Haemophilus influenzae with Non-Beta-Lactamase-Mediated Beta-Lactam Resistance: Easy To Find but Hard To Categorize

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Haemophilus influenzae is a major pathogen, and beta-lactams are first-line drugs. Resistance due to altered penicillin-binding protein 3 (rPBP3) is frequent, and susceptibility testing of such strains is challenging. A collection of 154 beta-lactamase-negative isolates with a large proportion of rPBP3 (67.5%) was used to evaluate and compare Etest (Haemophilus test medium [HTM]) and disk diffusion (EUCAST method) for categorization of susceptibility to aminopenicillins and cefuroxime, using MICs generated with broth (HTM) microdilution and clinical breakpoints from CLSI and EUCAST as the gold standards. In addition, the proficiency of nine disks in screening for the rPBPr genotype (N526K positive) was evaluated. By Etest, both essential and categorical agreement were generally poor (<70%), with high very major errors (VME) (CLSI, 13.0%; EUCAST, 34.3%) and falsely susceptible rates (FSR) (CLSI, 87.0%; EUCAST, 88.3%) for ampicillin. Ampicillin (2 μg) with adjusted (± 2 mm) zone breakpoints was superior to Etest for categorization of susceptibility to ampicillin (agreement, 74.0%; VME, 11.0%; FSR, 28.3%). Conversely, Etest was superior to 30 μg cefuroxime for categorization of susceptibility to cefuroxime (agreement, 57.1% versus 60.4%; VME, 2.6% versus 9.7%; FSR, 7.1% versus 26.8%). Benzylpenicillin (1 unit) (EUCAST screening disk) and cefuroxime (5 μg) identified rPBP3 isolates with highest accuracies (95.5% and 92.2%, respectively). In conclusion, disk screening reliably detects rPBP3 H. influenzae, but false ampillin susceptibility is frequent with routine methods. We suggest adding a comment recommending high-dose aminopenicillin therapy or the use of other agents for severe infections with screening-positive isolates that are susceptible to aminopenicillins by gradient or disk diffusion.

Non-typeable Haemophilus influenzae (NTHi) frequently causes acute otitis media, conjunctivitis, sinusitis, and respiratory tract infections, including pneumonia and exacerbations in chronic obstructive pulmonary disease and cystic fibrosis, and may also cause invasive disease (1). Beta-lactams are first-line drugs when systemic therapy is indicated, but resistance due to transferable beta-lactamase genes (blaTEM or blaOXB) and/or substitutions in penicillin-binding protein 3 (PBP3), encoded by the ftsI gene, is common (1, 2). Strains with low-level PBP3-mediated resistance (low-rPBP3) possess the R517H (group I) or the N526K (group II) substitution, whereas strains with high-level resistance (high-rPBP3) are characterized by the additional substitution S385T (2, 3). The distinction is clinically important because high-rPBP3 strains express higher resistance to extended-spectrum cephalosporins (1, 2, 4–6). Group II low-rPBP3 is the predominant genotype in Australia (7), Europe (8, 9), and North America (6, 10), whereas high-rPBP3 strains predominate in Japan and Korea (6, 11).

Susceptibility testing of H. influenzae has been characterized as “a tricky business” (12). Categorization of susceptibility to aminopenicillins (with or without beta-lactamase inhibitors) is particularly challenging (2, 13). First, the rPBP3 population overlaps the wild-type population in terms of MICs: bla-negative rPBP3 strains have ampicillin MICs of 0.5 to 16 mg/liter (1, 2, 13), whereas the epidemiological cutoff (ECOFF) value is ≤1 mg/liter (www.eucast.org/mic_distributions_ecoffs). Second, the clinical relevance of current breakpoints is debated (1, 2, 13). Ampicillin breakpoints from the Clinical and Laboratory Standards Institute (CLSI) were originally set to distinguish between bla-positive and bla-negative strains (13). Non-species-related (pharmacokinetic-pharmacodynamic [PK/PD]) breakpoints calculated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) suggest that isolates with ampicillin MICs of ≤2 mg/liter are susceptible to the standard dosage and that isolates with MICs of ≥8 mg/liter are susceptible to high-dose therapy (14). However, CLSI (15) and EUCAST (14) both use ECOFF to define susceptibility and divide the rPBP3 population by categorizing strains with ampicillin MICs of ≥1 mg/liter (EUCAST) or > 2 mg/liter (CLSI) as resistant irrespective of dosage. In addition, susceptibility testing is associated with uncertainty due to technical and biological variation. For broth microdilution (BMD), a precision of ±1 dilution is accepted (16). Thus, categorization of H. influenzae by MIC-based susceptibility to ampicillin will in some cases lead to categorization errors, even with reference methods.

Gradient tests and disk diffusion are commonly used for routine susceptibility testing (16). Several studies have indicated that gradient tests may be unreliable for H. influenzae and aminopenicillins (17–21). Irrespective of method, different media for susceptibility testing of H. influenzae to ampicillin may affect the results.
MATERIALS AND METHODS

Bacterial isolates. The test population consisted of 154 bla-negative H. influenzae isolates from a previous investigation (9) and comprised 50 (32%) R51H+, N52K+, and S385T-positive (rPBP3) isolates and 104 (68%) group II low-rPBP3 isolates (N52K+, positive and S385T negative). All rPBP3 isolates were susceptible to ampicillin (MIC range, 0.06 to 1 mg/liter), amoxicillin (MIC range, 0.125 to 2 mg/liter), and cefuroxime (MIC range, 0.125 to 1 mg/liter) by previous HTM (Oxoid, Basingstoke, United Kingdom) broth microdilution (BMD) MICs interpreted with breakpoints from CLSI (15) and EUCAST (14) breakpoints as the gold standards, and (ii) to evaluate selected beta-lactam disks as screening for the rPBP3 genotype in H. influenzae.

(These data were presented in part [PG1, PV10, and CEC30] at the 21st European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Milan, Italy, 2011 [26]).

Gradient MIC (Etest). Ampicillin, amoxicillin, and cefuroxime MICs were determined by Etest (bioMérieux, Marcy-l’Étoile, France) according to the manufacturer’s recommendations (in-house HTM; MHA from Oxoid). Essential agreement (±1 dilution), categorical agreement (S, I, and R), error rates (very major error [VME], major error [ME], and minor error [mE]) and falsely susceptible rates (FSR; proportions of R isolates categorized as S) were calculated with previously determined BMD MICs (9) interpreted according to CLSI (15) and EUCAST (14) clinical MIC breakpoints as the gold standards. For amoxicillin, “essential correlation” (defined as an amoxicillin MIC within ±1 dilution of the amoxicillin MIC + 1 dilution) and “categorical correlation” (identical categorization of amoxicillin and ampicillin according to MIC breakpoints) were also calculated. Because CLSI has not defined breakpoints for amoxicillin (15), categorical correlation was calculated only with EUCAST breakpoints (14).

Disk diffusion (EUCAST). Disk diffusion (in-house MH-F; MHA from Oxoid) was performed with EUCAST methods (23) and nine disks (Oxoid): 1 unit benzylpenicillin (PG1), 5 units benzylpenicillin (PG5), 10 μg phenoxymethylpenicillin (PV10), 2 μg ampicillin (AMP2), 2/1 μg amoxicillin-clavulanic acid (AMC3), 20/10 μg amoxicillin-clavulanic acid (AMC30), 30 μg cefaclor (CEC30), 5 μg cefuroxime (CXM5), and 30 μg cefuroxime (CXM30). All disks were evaluated for their ability to identify isolates with the rPBP3 genotype (N52K positive). Sensitivity and specificity at different zone breakpoints were used to construct receiver operating characteristic (ROC) curves. The disks were compared by sensitivity, specificity, and positive and negative predictive values with optimized screening breakpoints, i.e., the zone breakpoints resulting in the highest proportions of correct results.

Categorization was performed according to current EUCAST recommendations (14), using standard disks (AMP2 and CXM30) and zone breakpoints, and susceptibility to amoxicillin inferred from ampicillin. Categorization of susceptibility to amoxicillin, amoxicillin, and cefuroxime was also performed by using alternative disks (AMC3 and CXM5) and zone breakpoints and by using the PG1 disk and EUCAST screening breakpoints (14). Categorical agreement, error rates, and FSR for disk diffusion were calculated with previously determined BMD MICs (9) interpreted according to EUCAST clinical MIC breakpoints (14) as the gold standard (CLSI, not applicable). The results were compared with those obtained by Etest, and significance levels were calculated using the chi-square test and software at www.medcalc.net (MedCalc Software, Ostend, Belgium).

Quality control. All results for the reference strains H. influenzae ATCC 49247 and H. influenzae ATCC 49766 (MICs) and H. influenzae NCTC 8468 (zones) were within accepted ranges (15, 27).

RESULTS

MIC determination and susceptibility categorization (Etest). By Etest, 94.2% (145/154) of the isolates had ampicillin MICs of ≤1 mg/liter and were categorized as susceptible with both guidelines; 5.2% (8/154) had ampicillin MICs of 2 mg/liter and were categorized as intermediate (CLSI) or resistant (EUCAST); one isolate had a MIC of >2 mg/liter and was categorized as resistant. The corresponding proportions with BMD were 61.0%, 24.0%, and 14.9%. Essential correlation between ampicillin and amoxicillin MIC was 93.5% (144/154) by Etest and 86.4% (133/154) by BMD. With EUCAST breakpoints for ampicillin and amoxicillin (CLSI, not applicable), categorical correlation between the two agents was 88.3% (136/154) by Etest, compared to 89.6% (138/154) by BMD.

Table 1 shows correlations between Etest and BMD. Etest generally overestimated MICs at lower ranges and underestimated MIC at higher ranges, and agreement rates were low for all three agents. On average, Etest underestimated amoxicillin MIC by one dilution for isolates with BMD MICs of 2 mg/liter, by two dilutions for isolates with BMD MICs of 4 mg/liter, and by three dilutions for isolates with BMD MICs of 8 mg/liter, leading to high VME and FSR with both MIC guidelines. In contrast to ampicillin and amoxicillin, low categorical agreement rates with cefuroxime Etest (both guidelines) were caused mainly by high proportions of minor errors; however, FSR were high with CLSI breakpoints.

Susceptibility categorization (disk diffusion). Zone-MIC correlations showed considerable overlapping of susceptible and resistant isolates with AMP2, AMC3, CXM30, and CXM5 (see Fig. S1 in the supplemental material). Table 2 summarizes the performances of these four disks and PG1 for categorization of susceptibility to ampicillin, amoxicillin, and cefuroxime.

With current EUCAST zone breakpoints, categorical agreement was slightly higher with AMC3 than AMP2 (P = 0.1081), with significantly lower VME (P = 0.0047) and FSR (P = 0.0001). AMC3 was also superior to ampicillin Etest for categorization of susceptibility to amoxicillin. A minor zone breakpoint adjustment (+2 mm) for AMP2 slightly increased agreement with the BMD MIC for categorization of susceptibility to ampicillin (P = 0.1593) and amoxicillin (P = 0.0464). With adjusted zone breakpoints for AMP2 and AMC3 (+1 mm), there were no significant differences between the disks, and both were superior to Etest for categoriza-
TABLE 1  Categorization of susceptibility of beta-lactamase-negative isolates \((n = 154)\) to ampicillin, amoxicillin, and cefuroxime by Etest (HTM)\(^a\)

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\(^a\) HTM broth microdilution (BMD) MICs were interpreted according to breakpoints from CLSI (15) and EUCAST (14) as gold standards.

\(^b\) Difference between Etest and BMD MIC, expressed as the number of 2-fold dilutions by which the Etest MIC is higher (positive values) or lower (negative values) than BMD MIC. CA, categorical agreement between Etest MIC and BMD MIC (breakpoints indicated by vertical lines). mE, minor error (intermediate by Etest and susceptible/resistant by BMD MIC or susceptible/resistant by Etest and intermediate by BMD MIC); ME, major error (resistant by Etest and susceptible by BMD MIC). VME, very major error (susceptible by Etest and resistant by BMD MIC).

\(^c\) EA, essential agreement (proportion of Etest MICs within ± 1 dilution of BMD MIC) calculated by summarizing the values shown in bold in the adjacent column.

\(^d\) FSR, falsely susceptible rate: proportion of isolates resistant by BMD MIC categorized as susceptible by Etest. FSRs are calculated by dividing the number of VMEs ("n" column) by the number of isolates with BMD MICs above the resistance breakpoint (indicated by vertical lines).

\(^e\) Includes Etest MICs deviating from BMD MIC by more than three dilutions.
In recent years, altered PBP3 has surpassed beta-lactamase as the most frequent beta-lactam resistance mechanism in *H. influenzae*. Detection of susceptibility to amoxicillin was substantially reduced when hazy growth was ignored. For rPBP3 isolates, in particular with PG1 and CEC30; sensitivity and 2 most frequent beta-lactam resistance mechanism in recent years, altered PBP3 has surpassed beta-lactamase as the most frequent beta-lactam resistance mechanism in *H. influenzae*. In addition, categorical correlation between AMP2 (ampicillin) and AMC3 (amoxicillin) increased from 79.9% (123/154) with current EUCAST breakpoints to 90.9% (140/154) with adjusted zone breakpoints (*P* = 0.0121) (see Fig. S2 in the supplemental material).

For cefuroxime, categorical agreement with both disk potentials (and the PG1 screening disk) was poor and not significantly different from that obtained with Etest (EUCAST breakpoints). FSR and VME were significantly higher with CXM30 than Etest (and PG1), whereas there were no significant differences between Etest and CXM5 or between CXM5 and CXM30.

**Screening (disk diffusion).** The relative performance of the nine evaluated disks is shown in Fig. 1, and the correlation between zones and resistance genotype for selected disks is shown in Fig. S3 in the supplemental material. The optimized screening breakpoint for PG1 (5 ± 12 mm) was identical to the breakpoint recommended by EUCAST. PG1 identified PBP3 strains with the highest sensitivity and accuracy of all disks tested. CXM5 was superior to the other four disks with agents that are stable in the presence of beta-lactamase (Table 3).

Hazy growth within inhibition zones was frequently observed for rPBP3 isolates, in particular with PG1 and CEC30; sensitivity was substantially reduced when hazy growth was ignored.

**DISCUSSION**

In recent years, altered PBP3 has surpassed beta-lactamase as the most frequent beta-lactam resistance mechanism in *H. influenzae*.
in several geographical regions (6, 8–11), and reliable detection and susceptibility categorization of this organism have become increasingly important.

Commonly used acceptance criteria are >90% essential agreement for MIC determination and <1.5% VME and <3% ME for susceptibility categorization (16). Notably, VME and ME (and categorical agreement) are calculated with the complete test population as the denominator and thus strongly influenced by the proportion of resistant isolates (prevalence). These parameters may be used for comparison of methods for susceptibility testing with identical populations, but the usefulness of acceptance criteria based on defined categorical error rates without taking into account the representativeness of the test population is highly debatable. In contrast to VME, the falsely susceptible rate (FSR) is independent of prevalence and suitable for comparison of results obtained with different methods and test populations.

It should also be noted that categorical errors are dependent on MIC breakpoints and that rates achieved with different guidelines may not be compared.

In the present study, using a test population with 67.5% rPBP3 isolates, neither essential nor categorical agreement rates with Etest exceeded 70% for any of the tested agents. VME were particularly frequent for amoxicillin, and most resistant isolates were wrongly categorized as susceptible (both guidelines). Poor performance of gradient tests for susceptibility testing of *H. influenzae* to amoxicillin. The results may not be fully representative for susceptibility testing of *H. influenzae*. Using identical test populations, disk diffusion with current EUCAST breakpoints was non-inferior (AMP2) or superior (AMC3) to Etest for categorization of amoxicillin-susceptible isolates; with minor adjustments in zone breakpoints, both disks were superior to Etest. In a previous evaluation, Søndergaard et al. tested 135 bla-negative *H. influenzae* isolates (33% rPBP3) and reported that six isolates were ampicillin resistant by both EUCAST disk diffusion (MH-F medium) and HTM BMD (MIC > 1 mg/liter), whereas 10% (i.e., 13 or 14 isolates) were resistant by BMD only (29). Thus, more than half (7/13 or 8/14) of the ampicillin-resistant isolates were falsely categorized as susceptible with the AMP2 disk, compared to 76.7% (10/13) of the isolates (19). Finally, Tristram tested 15 bla-negative rPBP3 *H. influenzae* isolates with Etest and M.I.C.E., and despite 90% essential agreement, 47% (14/30) of ampicillin gradient MICs were 1 to 2 dilutions lower than the HTM BMD MIC (28).

To our knowledge, this is the first study comparing gradient MIC and EUCAST disk diffusion with reference methods for susceptibility testing of *H. influenzae*. Using identical test populations, disk diffusion with current EUCAST breakpoints was non-inferior (AMP2) or superior (AMC3) to Etest for categorization of susceptibility to amoxicillin, and AMC3 performance was significantly better than the other methods for categorization of resistance to amoxicillin. These results are consistent with previous studies comparing gradient tests for susceptibility testing of *H. influenzae*. In a more recent multicenter study by Kärpänoja et al., two ampicillin-susceptible and three ampicillin-resistant isolates were tested with Amp2 and HTM (24). Using the same interpretative criteria as Zerva et al. (S ≥ 16 mm and R < 16 mm) (25), the authors observed an overall FSR of 8% (sensitivity, 92%);
however, the individual FSR for one of the ampicillin-resistant strains (reference MIC = 8 mg/liter) was 24%. Higher frequencies of errors would be expected with clinical strains.

The observed 90% correlation between ampicillin and amoxicillin BMD MIC supports current recommendations that ampicillin may be used to infer susceptibility to amoxicillin (14, 15) and that ampicillin-sulbactam may be inferred from amoxicillin-clavulanic acid (14). Nevertheless, categorization of susceptibility to aminopenicillins with and without bla inhibitors should ideally be based on the same agent irrespective of bla status. With minor zone breakpoint adjustments, we found excellent correlation between AMP2 and AMC3, and AMC3 was noninferior to AMP2 for categorization of susceptibility to aminopenicillins, consistent with previous observations with HTM (24). Thus, AMC3 may be used for categorization of bla-negative as well as bla-positive H. influenzae isolates. Although the low antibacterial activity of clavulanic acid against H. influenzae (MIC range, 25 to 125 mg/liter) (30) is unlikely to affect bla-negative isolates by broth dilution with a fixed concentration at 2 mg/liter (14), the question of whether separate zone breakpoints are needed for bla-positive versus bla-negative isolates should be investigated.

The poor categorical agreement and high frequency of falsely susceptible results by categorization of rPB3 H. influenzae to aminopenicillins by disk diffusion and Etest observed in the present study are worrisome. The main reason for the discrepancy between AMP2 and the reference method was poor separation between rPB3 isolates with ampicillin BMD MICs of 1 mg/liter (susceptible) and 2 mg/liter (nonsusceptible). This observation is not surprising, as these isolates belong to the same population. Slightly improved performance with minor zone breakpoint adjustments may possibly reflect that susceptibility testing is method dependent. Differences in disks and media from different manufacturers may greatly affect the results, but such variation was not investigated in the present study. Further investigations are needed to decide whether a change in breakpoints is advisable. The data suggest that a change in ampicillin MIC breakpoints to avoid division of the rPB3 population would further improve categorical agreement with BMD MIC for both disk diffusion and Etest (data not shown). Definition of an intermediate category encompassing bla-negative isolates with ampicillin MICs up to 8 mg/liter is supported by PK/PD calculations (14); however, any changes in MIC breakpoints should be supported by clinical data, but such data are currently lacking (13).

With current breakpoints and methods for phenotypic susceptibility categorization, a useful approach to reduce errors is to screen for isolates with resistance mechanisms. A highly sensitive screening method practically eliminates ME, as wild-type isolates may be reported as being susceptible to beta-lactams without further testing. The PG1 disk recommended for screening by EUCAST (14) correctly categorized 95.5% of the isolates according to resistance genotype in the present study. Søndergaard et al. found similar accuracy (96%) but lower sensitivity (91%) using the same method (29). Diverging results may be due to different interpretation of hazy growth (24); this phenomenon may vary with MHA from different manufacturers (unpublished data). Notably, PG1 with current screening breakpoints was noninferior to Etest and superior to CXM30 for S/I/R categorization of susceptibility to cefuroxime (EUCAST breakpoints). As PG1 is unsuitable for rPB3 screening in bla-positive strains, we evaluated five disks with agents that are stable in the presence of beta-lactamase. The CXM5 disk categorized isolates according to resistance genotype with the highest accuracy (92.2%) and was superior to the previously evaluated CEC30, CXM30, AMC3, and AMC30 disks (29, 31); however, CXM5 is currently not available from all manufacturers.

In accordance with guidelines from the Nordic Committee on Antimicrobial Susceptibility Testing (NordicAST) (32), we suggest that H. influenzae isolates that are rPB3 positive by screening be categorized as cefuroxime resistant and always be reported as ampicillin resistant in cases of meningitis. In addition, to minimize the clinical consequences of falsely susceptible results, we suggest adding a comment recommending high-dose aminopenicillin therapy or the use of other agents in severe infections caused by screening-positive isolates categorized as susceptible to aminopenicillins by disk or gradient diffusion.

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