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GUEST COMMENTARY

In Vitro Susceptibility Testing of *Haemophilus influenzae*: Review of New National Committee for Clinical Laboratory Standards Recommendations

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In June 1987, the Antimicrobial Susceptibility Testing Subcommittee of the National Committee for Clinical Laboratory Standards (NCCLS) established a fastidious-organism working group expressly for the purpose of addressing issues related to in vitro susceptibility tests with aerobic or microaerophilic bacteria that do not grow well on unsupplemented Mueller-Hinton (MH) media. The fastidious-organism working group considered *Haemophilus influenzae*, for which no standardized method of susceptibility testing was available, for either disk diffusion or dilution susceptibility testing. Previously, numerous media with various inoculum sizes and conditions of incubation had been employed (24). Clearly, medium composition is crucial to susceptibility test results with *Haemophilus* spp. (1, 4–6, 17). Perhaps the most widely used medium for disk testing of *H. influenzae*, at least in the United States, has been chocolatized MH agar (MH-choc) (25). Indeed, the NCCLS had promulgated zone diameter interpretive criteria for four antimicrobial agents, ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, and chloramphenicol, when *H. influenzae* is tested on MH-choc (21). Similarly, the broth medium, MH supplemented with lysed horse blood (3 to 5%) (MH-LHB), appeared to be used more commonly than other media for dilution tests with this organism (25). MIC interpretive criteria existed for the same four antimicrobial agents (20).

The first issue considered by the NCCLS was the optimum medium composition for susceptibility tests with *Haemophilus* spp. Concerns pertaining to MH-choc included its opacity, its undefined chemical composition, and its lack of utility for trimethoprim-sulfamethoxazole (TMP-SMX) disk diffusion tests due to the presence of large amounts of thymidine and thymidine analogs. The major shortcomings of MH-LHB broth for dilution tests with *Haemophilus* spp. were its lack of commercial availability and the logistical complexities of preparing this medium in house. For these reasons, the NCCLS considered alternative media for standardized disk diffusion and dilution susceptibility tests with *Haemophilus* spp.

In January 1988, the use of Haemophilus Test Medium (HTM), a new medium first developed by Jorgensen and colleagues (16), was explored. The agar form of HTM consisted of MH agar, 15 μg of hematin per ml, 15 μg of NAD per ml, and 5 μg of yeast extract per ml. The broth version of HTM consisted of cation-adjusted MH broth, 0.2 IU of thymidine phosphorylase per ml, and the same supplements used in HTM agar. In preliminary studies with 15 antimicrobial agents, laboratory-prepared agar and broth versions of HTM were stable and appeared to support the growth of fresh clinical isolates of *H. influenzae* (16, 17). Other presumed advantages of HTM were its transparent nature, its ability to support disk diffusion tests with TMP-SMX, its lack of expense, and its chemical composition, which was more defined than that of MH-choc; in addition, HTM permitted the use of the same basal medium in both disk diffusion and broth dilution tests, and the broth version of the medium could be manufactured commercially.

On the basis of a series of preliminary studies (23a), the NCCLS adopted HTM as the recommended medium for disk diffusion and dilution susceptibility testing of *Haemophilus* spp. in January 1988 (22, 23). In addition, methods for both procedures were described. With disk diffusion tests, HTM plates should be incubated at 35°C in CO_2 (5 to 7%) for 16 to 18 h prior to zones of inhibition being read. Inocula are to be prepared from a saline suspension of the test organism equivalent in turbidity to a 0.5 McFarland standard. Because of the importance of accurate inoculum concentrations, it was recommended that this initial suspension be prepared with a bench-top nephelometric device directly from colony growth on a 20- to 24-h chocolate agar culture. The method chosen for dilution tests with HTM was a broth microdilution procedure (final volume per well, 100 μl) with trays incubated for 24 h at 35°C in ambient air and a final inoculum concentration of 5×10^5 CFU/ml.

Using HTM and these methods, a three-center collaborative study was conducted to develop interpretive criteria for disk diffusion and broth microdilution results (9). On the basis of the results of this study, interpretive criteria for 18 antimicrobial agents tested against *Haemophilus* spp. were adopted in June 1988 by the NCCLS (22, 23). These antimicrobial agents included ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, cefuroxime, cefamandole, cefonicid, cefaclor, ceftriaxone, cefotaxime, ceftizoxime, ceftazidime, imipenem, aztreonam, chloramphenicol, tetracycline, TMP-SMX, rifampin, and ciprofloxacin. In June 1989, interpretive criteria for cefixime were adopted, as were quality control (QC) criteria for the antimicrobial agents, which now numbered 19 (22, 23). These QC criteria had been developed in a six-center collaborative study using a non-typeable β-lactamase-negative, ampicillin-resistant (BLNAR) strain of *H. influenzae*, ATCC 49247, as a challenge organism (7). This strain was selected because it yielded adequate inter- and intralaboratory precision in the controlled, six-center collaborative study. It grew well on HTM and yielded zone sizes, and the MICs of most antimicrobial agents for this strain were midrange because of its BLNAR characteristics.

During the past 2 1/2 years, the NCCLS has adopted

interpretive criteria for HTM disk diffusion and broth microdilution tests with seven additional antimicrobial agents: loracarbef, cefprozil, cefetamet, ofloxacin, lomefloxacin, azithromycin, and clarithromycin. QC criteria have been adopted for all of these agents, plus the following nine others: cefmetazole, cefpirome, cefepime, cefdinir, cefpodoxime, ceftibuten, meropenem, trospectomycin, and fleroxacin. Although not yet specifically addressed by the NCCLS, the results of one study suggest that the media, methods, and interpretive criteria described above for *H. influenzae* are also applicable to other species of *Haemophilus* (8).

As might be expected with changes as extensive as those undertaken with *Haemophilus* susceptibility tests, several problems have arisen. In 1990 and early 1991, it became apparent that laboratories performing disk diffusion susceptibility tests with *H. influenzae* with HTM agar and 30-μg cefixime disks were reporting disproportionately large numbers of resistant strains (11b). Reanalysis of the results of the initial collaborative study for cefixime interpretive criteria indicated that a 30-μg disk had been used, whereas a 5-μg disk was common in clinical practice. As a result, a second study with 5-μg cefixime disks was performed to establish new cefixime zone diameter interpretive criteria (4a). The results of this study led to a revision of the disk diffusion interpretive criteria for cefixime in June 1991.

Questions concerning the adequacy of the zone diameter interpretive criteria that had been established for cefaclor with HTM and 30-μg disks have also been raised (24b). Specifically, it was contended that the initial NCCLS zone diameter criteria of ≤18 (resistant [R]) and ≥24 (S) were too conservative and would result in too many strains of *H. influenzae* being categorized as falsely resistant or intermediate. Zone diameter frequency distribution data from one laboratory indicated that the use of the initial criteria would result in a combined cefaclor resistance and intermediate rate of approximately 25% (24a). Rates of resistance of this magnitude seemed inconsistent with the apparent therapeutic efficacy of cefaclor. Therefore, in June 1992, on the basis of the results of a six-center collaborative study (11a) aimed at reassessing zone diameter interpretive criteria for relatively β-lactamase-labile cephalosporins such as cefaclor, the NCCLS modified the cefaclor disk diffusion interpretive criteria to ≤16 (R) and ≥20 (S). Provisional cefprozil and loracarbef zone diameter interpretive criteria were also changed, to ≤14 (R) and ≥18 (S) for cefprozil and ≤15 (R) and ≥19 (S) for loracarbef.

Ampicillin was used as an internal control agent in the six-center collaborative study noted above. Interestingly, the results of this study indicated that the ampicillin zone diameter interpretive criteria initially adopted in 1988 also needed to be revised. In June 1992, the following zone diameter criteria were adopted for HTM ampicillin disk diffusion tests: ≤18 (R) and ≥22 (S). This change is consistent with the observations of one recent investigation (11). Among 87 BLNAR clinical isolates of *H. influenzae*, 13 (15%) yielded ampicillin zone diameters of ≤24 mm on HTM, i.e., they would have been falsely categorized as resistant or intermediate. The new interpretive criteria would have correctly categorized the large majority of these 13 strains as susceptible.

Another issue concerns HTM QC testing. Initially, QC ranges of 4 to 5 mm were adopted for most antimicrobial agents when they were tested against ATCC 49247. While these ranges had been developed by using statistical analysis similar to that for non-fastidious organism–antimicrobial agent combinations, in practice they proved too narrow. As a result, QC ranges for HTM disk diffusion tests with ATCC 49247 were adjusted so that, in most cases, the range became 8 to 9 mm. Also, inconsistent QC results were noted with HTM agar when the antimicrobial agents cefaclor, cefuroxime, cefamandole, and cefonicid were tested against ATCC 49247. An observation that the MH base used to prepare HTM agar had a profound effect on disk diffusion results with these antimicrobial agents and ATCC 49247 was made (3, 11c). For these reasons, a study was done to identify an alternative strain for HTM disk diffusion QC tests with cefaclor, cefuroxime, cefamandole, cefonicid, and loracarbef (12). On the basis of the results of this study in June 1991, the NCCLS replaced ATCC 49247 with ATCC 49766 as the recommended QC strain for disk diffusion tests with cefaclor, cefuroxime, cefamandole, cefonicid, and loracarbef (23). In June 1992, QC criteria based on ATCC 49766 were also adopted for cefprozil. Although there was no evidence that MIC QC tests with ATCC 49247 were problematic, a second study was conducted in an effort to develop QC ranges for MIC tests with cefaclor, cefuroxime, cefamandole, cefonicid, and loracarbef against ATCC 49247 (2). The results of this study were also adopted by the NCCLS.

The consequence of these changes is that it is now easier to obtain "in-control" values with disk diffusion and possibly broth microdilution QC tests with the six antimicrobial agents noted in the previous paragraph. With the adoption of ATCC 49766, media-related problems that would have been observed with ATCC 49247 disappear. The question of which control strain yields the most valid results arises. Unfortunately, there currently exists no definitive answer to this important question. Clearly, however, the NCCLS has judged that ATCC 49247 provided too rigorous a test for cefaclor, cefuroxime, cefamandole, cefonicid, cefprozil, and loracarbef on HTM.

A variety of other concerns pertain to current NCCLS recommendations for *Haemophilus* susceptibility tests with HTM. With numerous antimicrobial agents, e.g., broad-spectrum cephalosporins, imipenem, aztreonam, and the fluoroquinolones, few or no clearly resistant strains exist for testing. As a result, the use of frequency distribution analysis to define interpretive criteria for results, as has been done by the NCCLS, may be questioned. Because of a lack of resistant strains, the NCCLS has appropriately elected to define only a susceptible category for these agents; however, even this conservative approach has problems. First, it has been suggested that in vitro testing of antimicrobial agents should be directed toward identifying resistant rather than susceptible strains. If so, a test that defines only susceptible organisms is of little value. Second, how are breakpoints best established for a unimodal susceptible population, close to the population or at some distance from the population? In the first case, the test is best able to detect organisms only slightly more resistant than the unimodal population, but it would also tend to overestimate resistance. In the second case, resistant strains might not be as easily detected; however, the problem of false resistance would be diminished. With regard to this problem, the NCCLS has adopted the following approach: the zone diameter breakpoint for the susceptible category is established at 6 mm smaller than the highest zone size capturing 99% of the unimodal population. The MIC breakpoint is established at 2 dilutions lower than the MIC for 90% of the strains of the unimodal population tested.

Several unresolved problems with HTM remain. Extensive anecdotal experience indicates frequent episodes of

poor growth or actual growth failures with HTM. Two published studies have alluded to this problem (1, 11). By contrast, the six-center study cited above, which was conducted with the intent of reassessing zone diameter interpretive criteria with antimicrobial agents such as cefaclor and cefprozil, did not experience major problems with growth failures. Inadequate growth on HTM, when it does occur, is probably due to the X-factor constituent of the medium, which is supplied in the form of purified hematin. Hematin is poorly soluble and extremely unstable. Some clinical isolates of *H. influenzae* may have very high X-factor growth requirements. Such strains are characterized by narrow zones of growth surrounding X- or V-factor-impregnated strips in satellitism tests. These strains may fail to grow on or in HTM if it has been prepared inadequately or if it has been stored for various periods of time. Recognizing this problem, in January 1990, the NCCLS officially adopted a recommendation that manufacturers of HTM routinely test production lots with *H. influenzae* ATCC 10211. This strain has a high X-factor growth requirement. A manufacturer's use of ATCC 10211 does not, of course, address the problem of poor shelf life. Therefore, individual laboratories might also employ ATCC 10211 as a growth control.

A further difficulty pertains to inter- and intramanufacturer variability with batches of HTM (2, 7, 11, 12). Such variability is perhaps not surprising, as the individual ingredients used to make HTM have profound effects on media performance. Problems related to manufacturing have prompted at least one national commercial media vendor to decline to offer HTM. Among those vendors selling HTM, Becton Dickinson Microbiology Systems (Cockeysville, Md.) has the most experience making and distributing this medium.

In addition to lot-to-lot variability, within-lot variability also exists with HTM-based susceptibility tests (2, 7, 12). The magnitude of this problem is unknown. It has been only casually addressed in the literature (1) and currently remains largely unexplained.

A third problem concerns testing BLNAR strains of *H. influenzae*. All such strains should be considered resistant to ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, and the relatively β-lactamase-labile cephalosporins, such as cefaclor, cefuroxime, cefonicid, cefamandole, cefprozil, and the new carbacephem, loracarbef (14, 18). A fairly convincing argument may be made for the assertion that it is detection of these strains that is the primary objective of in vitro susceptibility testing with *Haemophilus* spp. Excepting TMP-SMX and perhaps tetracycline, other antimicrobial agents are either predictably active or inactive, or they are best assessed by enzyme tests, such as β-lactamase or chloramphenicol acetyltransferase assays (9, 13). Unfortunately, current NCCLS HTM-based methods and interpretive criteria may not effectively identify BLNAR strains as ampicillin resistant (10, 19) or, for that matter, as resistant to the eight antimicrobial agents listed above (4a). First of all, technical problems often occur when BLNAR strains of *H. influenzae* are tested against beta-lactam antimicrobial agents with HTM. These include indistinct zones of inhibition and in growth with disk diffusion tests and trailing endpoints with broth microdilution tests. The recommended inoculum size of 5×10^5 CFU/ml may be a contributing factor with MIC tests. MH base appears to significantly influence the expression of the activities of cephalosporins such as cefaclor and cefuroxime against BLNAR strains (3, 11c). These technical problems are further complicated by the following observations. If resistance is defined according to MICs, then disk diffusion tests with certain beta-lactam antimicrobial agents on HTM do not adequately separate BLNAR strains of *H. influenzae* from strains which are susceptible. Establishing zone diameter breakpoints with compounds such as cefaclor so as to appropriately categorize BLNAR strains as cefaclor resistant would lead to a disproportionate number of susceptible strains being categorized as intermediate or, in a few cases, resistant. The same problem applies with cefprozil, loracarbef, and, to some extent, ampicillin, β-lactamase inhibitor combinations, cefuroxime, cefonicid, cefamandole, and perhaps cefixime.

As stated previously, one question arises: what is the objective of in vitro susceptibility tests, to detect resistance or to detect susceptibility? After much deliberation, the NCCLS decided to establish zone diameter interpretive criteria with these agents in such a way that susceptible strains would be correctly categorized as susceptible. As a consequence, BLNAR strains will commonly be falsely categorized as susceptible. This decision was predicated on commercial interests and on the observation that BLNAR strains currently remain distinctly uncommon in the United States. It may have to be reconsidered in the future.

In conclusion, the NCCLS has recently adopted a new approach to susceptibility testing of *H. influenzae*. While this approach appears to represent progress in the area of susceptibility testing of this fastidious bacterium, several problems have been identified, and these remain to be resolved. The obvious question is, what should laboratories do today when confronted with clinical isolates of *Haemophilus* spp.? One possibility is to not perform susceptibility tests at all. A strong case can be made for performing only a β-lactamase assay and possibly a test for chloramphenicol acetyltransferase, at least with the large majority of clinical isolates in the United States (8). When susceptibility tests with specific antimicrobial agents are judged necessary, one of two approaches can be taken. Employ the current NCCLS-recommended methods with HTM, excercising caution in ensuring that the medium used performs satisfactorily and that test results are in control. If for any reason an alternative method is deemed necessary, disk diffusion tests may be performed with MH-choc plates and disks containing ampicillin, chloramphenicol, amoxicillin-clavulanate, and ampicillin-sulbactam. Interpretive criteria are those originally published by the NCCLS for MH-choc (21). MICs of any agent may be determined with MH-LHB–10 μg of NAD per ml. A broth microdilution format with a 100-μl final volume, an inoculum concentration of 5×10 CFU/ml, and trays incubated for 20 to 24 h at 35°C in ambient air should be employed. The major limitation of disk diffusion tests with MH-choc plates and MICs with MH-LHB is the lack of published QC criteria. It may be, however, that laboratories that have traditionally used these media in the past have developed their own internal QC standards. Finally, it is clear that much more needs to be learned before any single method can be advocated with complete assurance for all *Haemophilus* susceptibility tests.

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