Detection of N526K-substituted penicillin-binding protein 3 conferring low-level mutational resistance to β-lactam antibiotics in Haemophilus influenzae by disc diffusion testing on Mueller-Hinton agar according to EUCAST guidelines

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Received 24 November 2011; returned 11 January 2012; revised 26 January 2012; accepted 8 February 2012

Objectives: EUCAST has recently authorized a new disc diffusion test for routine antimicrobial susceptibility testing of Haemophilus influenzae, calibrated to EUCAST MIC breakpoints. We investigated whether disc diffusion testing as recommended by EUCAST could discriminate strains of H. influenzae carrying the N526K substitution in penicillin-binding protein 3 from the wild-type population.

Methods: A total of 170 recent clinical isolates, genetically characterized for the presence of acquired and mutational resistance mechanisms, were tested by disc diffusion of β-lactam antibiotics on supplemented Mueller–Hinton agar. Tentative epidemiological breakpoint values for the presence of the N526K substitution were suggested for various β-lactams, and the performances were calculated.

Results: Epidemiological cut-off values of 19/20 mm for ampicillin (2 μg) and 11/12 mm for benzylpenicillin (1 U) accurately categorized 96% of the study strains, and outperformed cephalosporin-containing discs in the discrimination of mutational resistance in β-lactamase-non-producing isolates. Current EUCAST interpretative criteria for the categorization of clinical resistance showed concordance between resistance rates based on MIC and zone diameter breakpoints for both ampicillin and cefuroxime, but categorization of individual isolates was not consistent.

Conclusions: Disc diffusion testing of H. influenzae accurately identified β-lactamase-non-producing isolates with the N526K substitution by use of discs containing low amounts of penicillins. Cephalosporin-containing discs could detect mutational resistance in β-lactamase-producing isolates, but performed with reduced specificity.

Keywords: ampicillin resistance, susceptibility testing, ftsI

Introduction

Resistance to β-lactam antibiotics in Haemophilus influenzae is mediated by two separate mechanisms: production of constitutively expressed β-lactamases encoded on plasmids, and mutations of the β-lactam targets, the penicillin-binding proteins (PBPs).1 Production of β-lactamase usually confers high-level resistance to penicillins, and the mechanism can be confirmed by chromogenic assays or by overcoming of resistance with clavulanic acid.

Decreased susceptibility to β-lactams can also arise from mutations in PBP3,2,3 which is the sole essential PBP in H. influenzae.4 Substitution of lysine for asparagine-526 (N526K) results in a 2-fold increase in the MIC of ampicillin, and a 2- to 8-fold increase in resistance to various cephalosporins,5 typically conferring intermediate resistance (MIC = 2 mg/L) or resistance (MIC > 2 mg/L) to cefuroxime, according to current EUCAST clinical breakpoints.5 High-level mutational resistance to β-lactam antibiotics in H. influenzae is associated with additional substitutions in PBP3,2,3 but isolates of this genotype have only been sporadically detected in Europe.6

The N526K substitution in PBP3 may be present in 10% of clinical isolates in Europe,6,7 however, the identification and categorization of such strains is a challenge to the clinical microbiology laboratory. In 2011, EUCAST authorized a new disc diffusion test for routine antimicrobial susceptibility testing of H. influenzae using supplemented Mueller-Hinton agar.5 In the present study we tested the susceptibility of 170 recent clinical isolates...
to seven β-lactams by disc diffusion according to EUCAST guidelines, and correlated the results with mutations in the ftsI gene encoding PBP3, as well as MIC data obtained by the broth microdilution method.

**Methods**

**Bacterial isolates**

A total of 170 recent clinical isolates of *H. influenzae* were selected from an earlier study. Isolates were classified according to expression of β-lactamase and to the presence of the N526K substitution in PBP3: 93 strains were negative for both resistance mechanisms, 44 strains were positive for the N526K substitution only, 32 expressed β-lactamase only and 1 strain was positive for both resistance mechanisms. Two strains without acquired resistance could not be cultured on Mueller–Hinton agar plates with 5% horse blood and 20 mg/L nicotinamide adenine dinucleotide (MH-F), and the results of antimicrobial susceptibility testing were restricted to the 168 isolates that were culturable on MH-F.

**Determination of resistance mechanisms**

PCR amplification of the penicillin-binding locus of ftsI was carried out as described previously, and translated amino acid sequences were compared with the corresponding fragment of PBP3 from *H. influenzae* strain Rd. The N526K mutation was found in 45 strains, no strain was positive for group I or group III mutations as defined by Ubukata et al. and 33 strains produced β-lactamases as assessed by a chromogenic assay using nitrocefin as the substrate (BBL DrySlide; Becton–Dickinson, Franklin Lakes, NJ, USA). All β-lactamase-producing strains harboured the blatem-1 gene as revealed by multiplex PCR, or, in the case of absent or aberrant amplicons, by use of the internal primers TEM-1.338f (ATGGCATGACAGTAAGAGAATTATGC; ‘MGTEM101R’ in a previous study) and TEM-1.834r (TATCTAGCGATCTGTCTATTT; this study).

**Antimicrobial susceptibility testing and categorization of clinical resistance**

Two MH-F agar plates were inoculated with each strain according to EUCAST recommendations, and three or four paper discs containing ampicillin (2 μg), benzylpenicillin (1 U), phenoxymethylpenicillin (10 μg), cefaclor (30 μg), cefoxitin (30 μg), cefpodoxime (10 μg) and cefuroxime (30 μg) (Oxoid Ltd, Basingstoke, UK), were placed on each plate using an applicator. After incubation for 18 h at 35°C in a humidified atmosphere containing 5% CO₂, inhibition zones were measured using a ruler. MICs of ampicillin and cefuroxime were determined by the broth microdilution method using 2-fold serial dilutions of the drugs in Haemophilus Test Medium (HTM; TREK Diagnostic Systems, East Grinstead, UK) according to the CLSI method. Categorization of clinical resistance was performed according to EUCAST interpretative criteria, version 2.0 (1 January 2012).

**Results and discussion**

**Discrimination of mutational resistance by disc diffusion testing**

The results obtained with disc diffusion on MH-F agar using the different β-lactams are summarized in Table 1. Inhibition zones for the 168 isolates grown on MH-F were related to the presence or absence of the N526K mutation; for the penicillin class of antibiotics, the results are restricted to the 135 isolates negative for β-lactamase production. Suggested epidemiological cut-off (ECOFF) values to separate the wild-type phenotype (without acquired or mutational resistance mechanisms) from the non-wild-type are given in Table 1; these values were chosen to obtain optimal accuracy combined with sensitivity >85%. Positive and negative predictive values were calculated, assuming a true prevalence of 10% of the N526K mutation among clinical isolates of *H. influenzae*.

With the exception of cefpodoxime, disc diffusion testing was able to categorize 90%–96% of the isolates correctly. The

**Table 1.** AUCs, suggested ECOFF values and derived parameters for β-lactam antibiotics on MH-F plates; EUCAST ECOFFs are included for comparison

<table>
<thead>
<tr>
<th></th>
<th>Ampicillin, 2 μg</th>
<th>Penicillin G, 1 U</th>
<th>Penicillin V, 10 μg</th>
<th>Cefaclor, 30 μg</th>
<th>Cefoxitin, 30 μg</th>
<th>Cefpodoxime, 10 μg</th>
<th>Cefuroxime, 30 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>0.990</td>
<td>0.991</td>
<td>0.961</td>
<td>0.951</td>
<td>0.942</td>
<td>0.906</td>
<td>0.966</td>
</tr>
<tr>
<td>Suggested ECOFF (mm)</td>
<td>19/20</td>
<td>11/12</td>
<td>16/17</td>
<td>20/21</td>
<td>20/21</td>
<td>31/32</td>
<td>26/27</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.91</td>
<td>0.91</td>
<td>0.86</td>
<td>0.89</td>
<td>0.89</td>
<td>0.89</td>
<td>0.91</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.90</td>
<td>0.92</td>
<td>0.79</td>
<td>0.90</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>0.93</td>
<td>0.93</td>
<td>0.92</td>
<td>0.50</td>
<td>0.55</td>
<td>0.32</td>
<td>0.50</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>0.99</td>
<td>0.99</td>
<td>0.98</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Very major errors (N526K not identified)</td>
<td>0.030</td>
<td>0.030</td>
<td>0.044</td>
<td>0.030</td>
<td>0.030</td>
<td>0.030</td>
<td>0.024</td>
</tr>
<tr>
<td>Major errors (N526K erroneously identified)</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td>0.071</td>
<td>0.060</td>
<td>0.156</td>
<td>0.071</td>
</tr>
<tr>
<td>Accuracy</td>
<td>0.96</td>
<td>0.96</td>
<td>0.95</td>
<td>0.90</td>
<td>0.91</td>
<td>0.81</td>
<td>0.90</td>
</tr>
<tr>
<td>EUCAST ECOFF (mm)</td>
<td>15/16</td>
<td>11/12</td>
<td>14/15</td>
<td>18/19</td>
<td>—</td>
<td>—</td>
<td>25/26</td>
</tr>
</tbody>
</table>

Note: Only β-lactamase-negative strains (*N* = 135); penicillin G, benzylpenicillin; penicillin V, phenoxymethylpenicillin.

Positive/negative categorization for presence of resistance mechanism.

Assuming an N526K prevalence of 10% among all strains and 12% among β-lactamase-negative isolates.
performance of the different drugs can be described by the area under the receiver operating characteristic (‘ROC’) curve (AUC), which is independent of the prevalence and corresponds to the general accuracy of the method. Excellent performance was observed using ampicillin (2 µg) or benzylpenicillin (1 U), with AUC values of 0.990 and 0.991, respectively, corresponding to a categorization accuracy of 96% for this strain collection (Table 1). Cefaclor, cefoxitin and cefuroxime (30 µg each) showed acceptable performance, with AUC values in the range of 0.942–0.966, and accurately categorized 90%–91% of the tested strains. However, these cephalosporins performed with modest specificity (90%–92%), which resulted in positive predictive values of only 50%–55% when testing clinical material with an expected true prevalence of the N526K substitution of 10%.

Approximately 80%–85% of *H. influenzae* clinical isolates do not produce a β-lactamase,7,8 and disc diffusion testing using discs with low amounts of a penicillin could accurately discriminate isolates with the N526K substitution in this population. Identification of mutational resistance in β-lactamase-producing isolates was more challenging. The discs containing 30 µg of cefalosporin performed with decreased specificity compared with discs with a low content of a penicillin (Table 1). Furthermore, the N526K mutation may be less prevalent in β-lactamase-producing isolates,5,9 thus further decreasing the positive predictive value of the test. Finally, the ROB-1 β-lactamase showed some activity against cephalosporins, particularly cefaclor.8 Although this enzyme is rare in Europe, ROB-1-containing isolates may not reveal the simultaneous presence of the N526K mutation by disc diffusion testing using cephalosporins. Discs containing low amounts (1–2 µg) of amoxicillin in combination with clavulanic acid could potentially be used for the detection of N526K in β-lactamase-producing isolates and should be tested in future studies.

**Determination of clinical resistance**

The resistance rates according to EUCAST zone diameter and MIC breakpoints for determination of clinical resistance in *H. influenzae* are shown in Table 2. From 1 January 2012, EUCAST has changed the zone diameter breakpoint (S=R=C) for cefuroxime from 25/22 to 26/25 mm. With the present collection of strains, the new disc diffusion breakpoint increases the clinical resistance rate of cefuroxime from 9% to 23%. Similar resistance rates to ampicillin and cefuroxime were then determined by disc diffusion and broth microdilution (Table 2). However, categorization of individual isolates was inconsistent: of 12 isolates categorized as resistant to ampicillin by disc diffusion, only 6 were also categorized as resistant by MIC determination. The EUCAST clinical breakpoints of ampicillin and cefuroxime are close to the MICs for strains with mutational resistance to β-lactams, rendering antibiotic susceptibility testing subject to day-to-day variation.

**Algorithm for detection of resistance**

The Nordic committee for antibiotic susceptibility testing (NordicAST) has devised an algorithm for the detection of resistance to β-lactam antibiotics.10 The algorithm is based on EUCAST disc diffusion testing using an initial screening with benzylpenicillin (1 U); isolates with inhibition zones ≥12 mm are considered free of mutational or acquired resistance mechanisms and are categorized as susceptible to ampicillin, amoxicillin, co-amoxiclav, cefuroxime, ceftriaxone and carbapenems. Isolates with benzylpenicillin inhibition zones <12 mm are tested for the presence of β-lactamase; non-producing isolates are considered positive for mutational resistance and categorized as resistant to cefuroxime, while susceptibility to other β-lactam antibiotics is assessed by MIC determination. β-Lactamase-producing isolates are classified according to the cefaclor (30 µg) inhibition zone, and isolates with zones ≤23 mm are considered positive for mutational resistance and categorized as resistant to cefuroxime, while susceptibility to co-amoxiclav, ceftriaxone and carbapenems is assessed by MIC determination.

The suggested ECOFF value for benzylpenicillin (1 U) presented in Table 1 is identical to the screening breakpoint proposed by NordicCAST.10 The same breakpoint has also been introduced in version 2.0 of EUCAST interpretative criteria, where screening with benzylpenicillin has replaced screening with phenoxymethylpenicillin. Using this breakpoint on the present collection, 94 isolates were classified as free of mutational or acquired resistance mechanisms, and 74 as not wild-type. All of the 33 β-lactamase-producing strains were identified by the breakpoint, as were 40 of 44 β-lactamase-non-producing strains with the N526K substitution in PBP3. A single wild-type isolate was categorized with the resistant population. In total, the proposed benzylpenicillin breakpoint predicted mutational or acquired resistance mechanisms with a sensitivity of 95%, and wild-type susceptibility with a specificity of 99%.

Using our suggested breakpoint, categorization of clinical resistance to cefuroxime in strains harbouring the N526K substitution will reduce inconsistent categorizations of resistance to this drug by disc diffusion.

**Funding**

This study was supported in part by a grant from the Scandinavian Society for Antimicrobial Chemotherapy (SSAC) to N. N.-L.
Transparency declarations
K. F. has received lecture fees and sponsorship from Pfizer. N. N.-L. has received sponsorship from Pfizer. A. S. and M. T. P.: none to declare.

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5 EUCAST. http://www.eucast.org/clinical breakpoints/ (14 January 2012, date last accessed).