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Surveillance of Susceptibility Testing Methodologies for *Haemophilus influenzae* in Canada, Including Evaluation of Disk Diffusion Test

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We surveyed 75 clinical laboratories to determine if National Committee for Clinical Laboratory Standards (NCCLS) were being used for the susceptibility testing of *Haemophilus influenzae*. Of the 66 laboratories that performed susceptibility testing, all claimed to follow current NCCLS guidelines. However, upon further questioning, only 23, all of which used disk diffusion testing, accurately interpreted and followed the guidelines. Proficiency testing of 22 of these laboratories found that an unacceptable number of interpretive errors (>10%) occurred. These results query the merit of routine disk diffusion susceptibility testing of *H. influenzae* to β-lactam agents.

In an attempt to achieve standardization of in vitro susceptibility testing of *Haemophilus influenzae*, the National Committee for Clinical Laboratory Standards (NCCLS) developed standards based on the use of Haemophilus Test Medium (HTM) for both disk diffusion and broth microdilution testing (6, 7). However, the guidelines for both broth microdilution and disk diffusion testing have undergone several modifications since they were first published (8–11). The most recent NCCLS guidelines, published in December 1993 (11), contain major revisions for disk diffusion testing of *H. influenzae*, including the deletion of disk diffusion testing criteria for ampicillin-sulbactam, amoxicillin-clavulanate, and cefamandole (11). To determine the *H. influenzae* susceptibility testing practices in Canadian laboratories, we conducted a telephone survey of 75 clinical microbiology laboratories across Canada. As a result of that survey, we determined that more than 90% of the laboratories that performed susceptibility testing of *H. influenzae* used disk diffusion susceptibility testing. We therefore evaluated the accuracy of disk diffusion testing in 22 of those laboratories that purported to follow NCCLS guidelines for the susceptibility testing of *H. influenzae*.

A random sample of clinical laboratories was selected from a comprehensive listing of Canadian hospitals. The number of beds in the hospital-affiliated laboratories ranged from 50 to >500, and the hospital-affiliated laboratories were equally divided between university (n = 32) and non-university-affiliated (n = 33) centers. In addition to the 65 laboratories in hospital-affiliated centers, 10 nonhospital or private laboratories were included. The 75 laboratories were also chosen such that all regions of Canada (all 10 provinces and both territories) were represented. The technologist in charge of the microbiology laboratory or the technologist on the respiratory bench was identified, and a telephone poll was conducted. During the telephone interview, participants were initially asked if NCCLS guidelines were used for the susceptibility testing of fastidious and nonfastidious organisms. This was followed by specific questions on the methodologies used for the susceptibility testing of *H. influenzae*.

All laboratories that were contacted agreed to participate in the survey. Of the 75 laboratories contacted, 71 (95%) laboratories indicated that recommendations published by NCCLS were followed for susceptibility testing of both fastidious and nonfastidious organisms. The remaining four respondents indicated that they followed recommendations other than those published by NCCLS (n = 3) or did not follow any guidelines (n = 1).

Of the 75 respondents, 66 (88%) performed susceptibility testing in addition to a β-lactamase test for isolates of *H. influenzae*. Sixty of the 66 laboratories that performed additional susceptibility testing used the disk diffusion methodology. Only one center routinely performed broth microdilution tests. The participants in the survey indicated that they used either alone or in conjunction with disk diffusion testing, agar dilution (n = 2), the E test (n = 1) (AB Biodisk, Solna, Sweden), and various commercial test systems (n = 6).

Of the 60 centers performing disk diffusion testing, 46 centers used HTM and 36 of those used commercially prepared formulations. Of the 14 centers that used media other than HTM, 9 used chocolatized Mueller-Hinton medium, the medium recommended by NCCLS prior to the adaptation of HTM. Contrary to current NCCLS recommendations, two centers used standards other than a 0.5 McFarland for inoculation and 37 (80.4%) did not use a photometric device to standardize the inoculum. Twenty-nine of the 60 centers incubated disk diffusion plates in excess of the recommended 18 h, and 6 centers incubated these plates without the required increased CO_2 concentration. Nine centers did not use any of the recommended American Type Culture Collection controls when performing disk diffusion testing, and none of the 10 centers that prepared HTM in house used *H. influenzae* ATCC 10211. Therefore, of the 60 centers performing disk diffusion testing for the susceptibility testing of *H. influenzae*, only 23 actually followed the prescribed guidelines of NCCLS.

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TABLE 1. Total number of interpretive errors by 22 laboratories when performing disk diffusion testing in accordance to M2-A5 NCCLS guidelines

Type of error	No. (%) of errors with each antimicrobial agent tested		
	Ampicillin	Cefaclor	Cefuroxime
Very major	11 (6.1)	NA[a]	NA
Major	7 (3.9)	59 (32.6)	21 (11.6)
Minor	24 (13.3)	31 (17.1)	28 (15.5)

[a] NA, not applicable. The results of broth microdilution susceptibility testing negated error classification.

We evaluated the accuracy of disk diffusion testing in 22 of the 23 laboratories that were identified by the survey to be correctly following these guidelines. To conduct this proficiency test, 10 well-characterized isolates of *H. influenzae* were sent to the 23 laboratories identified by the survey to be correctly using the methodological guidelines of NCCLS. These 10 strains had been fully identified by standard methods (5). Susceptibility characterization was performed by broth microdilution according to NCCLS guidelines, and β-lactamase testing was performed by using a nitrocefin-impregnated disk (9). The 10 isolates of *H. influenzae* included 6 β-lactamase-positive strains. The centers enrolled in this part of the study were asked to perform disk diffusion testing and to report the resulting zone sizes for five antimicrobial agents: ampicillin, amoxicillin-clavulanate, cefaclor, cefamandole, and cefuroxime. At the time the study was carried out, the revised M2-A4 NCCLS guidelines for antimicrobial disk susceptibility were in use (6, 8, 9). Since then new guidelines have been published (11). Because the new guidelines do not contain methodologic changes but, rather, contain changes in zone interpretive criteria and the deletion of disk testing for certain antimicrobial agents, we were able to use the most recent guidelines for determining interpretive errors. The zone sizes for each isolate-drug combination were compared with the broth microdilution-derived interpretive categories, and very major, major, and minor errors were determined by using both M2-A4 (6, 8, 9) and the revised M2-A5 breakpoints (11) (Table 1). Very major errors are defined as those errors in which the "gold standard," broth microdilution, provides a resistant result, whereas the test, disk diffusion, provides a susceptible result.

When zone size determinations based on previous NCCLS criteria (6, 8, 9) were compared with the broth microdilution results, 318 interpretive errors occurred. There were 23 very major interpretive errors, or 5.2% of the 440 possible very major errors. Similarly, there were 173 major errors, or 15.7% of the 1,100 possible major errors, and 122 minor errors, or 13.9% of the 880 possible minor errors. When these errors rates were examined in terms of the total number of tests (n = 1,100), the very major error rate was 2.1% and the minor error rate was 11.1%. When interpretive errors were calculated by using the revised breakpoints for cefuroxime included in the M2-A5 document (11), there were 29 fewer errors with this agent. Since these guidelines have deleted the disk testing criteria for amoxicillin-clavulanate and cefamandole, the total number of interpretive errors was significantly decreased, to 181. However, the interpretive error rate remained unacceptably high (11.8%). There were statistically more interpretive errors with the single β-lactamase-negative ampicillin-resistant strain than with the β-lactamase-positive or β-lactamase-negative ampicillin-susceptible strains ($P < 0.001$) with both sets of guidelines (Table 2).

TABLE 2. Interpretive errors encountered with the three different ampicillin susceptibility phenotypes when tested by the 22 laboratories performing disk diffusion testing according to M2-A5 NCCLS guidelines

Type of error and ampicillin phenotype[a]	No. (%) of errors with each antimicrobial agent tested		
	Ampicillin	Cefaclor	Cefuroxime
Very major			
β-Lac^{neg} AmpR	6 (2.7)[b]	NA[c]	NA
β-Lac^{pos} AmpR	5 (2.3)	NA	NA
β-Lac^{neg} AmpS	NA[d]	NA	NA
Major			
β-Lac^{neg} AmpR	NA	13 (2.0)	4 (0.6)
β-Lac^{pos} AmpR	NA	10 (1.5)	5 (0.8)
β-Lac^{neg} AmpS	7 (1.1)[e]	36 (5.5)	12 (1.8)
Minor			
β-Lac^{neg} AmpR	4 (0.6)[d]	2 (0.3)	2 (0.3)
β-Lac^{pos} AmpR	2 (0.3)	19 (2.9)	9 (1.4)
β-Lac^{neg} AmpS	18 (2.7)	10 (1.5)	17 (2.6)

[a] β-Lac^{neg} AmpR, β-lactamase negative and ampicillin resistant (n = 1); β-Lac^{pos} AmpR, β-lactamase positive and ampicillin resistant (n = 4); β-Lac^{neg} AmpS, β-lactamase negative and ampicillin susceptible sensitive (n = 5).
[b] Percentages are based on 220 possible very major errors.
[c] NA, not applicable. The results of broth microdilution susceptibility testing negated error classification.
[d] Percentages are based on 660 possible minor errors.
[e] Percentages are based on 660 possible major errors.

NCCLS guidelines for the susceptibility testing of fastidious organisms, including *H. influenzae*, have undergone and continue to undergo significant modifications in an effort to improve accuracy and reproducibility. Although the adaptation by NCCLS of HTM for both broth microdilution and disk diffusion testing has alleviated some of the shortcomings of the previously recommended formulations, problems still exist with the susceptibility testing of *H. influenzae* (4).

Many of these problems may be due to the fact that susceptibility testing of *H. influenzae* is highly method dependent. This problem is compounded if laboratories are not following recommended guidelines. We found that 43 of 66 laboratories that performed susceptibility testing failed to perform susceptibility testing in accordance with the methods described by NCCLS. In a College of American Pathology (CAP) survey questionnaire on in vitro susceptibility test practices with *H. influenzae*, Doern and Jones (2) found that the recently adopted NCCLS guidelines have had an impact upon laboratory practices in the United States. However, like the present study, CAP survey participants were found not to comply with all of the specific methodologic recommendations of NCCLS (2). It is not completely obvious why laboratories fail to comply with current methodologic recommendations; however, it may be that laboratories fail to recognize the highly method-dependent testing required to obtain accurate, reproducible results when testing fastidious gram-negative organisms such as *H. influenzae*.

Since HTM was introduced in 1990 (M2-A4), interpretive breakpoints for ampicillin, cefaclor, cefamandole, and cefuroxime have been modified because of the high rates of false-positive resistance (6, 8, 9). More recently, NCCLS guidelines have deleted interpretive breakpoints for ampicillin-sulbactam, amoxicillin-clavulanate, and cefamandole because of excessive interpretive errors (11). The results of the present study support these recommendations. However, ampicillin disk diffusion testing continues to be problematic. The oc-

currence of significantly more interpretive errors with the ampicillin-resistant β-lactamase-negative strain than with the β-lactamase-positive and β-lactamase-negative ampicillin-susceptible strains is of particular concern since these interpretive errors included very major errors with ampicillin. The identification of these strains must be one of the primary objectives of in vitro susceptibility testing of *H. influenzae* because the ineffectiveness of β-lactam agents against these strains cannot be predicted by an enzymatic test. Heelan et al. (3) found that disk diffusion testing of ampicillin with either chocolate Mueller-Hinton medium or HTM resulted in frequent intermediate zone sizes for non-β-lactamase-producing strains. When these isolates were further tested by the broth microdilution method, ampicillin MICs in the susceptible range (≤1.0 μg/ml) were obtained. These results prompted the authors to caution that the results for β-lactamase-negative isolates that produce zone sizes indicating "intermediate" and "resistant" should be confirmed by the broth microdilution method. In the present survey, we found that only three laboratories had the facilities to perform this type of susceptibility testing.

In conclusion, HTM has been widely adopted as the preferred susceptibility test medium for *H. influenzae*. However, the failure of laboratories to comply with the methodologic recommendations of NCCLS and the lack of interlaboratory reproducibility with HTM suggest that other medium formulations should be evaluated. Laboratories may also want to reevaluate the necessity of performing susceptibility testing of *H. influenzae* to β-lactams. Since there is no demonstrable resistance to broad-spectrum cephalosporins and the prevalence of β-lactamase-negative ampicillin-resistant strains is <1.0%, the present study supports the suggestion made by Doern (1) that other than a β-lactamase, susceptibility testing of *H. influenzae* to β-lactams should not be done on a routine basis.

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