Ampicillin Resistance Mechanisms in Clinical Haemophilus influenzae: What is Happening in Portugal?

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Antibiotic Resistance

- **Antibiotics** are used to prevent and treat bacterial infections

- **Antibiotic resistance** occurs when bacteria change in response to the use of antibiotic treatment

- **Bacteria**, not humans or animals, become antibiotic-resistant. These resistant bacteria may infect humans and animals, and the infections they cause are harder to treat.

- **Antibiotic resistance** leads to higher medical costs, prolonged hospital stays, and increased mortality.
CAUSES OF ANTIBIOTIC RESISTANCE

Antibiotic resistance happens when bacteria change and become resistant to the antibiotics used to treat the infections they cause.

- Over-prescribing of antibiotics
- Patients not finishing their treatment
- Over-use of antibiotics in livestock and fish farming
- Poor infection control in hospitals and clinics
- Lack of hygiene and poor sanitation
- Lack of new antibiotics being developed

www.who.int/drugresistance

#AntibioticResistance
Antibiotic Resistance

Antibiotic Resistance ECDC video

https://vimeo.com/181041782
Antibiotic Resistance

We urgently need to change the way antibiotics are used and prescribed

Even if new antibiotics are developed, without behavior change, antibiotic resistance will remain a major threat.
Haemophilus influenzae

H. influenzae, a human-restricted pathogen, can cause life-threatening infections such as pneumonia, bacteremia, and meningitis.

It is also a key etiological agent of upper and lower respiratory tract infections in both adults and children.

It is classified as encapsulated (serotypes a-f) or no capsulated (NCHi), depending on the presence or absence of a polysaccharide capsule.
Hib has long been a major cause of morbidity and mortality, especially in children ≤5 years old.
In Portugal, Hib vaccination was implemented in NIP in June 2000, for all children of pre-school age (≤5 years old)
- nearly extinction of Hib disease
- increase in non-Hib disease, especially of NCHi in all age groups
Most cases of *H. influenzae* invasive disease occurring in Portugal are now due to fully susceptible NCHi strains

Ampicillin Resistance Mechanisms in Haemophilus influenzae

Beta-lactams have been extensively used in the therapy for *H. influenzae* infection, but, ampicillin-resistant strains have emerged and spread in early 70s

Two major mechanisms are involved in ampicillin resistance

- the enzymatic mechanism: β-lactam hydrolysis due to the production of β-lactamase, either TEM-1 type or ROB-1 type (rarely)

- the non-enzymatic mechanism: decreasing affinity of β-lactams for altered penicillin-binding proteins (PBPs)

Ampicillin Resistance Mechanisms in *Haemophilus influenzae*

Strains exhibiting the non-enzymatic mechanism of resistance are called β-lactamase-nonproducing ampicillin resistant strains (BLNAR) and have been increasingly described worldwide.

Strains possessing both mechanisms, β-lactamase and altered PBPs, are defined as β-lactamase-positive amoxicillin/clavulanic acid-resistant (BLPACR) and seem to be increasing, after their first description in the USA.

BLNAR strains show reduced susceptibility not only to ampicillin but also to other β-lactam antibiotics, particularly cephalosporins.

It is recommended that we should considered these strains resistant to all β-lactam antibiotics, despite apparent in vitro susceptibility.

Ampicillin Resistance Mechanisms in *Haemophilus influenzae*

In the microbiological point of view it is difficult to define BLNAR strains:
- the strain must be β-lactamase negative and have an ampicillin MIC equal or above the CLSI resistant breakpoint (≥4.0 mg/L)
- strains with an MIC of 2.0 mg/L present difficulties, as they fall in the intermediate category
- In addition, there is no international consensus on ampicillin breakpoints; for example, EUCAST has a different resistance breakpoint (≥1.0 mg/L) and other systems have their own resistance breakpoints

Since we are in a World Congress I will consider CLSI breakpoints.

The lack of a consensus and the broad range of ampicillin MICs associated with BLNAR strains is demonstrated by the range of ampicillin breakpoints used in various surveillance studies: from ≥1.0 mg/L to ≥4.0 mg/L. A few researchers start at ≥0.5 mg/L.
### CLSI and EUCAST breakpoints

Breakpoints used to determine susceptible, intermediate, and resistant categories for *H. influenzae* based on CLSI and EUCAST interpretative breakpoints

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>CLSI</th>
<th>EUCAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>$S \leq 1$</td>
<td>$S \leq 1$</td>
</tr>
<tr>
<td>Amoxicillin/Clavulanate</td>
<td>$S \leq 4$</td>
<td>$R \geq 8$</td>
</tr>
</tbody>
</table>
Overlap of MIC ranges according to different resistance mechanisms

# Susceptibilities to β-lactam antibiotics of 240 *H. influenzae* (2001-2008) isolates grouped in genotypes based on the presence/absence of *ftsI* mutations

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Genotype (n)</th>
<th>MIC (mg/L)</th>
<th>Susceptibility class (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC$_{50}$</td>
<td>MIC$_{90}$</td>
<td>MIC-range</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>gBLNAS (66)</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>gBLPAR (33)</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>gBLPACR (47)</td>
<td>128</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>gBLNAR (94)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Amoxicillin/</td>
<td>gBLNAS (66)</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>clavulanate</td>
<td>gBLPAR (33)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>gBLPACR (47)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>gBLNAR (94)</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>
Methods

Determination of Antimicrobial Susceptibility (MiCroSTREP plus Panels) - Minimum Inhibitory Concentration - MIC - mg/L

- Ampicillin
- Amoxicillin/Clavulanate
- Cefepime
- Cefotaxime
- Cefuroxime
- Cefaclor
- Meropenem
- Ciprofloxacin
- Chloramphenicol
- Tetracycline
- Trimethoprim-
- Sulfamethoxazole
- Rifampin
- Azithromycin

Siemens Healthcare Diagnostics Inc. / Beckman Coulter
Methods

- β-lactamase characterization by nitrocefin or PCR (TEM-1 or ROB-1)
- Serotyping: Presence/absence of capsule by PCR according to Falla et al., 1994
- Amplification and Sequencing of *ftsI* gene (1832 bp) according to Cerquetti et al., 2007

Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER (1994); J Clin Microbiol 32 (10):2382-2386
Analysis

Analysis of substitutions in *ftsI* gene

Bioinformatic tools


- *Expasy Translate Tool (Translate nucleotide sequence)* *(http://web.expasy.org/translate/)*

β-lactamase production

Portuguese *H. influenzae* collection- more than 13500 strains, collected since 1989

β-lactamase production have been determined with Nitrocefin and/or PCR multiplex TEM/ROB

- Percentage of β-lactamase producers are ~10%, with variations from year to year
- Most of characterized strains by PCR are TEM-1
- Two strains are ROB-1 producers
- One strain is TEM-2 producer
Groups and subgroups

BLNAR strains can be categorized by sequencing the \textit{ftsI} gene that encodes the transpeptidase region of PBP3; \textit{ftsI H. influenzae} gene have 3 highly conserved amino acid motifs which are essential for function: \textit{STVK- \textit{S}\textsuperscript{327}TVK, SSN- \textit{S}\textsuperscript{379}SN, and KTG- K\textsuperscript{512}TG}

The BLNAR strains are categorized into 3 groups (I, II, and III) based on the presence/absence of different amino acid substitutions in the neighborhood of these conserved PBP3 motifs (Ubukata et al., 2001)

Most isolates had Asp350Asn substitution

In addition:

\textbf{Group I} strains had Arg517His near the conserved Lys-Thr-Gly (KTG) motif

\textbf{Group II} strains had Asn526Lys near the conserved Lys-Thr-Gly (KTG) motif

\textbf{Group III} strains had Met377Ile, Ser385Thr, and Leu389Phe near the conserved Ser-Ser-Asn (SSN) motif, in addition to Asn526Lys

\textit{Ubukata K, Shibasaki Y, Yamamoto K et al.; Antimicrob Agents Chemother 2001; 45: 1693-99}
Groups and subgroups

Group II strains were further divided into subgroups a, b, c, and d, according to the presence of other substitutions (Dabernat et al., 2002)

- Subgroup IIa: the only observed substitution was Asn526Lys.
- Subgroup IIb: Ala502Val, along with other substitutions: Asp350Asn and Gly490Glu, Asp350Asn and Ala437Ser
- subgroup IIc, Ala502Thr
- subgroup IId, Ile449Val

Later, in 2007, group III-like was described (Garcia-Cobos et al., 2007)

Met377Ile e Ser385Thr in SSN motif
Arg517His and Thr532Ser in the KTG motif
Asp350Asn and Ser357Asn in (Ser- Thr- Val-Lys) STVK motif

Antibiotic resistance vs Groups

Strains with decreased ampicillin susceptibility (MIC≥1 mg/L) are commonly found in group I and II.

Strains belonging to group III and group III like are normally associated with high resistance levels to ampicillin, as well as cephalosporins.

# Resistance genotypes

*Haemophilus influenzae* genotypes distribution among the two periods: 2001-2008 and 2009-2011

<table>
<thead>
<tr>
<th>Genotype 2001-2008</th>
<th>N (%)</th>
<th>Genotype 2009-2011</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gBLNAS</td>
<td>66 (27.5%)</td>
<td>gBLNAS</td>
<td>46 (18.5%)</td>
</tr>
<tr>
<td>gBLPAR</td>
<td>33 (13.7%)</td>
<td>gBLPAR</td>
<td>22 (8.9%)</td>
</tr>
<tr>
<td>gBLNAR</td>
<td>94 (39.2%)</td>
<td>gBLNAR</td>
<td>136 (54.9%)</td>
</tr>
<tr>
<td>gBLPACR</td>
<td>47 (19.6%)</td>
<td>gBLPACR</td>
<td>44 (17.7%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>240 (100.0%)</strong></td>
<td><strong>Total</strong></td>
<td><strong>248 (100.0%)</strong></td>
</tr>
</tbody>
</table>


Resistance genotypes: 2001-2008

240 strains
141 with mutations in *ftsI*
44 without mutations in *ftsI*

* Mutations not associated to β-lactams resistance mechanism
Resistance genotypes: 2009-2012

42 BLNAS (MIC<1mg/L)
140 BLNAR (MIC≥1mg/L)
66 BLPAR (MIC≥2mg/L)

18 gBLNAS
24 gBLNAR* 57%
4 gBLNAS
136 gBLNAR 97%
22 gBLPAR
44 gBLPACR 67%

248 strains
180 with mutations in ftsI
68 without mutations in ftsI

* Mutations not associated to β-lactams resistance mechanism
Discussion

Emergence and dissemination of a non-enzymatic resistance mechanism among Portuguese *H. influenzae* clinical isolates: a high percentage of β-lactamase negative strains with reduced susceptibility or resistance to ampicillin carry mutations in the *ftsI* gene (>95%)

High percentage of gBLPACR strains among our BLPAR strains (>50%)

Revision of the ampicillin breakpoints allow a more efficient detection and characterization of BLNAR strains, in the susceptibility routine testing

Ampicillin EUCAST breakpoints are more accurate than CLSI, since intermediate strains are now considered resistant but still that most strains with ampicillin MIC ≥1mg/L are not correctly characterized

Comparing the first study with the second one we observed:

- Increasing of BLNAR and BLPACR resistance genotypes
- Increasing of strains characterized in mutational group III like
- Increasing in genetic diversity
Conclusion

Although a lot of studies have been performed on this subject more studies are needed to establish adequate therapeutic and preventive measures to avoid selection and dissemination of resistant strains.

The inappropriate use of oral antibiotics may be responsible for the selection of this new resistance trait.

We would like to emphasize the importance of continuing surveillance studies as essential tools to define trends in the antimicrobial resistance of Haemophilus influenzae.
Thanks Very Much for your attention