medium other than Haemophilus test medium for disk diffusion testing of *H. influenzae*. Another study found that a similar number of laboratories (23.3%) did not use Haemophilus test medium (31). Although Mueller-Hinton agar plus chocolate agar was previously accepted by NCCLS for *H. influenzae*, Haemophilus test medium was adopted in 1988 (28). While there were some problems early on with Haemophilus test medium (including limited shelf life, growth failures, few sources of commercially available media, and difficulties with certain drugs and corresponding zone sizes) (see reference 9 for a review), all other media have been eliminated from NCCLS recommendations, making it necessary to use Haemophilus test medium to ensure accurate results.

***N. gonorrhoeae*.** Our survey found that antimicrobial susceptibility testing of *N. gonorrhoeae* was usually limited to checking of isolates for the presence of a beta-lactamase enzyme. While 37.4% of the respondents in the current study used the disk diffusion method for in vitro susceptibility testing, the remainder performed only a beta-lactamase test. A positive beta-lactamase test result predicts resistance to penicillin, ampicillin, and amoxicillin by plasmid-mediated mechanisms but fails to detect chromosomally mediated resistance to penicillin and to select cephalosporins (see reference 13 for a review). Altaie et al. (2) found that of 51 penicillin-resistant isolates detected by the disk diffusion method, 17 (33%) were beta-lactamase negative and therefore would have been missed by beta-lactamase testing alone (2). Fox et al. (14) showed a lower but increasing level of chromosomally mediated resistance to penicillin in the United States between 1988 and 1994. In addition, beta-lactamase testing would not detect resistance to non-beta-lactam classes of antimicrobial agents such as the fluoroquinolones or the macrolides to which resistance may be emerging (14, 24).

We are concerned that only 29.3% of the laboratories surveyed in this study use the recommended supplemented GC base medium. Most other laboratories indicated the use of either chocolate agar or Mueller-Hinton plus chocolate agar. However, Mueller-Hinton agar might contain inhibitors of growth of gonococcal strains (13). Moreover, zone sizes on Mueller-Hinton plus chocolate agar may be inconsistent with (usually smaller than) those of GC agar plus 1% supplement (2) and between chocolate agar and GC base medium (2).

Several limitations exist in the present study. First, our study

strictly assesses compliance with NCCLS guidelines and does not address their validity. Second, the data presented here are based on results reported to us, and we did not determine the actual practices used in each laboratory, address any errors made in filling out the questionnaire, or determine if the fact that the survey was included with proficiency test samples had an impact on their responses. In addition, the rates of compliance reported in this study may be higher because of the two questions in which response choices overlapped (temperature of incubation and length of incubation), and therefore, we accepted additional answers as compliance with NCCLS guidelines. For *S. aureus* and *Enterococcus* species, the data regarding the length of incubation were difficult to analyze as the 1993 (27) and 1997 (26) NCCLS guidelines mandate only that plates be incubated for a full 24 h for detection of methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus* species, respectively. Therefore, one could argue that 16 to 18, 20 to 24, and 24 h should be considered acceptable, as antimicrobial susceptibility tests with other antimicrobial agents do not necessarily require the full 24-h incubation period. However, 95.4% of laboratories reporting 16 to 18 h of incubation for the disk diffusion method reported testing methicillin, oxacillin, or nafcillin against *S. aureus* and 86.1% reported testing vancomycin against *Enterococcus* species (data not shown).

Follow-up. As our study showed that the rate of compliance with NCCLS guidelines for susceptibility testing of these problem and fastidious organisms was suboptimal in some categories, we implemented several different follow-up approaches to improve susceptibility testing of these organisms. After the survey was evaluated, written results from the questionnaire and abbreviated guidelines outlining the correct methodology for disk diffusion testing of each organism were distributed to all participants. Likewise, on three occasions after this survey was taken, educational information was provided to laboratories regarding AST of problem and fastidious organisms along with proficiency testing materials. In addition, specific guidelines regarding the susceptibility testing of problem and fastidious organisms were incorporated into the *Laboratory Standards for New York State Department of Health* in September 1998 (28a). Lastly, the importance of reporting of antimicrobial resistance for several of these pathogens (*S. pneumoniae*, *H. influenzae*, *N. gonorrhoeae*, as well as vancomycin-resistant *S. aureus*) was emphasized in an educational update on *Laboratory Reporting of Communicable Diseases 1999* (28b) provided by the NYS Department of Health to all permitted laboratories. Although we believe that it is too early to assess the impact of these approaches, we plan to continue our emphasis on the need to use accurate and reliable susceptibility testing methods.

ACKNOWLEDGMENTS

We acknowledge the valuable contributions of all NYS permitted laboratories for responding to the questionnaire.

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