Evaluation of the EUCAST disc diffusion susceptibility testing method for *Haemophilus influenzae* based on the resistance mechanism to β-lactam antibiotics

S. García-Cobos¹, M. Arroyo¹, M. Pérez-Vázquez¹, B. Aracil¹, J. Oteo¹ and J. Campos¹,²*

¹Antibiotic Laboratory, Bacteriology Service, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; ²Consejo Superior de Investigaciones Científicas, Madrid, Spain

*Corresponding author: Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera Pozuelo a Majadahonda, 28220 Majadahonda, Madrid, Spain. Tel: +34 918223650; Fax: +34915097966; E-mail: jcampos@isciii.es

Received 15 June 2012; returned 17 July 2012; revised 1 August 2012; accepted 22 August 2012

Objectives: EUCAST developed an antibiotic susceptibility testing method for *Haemophilus influenzae*. We assessed the EUCAST testing method and EUCAST clinical breakpoints and newly proposed epidemiological cut-off values against *H. influenzae* clinical isolates with known molecular mechanisms of resistance to β-lactam antibiotics.

Methods: In total, 89 clinical isolates were used: 30 were β-lactamase negative with PBP3 mutations (gBLNAR), 20 were β-lactamase positive without PBP3 mutations (gBLPAR), 15 were β-lactamase positive with PBP3 mutations (gBLPACR), and 24 were β-lactamase negative without resistance mechanism (gBLNAS). Twelve different β-lactam antibiotics and disc charges were tested.

Results: None of the discs tested fully separated between gBLNAS and gBLNAR populations. According to EUCAST clinical zone diameter breakpoints, overall the best values of sensitivity and specificity were obtained with cefuroxime 30 μg and amoxicillin/clavulanic acid 2/1 μg discs for detection of gBLNAR and gBLPACR populations, although a previous β-lactamase test was needed. Other antibiotic discs could be suitable for epidemiological purposes, such us penicillin 10 U for separating gBLNAR isolates and cefoxitin 30 μg for detection of gBLPACR isolates. By Etest using the EUCAST method, the EUCAST MIC clinical breakpoints for ampicillin and amoxicillin/clavulanic acid showed high specificity, but low sensitivity, for the detection of genotypes with mutations in PBP3.

Conclusions: The main genotypes of β-lactam-resistant *H. influenzae* can be separated by using the EUCAST disc diffusion method, although it should be noted that overlapping between populations with and without PBP3 mutations is common.

Keywords: β-lactamases, gBLNAR, PBP3

Introduction

*Haemophilus influenzae* is a human pathogen that causes invasive and respiratory infections in children and adults. Resistance or decreased susceptibility to β-lactam antibiotics in this pathogen is mostly due to β-lactamase production and decreased PBP3 affinity (BLNAR phenotype); both mechanisms can be found in the same isolate.¹

In vitro detection of aminopenicillin resistance due to the BLNAR phenotype may be challenging because strains with altered PBP3 exhibit a range of ampicillin MICs that cluster around the resistance breakpoint.²,³ Different growth media have been developed for susceptibility testing of *H. influenzae*, with Haemophilus Test Medium (HTM), which was adopted by the CLSI,⁴,⁵ being the reference culture method.

EUCAST has proposed a disc diffusion testing method for *H. influenzae*.⁶ The aim of this study was to evaluate the *H. influenzae* EUCAST disc susceptibility testing method for β-lactam antibiotics by challenging it with a well-characterized collection of clinical isolates with known mechanisms of resistance.⁷ We studied whether the EUCAST method could separate *H. influenzae* resistance classes using either EUCAST clinical breakpoints or newly proposed epidemiological cut-off values.
Materials and methods

Test isolates

We used a study collection of 89 *H. influenzae* clinical isolates for which the resistance mechanisms to β-lactam antibiotics have been determined previously by our group. In the collection, four genotypes of ampicillin susceptibility were represented: 30 isolates were β-lactamase negative, but had mutations in their *ftsI* gene causing reduced susceptibility to ampicillin (genotype gBLNAR); 20 isolates were β-lactamase positive and ampicillin resistant, but had no *ftsI* mutation (genotype gBLPACR); 15 isolates were β-lactamase positive and had mutations in the *ftsI* gene (genotype gBLPACR); and 24 isolates were β-lactamase negative and ampicillin susceptible without resistance mechanism (genotype gBLNAS). All β-lactamase-positive isolates were TEM-1.

Isolates belonging to gBLNAR and gBLPACR genotypes were selected according to their PBP3 amino acid substitutions in such a way that the most common mutation patterns described in European gBLNAS isolates were represented. Four isolates belonged to group I1a, 12 to group I1b (7 gBLNAR and 5 gBLPACR), 22 to group I1c (12 gBLNAR and 10 gBLPACR) and 7 to group III-like.

Susceptibility testing

Disc diffusion

Antibiotic susceptibility testing was performed by the disc diffusion method following the EUCAST guidelines; Mueller–Hinton agar was supplemented with 5% horse blood and 20 mg/L β-NAD (Sigma-Aldrich). Plates were inoculated with samples of each strain and adjusted to a turbidity of 0.5 McFarland with a spectrophotometer (SmartSpec Plus, Bio-Rad). Antibiotic discs were applied to the dried surface of the inoculated agar and further incubated at 37°C for 18 ± 2 h in a 5% CO2 atmosphere.

Antibiotic discs tested and charges were penicillin (10 U), ampicillin (2 and 10 μg), amoxicillin (2 and 10 μg), amoxicillin/clavulanic acid (2/1 and 20/10 μg), cefaclor (30 μg), cefuroxime (30 μg), cefotaxime (30 μg), cefoxitin (5 μg), and cefotaxime (30 μg).

*H. influenzae NCTC 8468* was used as a susceptibility quality control strain, as recommended by EUCAST.

The sizes of inhibition zone diameters were independently read by at least two of the authors. Final inhibition zone diameters (in millimetres) were the averages of the independent readings.

Definitions

In this study the terms clinical breakpoint and epidemiological cut-off value were used according to EUCAST definitions and guidelines.

MIC determination by Etest

The MICs of ampicillin and amoxicillin/clavulanic acid were determined by Etest (AB-Biodisk, Solna, Sweden) using the EUCAST testing method as described previously.

Analysis of results

Genotype determination as determined by PCR amplification and DNA sequencing of *ftsI* and *blaTEM* genes was considered the gold standard method against which the EUCAST disc diffusion method was tested.

Very major errors were defined as results that indicated a lack of modifications in PBP3 (gBLNAS or gBLPAR) by disc diffusion when they were gBLNAR or gBLPACR by the gold standard method. Major errors were classified as results that indicated the presence of mutations in the *ftsI* gene (gBLNAS or gBLPACR) by disc diffusion when they had no mutations in PBP3 (gBLNAS or gBLPAR) by the gold standard method.

Results and discussion

Population distribution of inhibition zone diameters by resistance mechanisms

In general, none of the 12 discs tested fully separated between the gBLNAS and gBLNAR populations (Figure S1, available as Supplementary data at *JAC* Online); due to the high degree of overlap, the following discs were found to be of little use for this purpose: ampicillin 10 μg, amoxicillin 10 μg, amoxicillin/clavulanic acid 20/10 μg, cefaclor 30 μg, cefotaxime 30 μg, and cefoxitin 5 μg. Usually low-chance discs offered better separation between these two populations, but none of them was perfect; the following discs had fewer overlapping zones: penicillin 10 U, ampicillin 2 μg, amoxicillin 2 μg, amoxicillin/clavulanic acid 2/1 μg, and cefuroxime 30 μg, and they were considered for further analysis.

Separation between fully susceptible (gBLNAS) and β-lactamase-producing (gBLPAR and gBLPACR) isolates can be easily accomplished by performing a simple β-lactamase test; however, further separation between the gBLPAR and gBLPACR populations can be challenging, although they could be tentatively distinguished by the amoxicillin/clavulanic acid 2/1 and 20/10 μg, cefuroxime 30 μg, and cefoxitin 30 μg discs (Figure S1, available as Supplementary data at *JAC* Online).

Clinical breakpoints and resistance mechanisms

**gBLNAR detection by the disc method**

The following discs had better balanced values for the gBLNAR identification: ampicillin 2 μg, amoxicillin/clavulanic acid 2/1 μg disc, and cefuroxime 30 μg (Table 1). The clinical ampicillin 2 μg disc breakpoint, currently proposed by EUCAST (R<:16 mm), had a sensitivity of 73.3% and a specificity of 79.2% (Table 1).

**gBLPAR detection by the disc method**

The EUCAST clinical zone diameter breakpoint recommended with the cefuroxime 30 μg disc showed very high values of sensitivity, specificity and positive predictive value (PPV) for the detection of gBLPACR isolates (Table 1).

**gBLNAR and gBLPACR detection by Etest**

All the proposed EUCAST clinical MICs breakpoints have excellent specificity and PPV, but very low sensitivity, and then high very major errors because many gBLNAR isolates had MICs of 1 mg/L for ampicillin or 2 mg/L for amoxicillin by Etest (Table 1) and also by microdilution, as previously published.

**Newly suggested epidemiological cut-offs**

**gBLNAR epidemiological cut-offs**

By decreasing the zone diameter breakpoint for amoxicillin 2 μg to 14 mm, specificity improved to 83.3% (Table 1). Søndergaard et al. suggested an epidemiological cut-off value of 19/20 mm
with amoxicillin 2 μg disc for the detection of isolates with the
NS26-K mutation in ftsI with a specificity of 99%.
A penicillin 10 U (currently not recommended by EUCAST)
breakpoint <24 mm had sensitivity, specificity, and PPVs >85%
(Table 1). A benzylpenicillin disc of 1 U, not tested in this study,
is now being proposed by EUCAST.9
Detection of gBLNAR for epidemiological purposes can also be
improved by using an MIC cut-off value (as determined by Etest)
of R > 1 mg/L for amoxicillin (Table 1).

gBLPACR epidemiological cut-offs
Our tentatively proposed epidemiological cut-off values for
amoxicillin/clavulanic acid 2/1 μg (R < 16 mm) and 20/10 μg
(R < 25 mm) discs had good sensitivity and specificity values
(Table 1). Also, the cefotaxime and cefoxitin discs may have
some interest according to our results (Table 1). Some cephalosporin discs, such as cefaclor, cefoxitin and
cefuroxime (30 μg each), had been proposed for the detection of the ftsI mutation NS26-K.10 In the present study, the cefoxitin
30 μg disc (and a zone diameter breakpoint of 22 mm) was
found to be useful for the detection of gBLPACR isolates (Table 1).

Considering the above data, Figure 1 shows a suggested
easy-to-use algorithm for screening of gBLNAS, gBLNAR,
gBLPACR and gBLPAR populations of H. influenzae using the
EUCAST test method. The algorithm utilizes a β-lactamase test
and a cefuroxime 30 μg disc; other antibiotic discs could also
be used according to data depicted in Table 1. It should be
emphasized that the final detection of resistance genotypes,
such as gBLNAR, requires PCR amplification and DNA sequencing of the ftsI gene.

Conclusions
In this study, we provide several findings of microbiological and
epidemiological interest: (i) the EUCAST proposed susceptibility
testing method for H. influenzae may separate the three most

### Table 1. Detection of gBLNAR and gBLPACR H. influenzae isolates by using currently recommended EUCAST clinical MIC breakpoints and newly suggested epidemiological cut-offs

<table>
<thead>
<tr>
<th>Genotype population</th>
<th>Susceptibility method</th>
<th>Antibiotic (disc charge, μg)</th>
<th>Inhibition zone diameter (mm)/MIC (mg/L)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>Test error (%)</th>
</tr>
</thead>
</table>

#### Using currently recommended EUCAST clinical breakpoints

<table>
<thead>
<tr>
<th>gBLNAR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>disc</th>
<th>AMP (2)</th>
<th>R &lt; 16, S ≥ 16</th>
<th>73.3</th>
<th>79.2</th>
<th>81.5</th>
<th>9.2</th>
<th>14.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>gBLPACR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>disc</td>
<td>AMC (2/1)</td>
<td>R &lt; 25, S ≥ 26</td>
<td>86.9</td>
<td>78.9</td>
<td>86.7</td>
<td>8.3</td>
<td>6.25</td>
</tr>
<tr>
<td>gBLNAR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Etest</td>
<td>AMC</td>
<td>R &gt; 1, S ≤ 1</td>
<td>16.7</td>
<td>100</td>
<td>100</td>
<td>46.3</td>
<td></td>
</tr>
<tr>
<td>gBLPACR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Etest</td>
<td>AMC</td>
<td>R ≤ 2</td>
<td>6.7</td>
<td>100</td>
<td>100</td>
<td>40.7</td>
<td></td>
</tr>
</tbody>
</table>

#### Newly suggested epidemiological cut-offs

<table>
<thead>
<tr>
<th>gBLNAR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>disc</th>
<th>AMX (2)</th>
<th>R &lt; 14, S ≥ 14</th>
<th>90</th>
<th>83.3</th>
<th>87.1</th>
<th>7.4</th>
<th>5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>gBLPACR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>disc</td>
<td>PEN (10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>R &lt; 24, S ≥ 24</td>
<td>86.7</td>
<td>91.7</td>
<td>92.8</td>
<td>3.7</td>
<td>7.4</td>
</tr>
<tr>
<td>gBLNAR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Etest</td>
<td>AMC</td>
<td>R ≥ 1, S ≤ 1</td>
<td>86.7</td>
<td>96</td>
<td>96</td>
<td>1.85</td>
<td>7.4</td>
</tr>
<tr>
<td>gBLPACR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Etest</td>
<td>AMC</td>
<td>R ≥ 1</td>
<td>73.3</td>
<td>90</td>
<td>84.6</td>
<td>5.7</td>
<td>11.4</td>
</tr>
</tbody>
</table>

#### Notes

- gBLNAS (n = 24), β-lactamase negative and amoxicillin susceptible without resistance mechanism; gBLNAR (n = 30), β-lactamase-negative isolates with PBP3 mutations; gBLPAR (n = 20), β-lactamase-positive isolates without PBP3 mutations; gBLPACR (n = 15), β-lactamase-positive isolates with PBP3 mutations; AMP, amoxicillin; AMX, amoxicillin; CEC, cefadroxil; CXM, cefuroxime; AMC, amoxicillin/clavulanic acid; PEN, penicillin; FOX, cefoxitin.

- <sup>a</sup>Calculated for the gBLNAS (n = 24) and gBLNAR (n = 30) populations.
- <sup>b</sup>Calculated for the gBLPAR (n = 20) and gBLPACR (n = 15) populations.
important populations of β-lactam-resistant *H. influenzae*, although inhibition zone diameters of the gBLNAS and gBLNAR populations often overlap; (ii) the clinical breakpoints currently proposed by EUCAST are also useful for the initial distinction between different β-lactam resistance mechanisms; (iii) newly proposed epidemiological cut-offs may improve the screening of β-lactam resistance mechanisms using the EUCAST method; and (iv) in addition to low-charge discs, antibiotic discs of standard charges such as cefuroxime 30 μg may also be useful for clinical and epidemiological purposes.

**Acknowledgements**

We thank all the hospital participants for submitting isolates for the study.

**Funding**

This study was supported by the Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III, the Spanish Network for Research in Infectious Diseases (REIPI C03/14, REIPI RD06/0008) and the Network of Excellence GRACE (PL 518226). S. G.-C. is the recipient of a research contract from Fondo de Investigación Sanitaria (CA09/00031).

**Supplementary data**

Figure S1 is available as Supplementary data at JAC Online (http://www.jac.oxfordjournals.org/).

**References**


