JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1989, p. 192–195
0095-1137/89/010192-04$02.00/0

Vol. 27, No. 1

Quality Control Limits for the Standard Anaerobic Reference Agar Dilution Susceptibility Test Procedure of the National Committee for Clinical Laboratory Standards

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Received 1 August 1988/Accepted 3 October 1988

Multilaboratory studies were performed to develop MIC quality control limits for the National Committee for Clinical Laboratory Standards reference agar dilution method for anaerobic susceptibility tests. Acceptable MICs were defined as those which include >95% of all 100 MICs generated by the study. Most MIC control limits included either 2- or 3-dilution intervals rather than the more traditional 3-dilution intervals that are described as the mode ± 1 doubling dilution.

The problems associated with determining antimicrobial susceptibility of anaerobic bacteria and the clinical relevance of such tests have been reviewed recently by Finegold et al. (2). Inconsistencies in test results underscore the need for strict quality assurance programs and standardization of methods.

To provide guidelines for monitoring the accuracy and precision of anaerobic susceptibility tests, we have performed several collaborative evaluations during the past year. The study protocols were designed to fulfill the requirements outlined by the National Committee for Clinical Laboratory Standards (NCCLS; 5). Three established control strains of anaerobic microorganisms were tested by five independent laboratories against selected study drugs using the standard NCCLS reference agar dilution procedure (4). For each control strain, 20 MICs (different inoculum preparations) were generated by each of five laboratories by using the Wilkins-Chalgren agar that was available for routine use at the time this work was performed. Consequently, 100 MICs were available for each antimicrobial agent-microorganism combination. The distribution of those MICs was examined, and acceptable ranges of MICs were then calculated by applying two different statistical methods. The data included in this report were used to develop the control limits that are described in the recent NCCLS supplement (6). In this report, the data are described in detail and an alternative method of data analysis is applied to propose more stringent control limits.

Studies were performed with amoxicillin and ticarcillin with or without clavulanic acid as well as ampicillin and cefoperazone with or without sulbactam. Doubling dilutions of ticarcillin were prepared with a constant 2.0 μg of clavulanic acid per ml. The other β-lactamase inhibitor combinations were prepared as 2:1 ratios (2 parts active β-lactam plus 1 part inhibitor). Cefmetazole is a new parenteral cephamycinlike antimicrobial agent which was also evaluated. Data which our group has previously published were also reviewed to evaluate the effect of applying two different statistical criteria. The same protocol was used for the latter studies and included tests with cefotetan (9), cefoxitin (3), ceftriaxone (1), and ceftizoxime (3).

The results of previously unpublished tests with each of the three standard control strains are shown in Tables 1 to 3. One participant reported aberrant MIC test results with the ticarcillin-clavulanic acid combination, and those data were excluded from subsequent analyses. No obvious explanation for those aberrant results was found, but that laboratory data were outside of control limits that would have been defined as the all-laboratory mode ± 1 doubling dilution. Useful control limits could not be defined for testing the *Bacteroides fragilis* control strain against the cefoperazone-sulbactam and ticarcillin-clavulanic acid combinations. Agar dilution tests with *Clostridium perfringens* ATCC 13124 were too variable to be useful for controlling the quality of tests with drugs other than ticarcillin or ticarcillin-clavulanic acid. Difficulties in obtaining reproducible results when testing the *C. perfringens* strain against β-lactam antibiotics have been reported elsewhere (8, 9). With the *C. perfringens* strain, inconsistent results might be related to difficulties in standardizing the inoculum (7) or in defining endpoints (8). These technical problems limit the usefulness of the *C. perfringens* control strain, and alternative control strains are currently being sought. Extreme variability in tests with certain drug-microorganism combinations are very real problems (2), which are not resolved by simply selecting control strains that provide reproducible results. On the other hand, control strains with very broad ranges of acceptable MICs are of little practical value for monitoring such tests on a day-to-day basis.

Traditionally, MIC quality control limits have been defined as a range which includes 3 doubling dilutions. A multilaboratory study is first needed in order to identify a measure of central tendency, and that idealized target value is normally defined as the mode (the most commonly occurring value in a series of tests). The target value is difficult to identify when there are approximately equal numbers of MICs distributed evenly between two adjacent concentrations. In that case, the real target value is actually somewhere between the two concentrations that are normally tested. The range of acceptable MICs is often defined as that which includes concentrations 1 doubling dilution on either

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TABLE 1. Replicate agar dilution susceptibility tests with *B. thetaiotaomicron* ATCC 29741: summary of 20 tests in five participating laboratories

Antimicrobial agent(s)[a]	Geometric mean MIC (μg/ml)	No. of times each MIC (μg/ml) was reported[b]									
		0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	128	256
Amoxicillin	32.0							[0	100	0]		
+ CA (2:1)	0.8		[32	64	0]							
Ampicillin	30.1							[9	91	0]		
+ Sulb (2:1)	1.2		[0	74	26]							
Cefoperazone	81.6								[10	45	45]	
+ Sulb (2:1)	14.5						[34	46	20]			
Ticarcillin[c]	39.7							[0	55	25]		
+ CA[c] (2.0 μg/ml)	1.1		[0	70	10]							
Cefmetazole	68.6								[10	70	20]	

[a] β-lactamase inhibitors (clavulanic acid [CA] or sulbactam [Sulb]) were added to the designated compounds, and MICs are expressed as the inhibitory concentration of the active component in the combination.
[b] Current NCCLS control limits are enclosed in brackets.
[c] Excludes aberrant data from one laboratory. That laboratory reported 20 ticarcillin MICs at 32 μg/ml, but ticarcillin + clavulanic acid MICs were either 0.12/2.0 or 0.25/2.0 μg/ml (10 of each).

side of the modal value. When the mode falls between two tested concentrations, the range might actually include 4 doubling dilution steps (midpoint mode ± 2 doubling dilutions). However, the observed MICs may not vary that greatly. Most in vitro tests which involve a series of twofold dilutions are generally assumed to be adequately controlled if they vary no more than 1 dilution interval from the mode, and that is the basis for applying a mode ± 1 dilution statistical criterion. However, in our experience, broth or agar dilution susceptibility tests can be even more precisely performed, and more stringent control limits might be applicable. With a three-dilution step range of acceptable MICs, rather major deviations from the norm will be required before the possibility of technical problems will be detected. On the other hand, extremely stringent control limits might cause too many false alarms resulting from the predictable number of test results that are expected to fall just outside the very narrow control limits. We have attempted to develop an alternative criterion that should define more practical control limits for antimicrobial dilution tests.

In Tables 1 to 3, we present control statistics for 18 different drug-microorganism combinations (100 MICs for each combination). Less than half of the combinations provided MICs that were distributed on either side of a readily identified modal value. Of the 18 drug-microorganism combinations, 10 demonstrated much greater precision since all 100 MICs were distributed over two adjacent MICs, and in one case (amoxicillin with the *Bacteroides thetaiotaomicron* strains), all 100 MICs were identical (32 μg/ml). For such drug-microorganism combinations, a 3-dilution control range seems to be too broad. More stringent control limits should permit earlier detection of testing deficiencies. Specific criteria for defining such control limits were needed.

An alternative criterion is needed for analyzing MICs generated by the type of multilaboratory evaluation that is described in this report. We propose that MIC control limits should be the smallest range that includes more than 95% of the 100 or more MICs that are available from such an evaluation. This should provide a 95% confidence interval, i.e., at least 19 of every 20 replicate tests would be expected to fall within these narrower control limits. Of every 20 tests, 1 might fall just outside of those limits, but such tests should fall within the limits when repeated. The possibility of a statistical outlier occurring on 2 consecutive test days is very small. Consequently, corrective measures would be needed only when two consecutive tests fall just outside the limits or when a single test gives MICs ≥2 doubling dilutions beyond the maximal or minimal MICs that are allowed by this criterion.

Table 4 summarizes the control limits that may be defined by applying the two different types of statistical criteria. Previously reported data (1, 3, 9) with cefoxitin, cefotetan,

TABLE 2. Replicate agar dilution susceptibility tests with *B. fragilis* ATCC 25285: summary of 20 tests in five participating laboratories

Antimicrobial agent(s)[a]	Geometric mean MIC (μg/ml)	No. of times each MIC (μg/ml) was reported[b]										
		0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	128	256
Amoxicillin	34.3							[0	90	10]		
+ CA (2:1)	0.5	[13	87	0]								
Ampicillin	37.8							[0	80	20]		
+ Sulb (2:1)	1.3		[0	60	40]							
Cefoperazone	64.9								[21	57	21]	1
+ Sulb (2:1)	8.8	—[c]	—	—	—	—	—	—	—	—	—	—
Ticarcillin	34.5							[20	49	31]		
+ CA (2:0 μg/ml)	0.3	—[d]	—	—	—	—	—	—	—	—	—	—
Cefmetazole	13.1						[34	61	5]			

[a] β-Lactamase inhibitors (clavulanic acid [CA] or sulbactam [Sulb]) were added to the designated compounds and MICs are expressed as the inhibitory concentration of the active component in the combination.
[b] Current NCCLS control limits are enclosed in brackets.
[c] —, Not recommended. MICs for cefoperazone + sulbactam ranged from 2.0/1.0 to 32/16 μg/ml; useful control limits cannot be defined.
[d] —, Not recommended. MICs for ticarcillin + clavulanic acid ranged from 0.06/2.0 to 1.0/2.0 μg/ml; useful control limits cannot be defined.

TABLE 3. Replicate agar dilution susceptibility tests with *C. perfringens* ATCC 13124: summary of 20 tests in five participating laboratories

Antimicrobial agent(s)[a]	Geometric mean MIC (μg/ml)	No. of times each MIC (μg/ml) was reported[b]					
		0.06	0.12	0.25	0.5	1.0	2.0
Ticarcillin							
5 laboratories	0.55			[24	37	39]	
4 of 5 laboratories	0.66			[6	35	35]	
Ticarcillin + CA (2.0 μg/ml)							
5 laboratories	0.16	21	[27	46	6]		
4 of 5 laboratories	0.20	1	[27	46	6]		

[a] Of the nine drugs or drug combinations included in this report, only ticarcillin and ticarcillin + clavulanic acid (CA) provided useful control limits; the other drugs or drug combinations were too variable when tested against the *C. perfringens* control strain.

[b] Current NCCLS control limits are enclosed in brackets.

ceftriaxone, and ceftizoxime are also reexamined by both types of criteria. The current NCCLS control limits (6) are those that were defined as a mode ± 1 doubling dilution. When ticarcillin was tested against the *C. perfringens* strain, a clear-cut mode was not well defined (Table 3), but a 3-dilution range did include all 100 MICs. With ticarcillin-clavulanic acid, 98.8% of the MICs generated by four of the five laboratories fell within the proposed range. The laboratory reporting aberrant results was "out of control," regardless of the criterion used for defining control limits. When ceftriaxone was tested against the *B. fragilis* strain, the mode was clearly defined as being 64 μg/ml and a range of 32 to 128 μg/ml was proposed, although there were no values at 128 μg/ml and 5% of the MICs were 16 μg/ml (outside the proposed limits). The >95% rule would include all 100 MICs within a range of 16 to 64 μg/ml, and those limits seem to be more appropriate, although the majority of observed MICs were in the upper end of the new control limits. When cefmetazole was tested against the *B. fragilis* strain, 95% of the MICs were either 8.0 or 16 μg/ml and 5% were 32 μg/ml. The >95% rule would lead to a 3-dilution control limit (8.0 to 32 μg/ml) but a 2-dilution range would have been proposed if there had been one less MIC at 32 μg/ml. With 12 of the 25 drug-microorganism combinations listed in Table 4, both criteria supported control limits which included 3 doubling dilutions and all but one of those combinations had the same upper and lower limits identified by the two types of criteria. The one exception is ceftriaxone and *B. fragilis*, which is described above. The >95% rule led us to propose more stringent control limits which included only 2 doubling dilutions for 12 of the 25 drug-microorganism combinations, and in all 12 of these cases, 100% of our MICs fell within the narrower limits. It is important to stress the fact that the 2-dilution range should include at least 95% of all control test results and a 4-dilution range should never be exceeded. When amoxicillin was tested against the *B. thetaiotaomicron* strain, all 100 MICs were 32 μg/ml, and thus we propose that any deviation from 32 μg/ml might be considered a deviation from the expected MIC. Ampicillin and amoxicillin are very similar in their antimicrobial activities, and since ampicillin control limits are 16 to 32 μg/ml, the same limits might prove to be appropriate for amoxicillin; but our data neither support nor reject that assumption.

In summary, with the new statistical criterion that is proposed here, approximately half of the drug-microorganism combinations that we examined would have control limits narrower than the usual 3-dilution range. MICs that fell 1 dilution above or below those more stringent control limits should not occur more than once in every 20 consecutive tests. They were rarely observed among the 100 values that were generated by our five-laboratory collaborative studies. This approach also proved to be useful when dealing with the data base that occasionally provides a poorly defined mode or a mode that is not in the center of a 3-dilution range. It should be pointed out that these observations apply only to tests that are performed with the NCCLS reference agar dilution procedure (4). Modified agar dilution tests (different media) or broth microdilution tests would normally be expected to give MICs that fall within the same limits, but this must be confirmed for each antimicrobial agent tested.

TABLE 4. Summary of anaerobic MIC control limits that can be defined by two different statistical criteria, including previously reported results with four additional drugs

Control strain and antimicrobial agent(s) (reference)[a]	Minimal and maximal acceptable MICs (μg/ml)	
	Mode ± 1 dilution[b]	95% confidence limits (% included)[c]
B. thetaiotaomicron ATCC 29741		
Amoxicillin	16–64	32 (100)
+ CA	0.5–2.0	0.5–1.0 (100)
Ampicillin	16–64	16–32 (100)
+ Sulb	0.5–2.0	1.0–2.0 (100)
Cefoperazone	32–128	32–128 (100)
+ Sulb	8.0–32	8.0–32 (100)
Ticarcillin	16–64	32–64 (100)
+ CA	0.25–2.0	1.0–2.0 (100)
Cefmetazole	32–128	32–128 (100)
Cefoxitin (2)	8.0–32	16–32 (100)
Cefotetan (8)	32–128	64–128 (100)
Ceftriaxone (1)	64–256	64–128 (100)
C. perfringens ATCC 13124[d]		
Ticarcillin	0.25–1.0	0.25–1.0 (100)
+ CA	0.12–0.5	0.12–0.5 (79)[e]
B. fragilis ATCC 25285		
Amoxicillin	16–64	32–64 (100)
+ CA	0.25–1.0	0.25–0.5 (100)
Ampicillin	16–64	32–64 (100)
+ Sulb	0.5–2.0	1.0–2.0 (100)
Cefoperazone	32–128	32–128 (99)
Ticarcillin	16–64	16–64 (100)
Cefmetazole	8.0–32	8.0–32 (100)
Cefoxitin (2)	4.0–16	4.0–16 (100)
Cefotetan (8)	4.0–16	4.0–16 (100)
Ceftriaxone (1)	32–128	16–64 (100)
Ceftizoxime (2)	32–128	32–128 (100)

[a] Only those drug-microorganism combinations for which useful MIC control limits can be defined are listed. Data with cefoxitin, cefotetan, ceftriaxone, and ceftizoxime are from the references cited. CA, Clavulanic acid; Sulb, sulbactam.

[b] The ranges that include 3 doubling dilution steps are those recommended by the NCCLS (6), but ticarcillin-clavulanic acid control limits are not yet published in the current tables.

[c] By these statistical criteria, control limits represent a range that includes >95% of the MICs reported in the initial collaborative study. The percentage of the first 100 MICs that actually fell within the proposed limits is in parentheses.

[d] The NCCLS document recommends cefoxitin control limits of 0.25 to 1.0 μg/ml, but a previous report (3) failed to confirm the utility of those MIC limits.

[e] 99% if one laboratory omitted (see Table 3).

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