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

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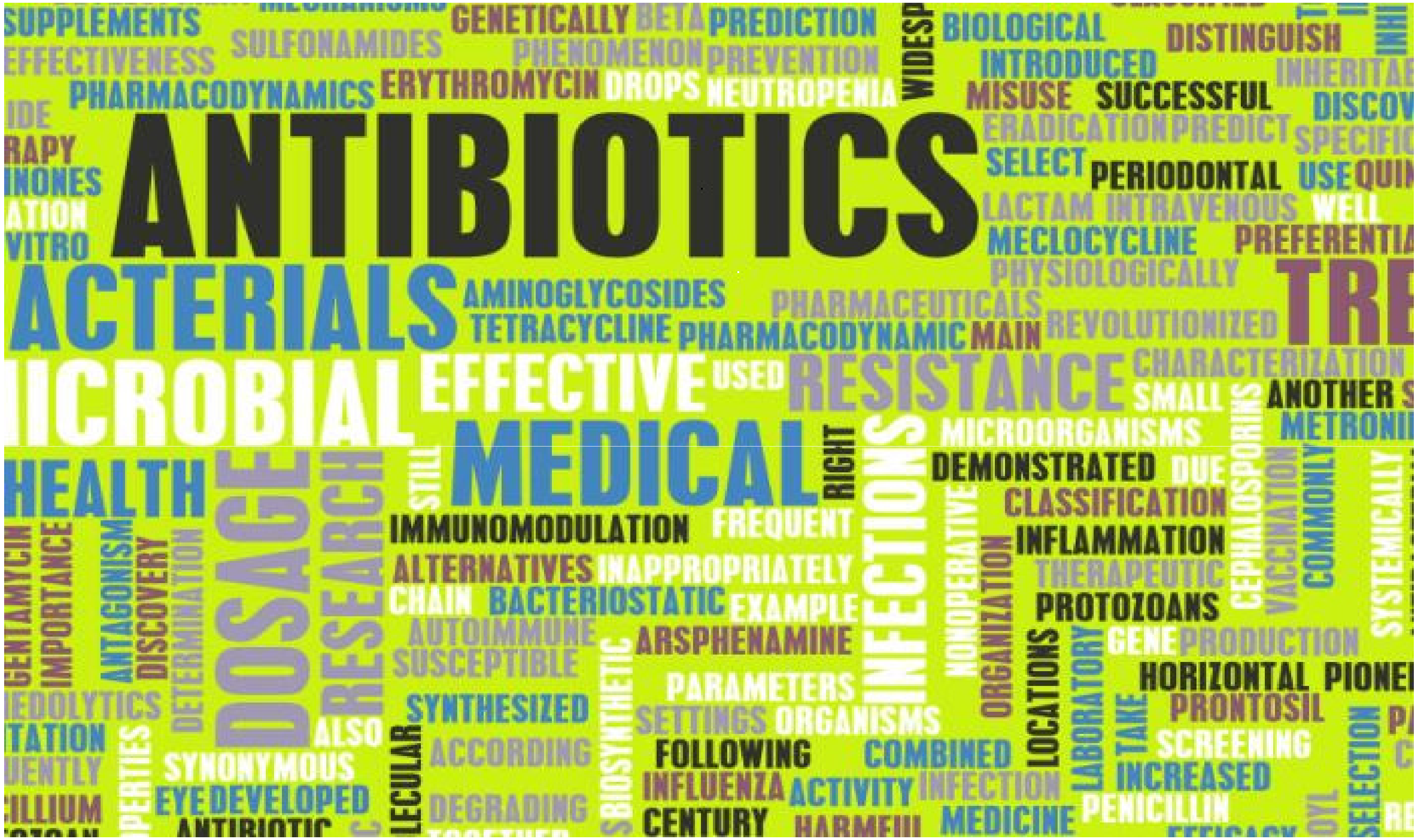
www.who.int/drugresistance

#AntibioticResistance

World Health Organization

Antibiotic Resistance



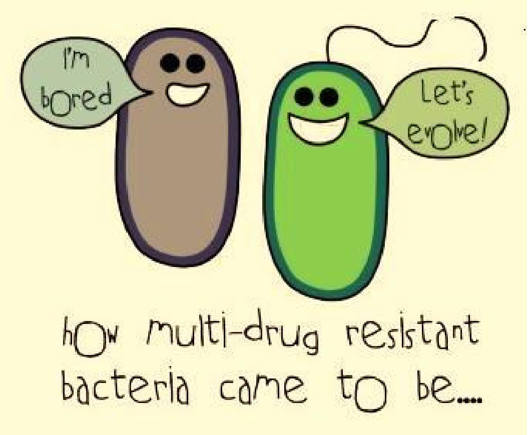
Antibiotic Resistance ECDC video

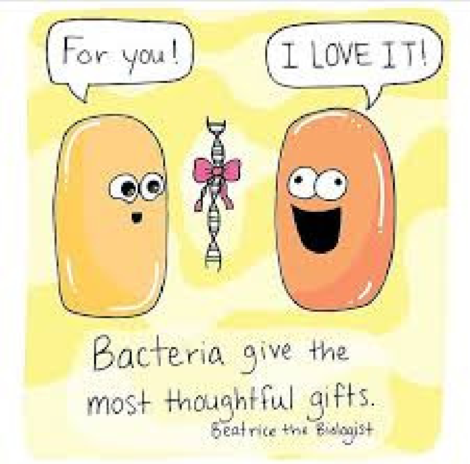
https://vimeo.com/181041782

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



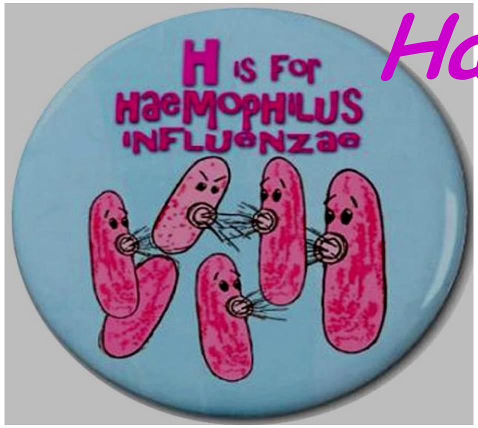


Haemophilus influenzae

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

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



Haemophilus influenzae

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

Bajanca P, Canica M (**2004**) Emergence of nonencapsulated and encapsulated non-b-type invasive *Haemophilus influenzae* isolates in Portugal (1989-2001). J Clin Microbiol 42 (2):807-810

Bajanca-Lavado, M.P. Simões A.S. Betencourt C.R. Sá-Leão R. The Portuguese Group for the Study of *Haemophilus influenzae* infection. Characteristics of *Haemophilus influenzae* invasive isolates from Portugal following routine childhood vaccination against *H. influenzae* serotype b (2002-2010). *Eur J Clin Microbiol Infect Dis*, **2014**;

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)

Medeiros, A. A., and T. F. O'Brien. **1975**. Lancet i:716-719
Rubin LG, Medeiros AA, Yolken RH, Moxon ER (**1981**) Lancet 2 (8254):1008-1010
Mendelman, P. M., D. O. Chaffin, and G. Kalaitzoglou. **1990**. J. Antimicrob. Chemother. 25:525-534

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

Doern GV, Brueggemann AB, Pierce G *et al. Antimicrob Agents Chemother* **1997**; **41**: 292-97
Tristram S, Jacobs MR, Appelbaum PC (**2007**) ;Clin Microbiol Rev 20 (2):368-389

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

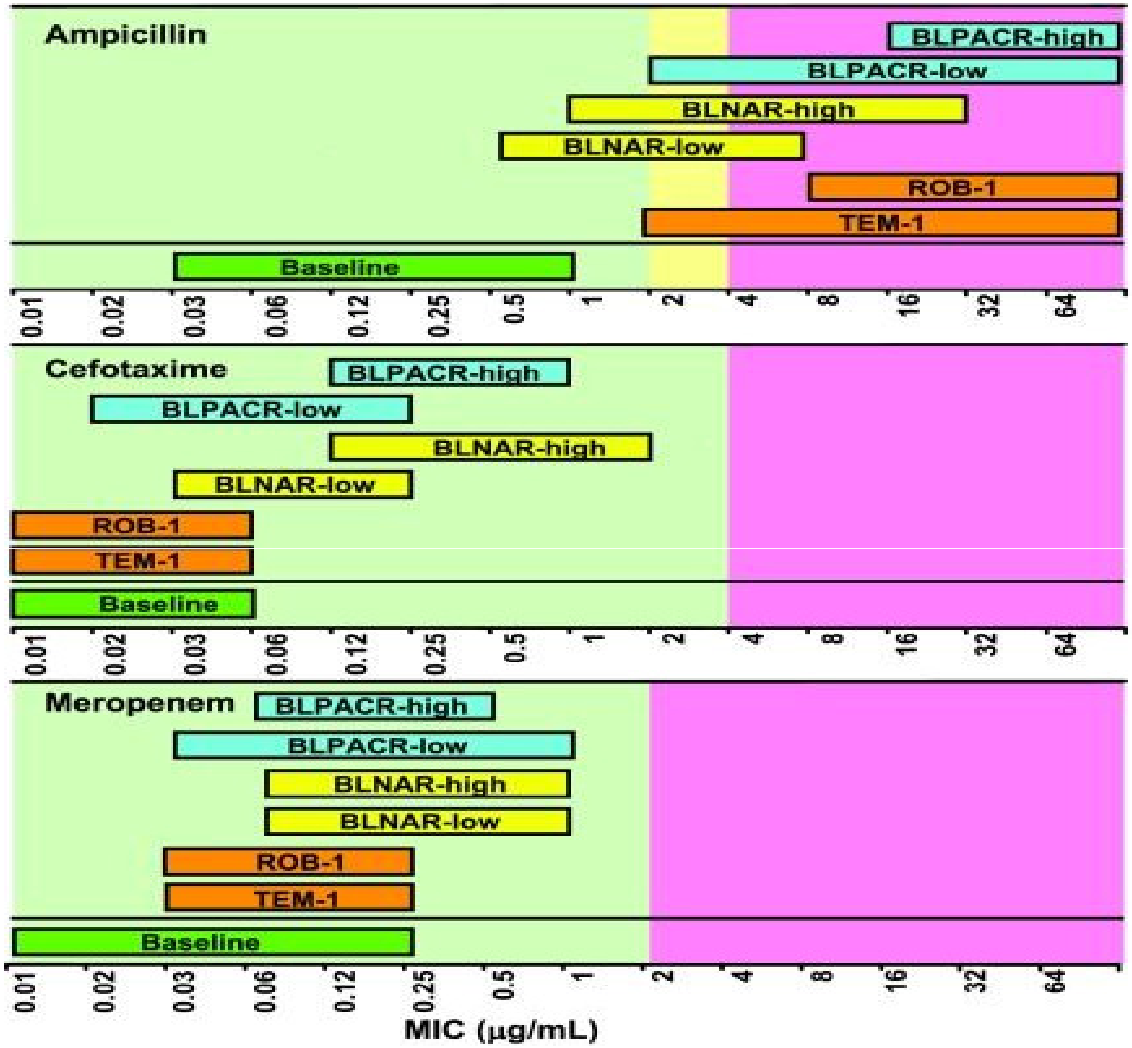
The lack of a consensus and the broad range of ampicillin MICs associated with BLNAR strains is demonstrated by the range of ampicillin breakpoints used in various surveillance studies: from ≥1.0 mg/L to ≥4.0 mg/L. A few researchers start at ≥0.5 mg/L

CLSI and EUCAST breakpoints

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

Antimicrobial	Breakpoint mg/L				
	CLSI			EUCAST	
Ampicillin	**S≤1**	**I=2**	**R≥4**	S≤1	R>1
Amoxicillin/ Clavulanate	**S≤4**		**R≥8**	S≤2	R≥4

Overlap of MIC ranges according to different resistance mechanisms



Tristram S, Jacobs MR, Appelbaum PC (**2007**);Clin Microbiol Rev 20 (2):368-389. **Adapted from Hasegawa *et al.*, 2006 and Sanbongi *et al.*, 2006**
Hasegawa K, Chiba N, Kobayashi R *et al. Antimicrob Agents Chemother* **2004**; 48: 1509-14.
Sanbongi Y, Suzuki T, Osaki Y *et al. Antimicrob Agents Chemother* **2006**; 50: 2487-92

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

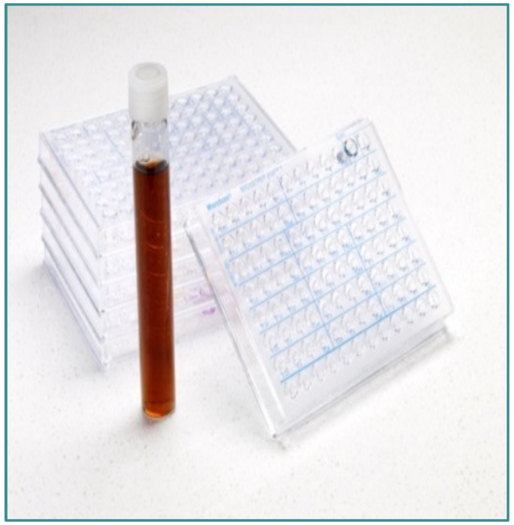
	Genotype (n)	MIC (mg/L)			Susceptibility class (%)		
Antibiotic		**MIC$_{50}$**	**MIC$_{90}$**	**MIC-range**	**S**	**I**	**R**
Ampicillin	gBLNAS (66)	0.25	1	0.12-2	100	0	0
	gBLPAR (33)	64	256	4-512	0	0	100
	gBLPACR (47)	128	512	8->512	0	0	100
	gBLNAR (94)	2	2	1-8	42.6	52.1	5.3
Amoxicillin/ clavulanate	gBLNAS (66)	0.5	1	≤0.25-4	100	0	0
	gBLPAR (33)	2	2	0.5-4	100	0	0
	gBLPACR (47)	2	4	1-4	100	0	0
	gBLNAR (94)	2	4	≤0.25-4	100	0	0

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
Cerquetti M, Giufre M, Cardines R, Mastrantonio P (**2007**); Antimicrob Agents Chemother 51 (9):3155-3161

Analysis

- **Analysis of substitutions in *ftsI* gene**

  Bioinformatic tools

- *Reverse Complement*

  (http://www.bioinformatics.org/sms/rev_comp.html)

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

- *BLAST*

  (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

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 multiplexTEM/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 *ftsI* gene that encodes the transpeptidase region of PBP3; *ftsI H. influenzae* gene have 3 highly conserved amino acid motifs which are essential for function: **STVK- S327TVK**, **SSN- S379SN**, and **KTG- K512TG**

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:

Group I strains had Arg517His near the conserved Lys-Thr-Gly (**KTG**) motif

Group II strains had Asn526Lys near the conserved Lys-Thr-Gly (**KTG**) motif

Group III strains had Met377Ile, Ser385Thr, and Leu389Phe near the conserved Ser-Ser-Asn (**SSN**) motif, in addition toAsn526Lys

Ubukata K, Shibasaki Y, Yamamoto K *et al.*; *Antimicrob Agents Chemother* **2001**; 45: 1693-99

Groups and soubgroups

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

Dabernat H, Delmas C, Seguy M *et al.*; *Antimicrob Agents Chemother* **2002**; 46: 2208-18

García-Cobos S, Campos J, Lázaro E *et al.*;*Antimicrob Agents Chemother* **2007**; 51: 2564-73

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

García-Cobos S, Campos J, Lázaro E *et al.*; *Antimicrob Agents Chemother* **2007**; 51: 2564-73

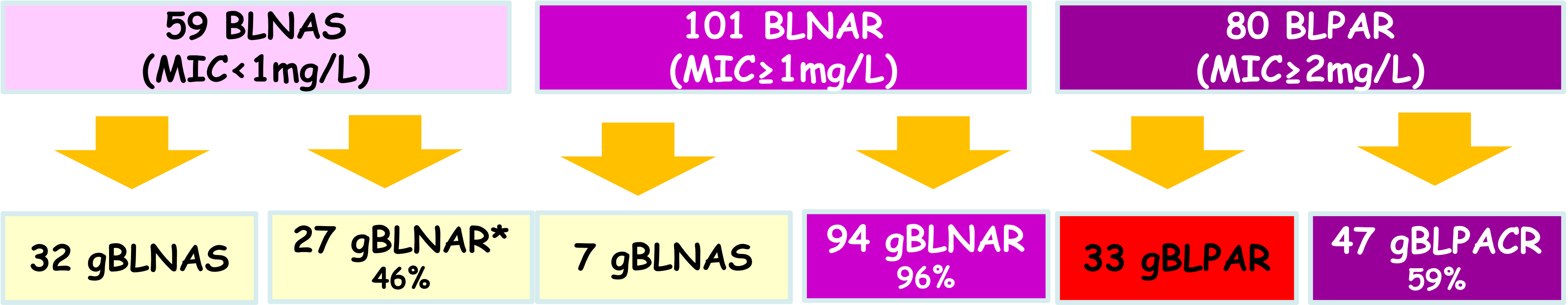
Resistance genotypes

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

Genotype 2001-2008	N (%)	Genotype 2009-2011	N (%)
gBLNAS	66 (27.5%)	gBLNAS	46(18.5%)
gBLPAR	33 (13.7%)	gBLPAR	22 (8.9%)
gBLNAR	94 (39.2%)	gBLNAR	136 (54.9%)
gBLPACR	47 (19.6%)	gBLPACR	44 (17.7%)
Total	**240 (100.0%)**	**Total**	**248 (100.0%)**

Barbosa AR, Giufre M, Cerquetti M, **Bajanca-Lavado MP** (**2011**); J Antimicrob Chemother 66 (4):788-796
Elsa Guilherme. Master thesis in *H. influenzae* ampicillin resistance mechanisms in Portugal: 2009-2012; Universidade de Lisboa, **2013**

Resistance genotypes: 2001-2008



59 BLNAS
(MIC<1mg/L)

32 gBLNAS

27 gBLNAR*
46%

101 BLNAR
(MIC≥1mg/L)

7 gBLNAS

94 gBLNAR
96%

80 BLPAR
(MIC≥2mg/L)

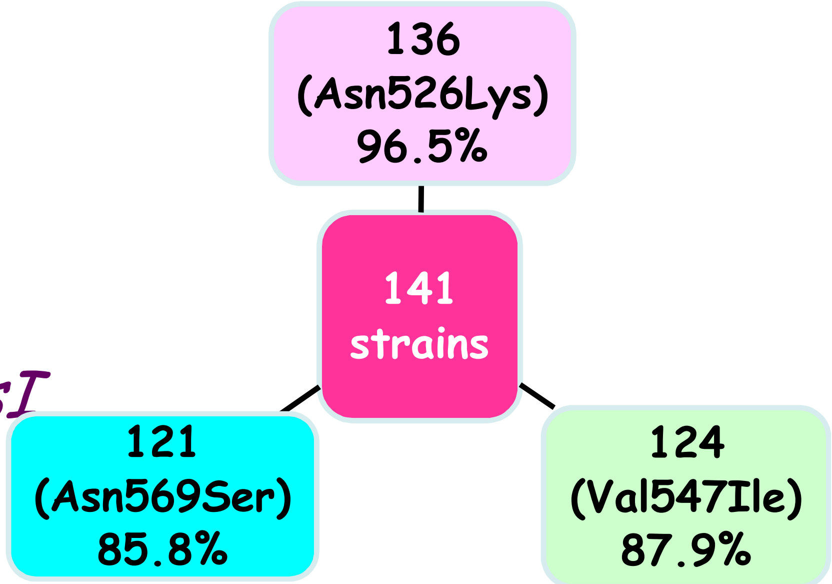
33 gBLPAR

47 gBLPACR
59%

240 strains

141 with mutations in *ftsI*

44 without mutations in *ftsI*



141
strains

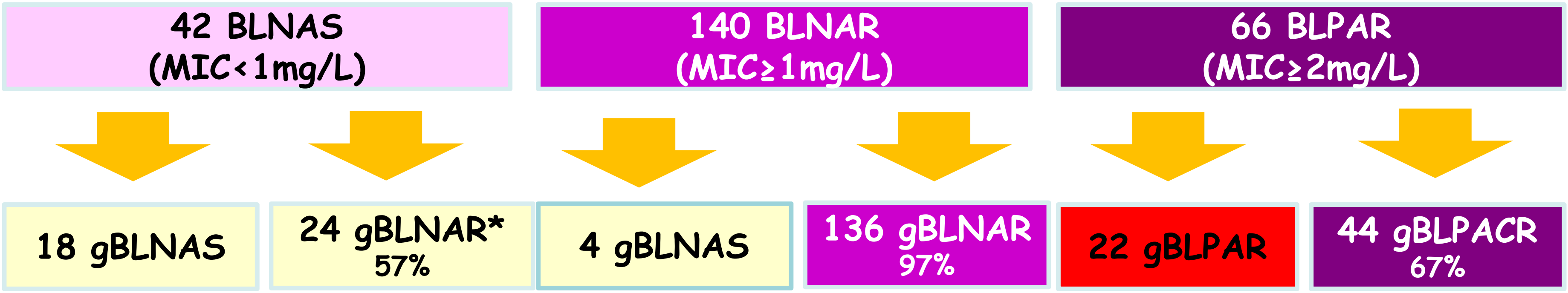
136
(Asn526Lys)
96.5%

121
(Asn569Ser)
85.8%

124
(Val547Ile)
87.9%

* 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%

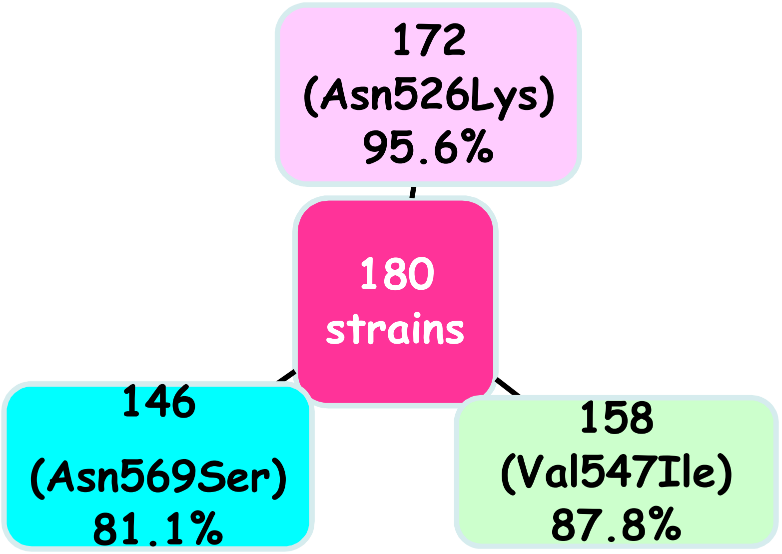
22 gBLPAR

44 gBLPACR
67%

248 strains

180 with mutations in *ftsI*

68 without mutations in *ftsI*



172
(Asn526Lys)
95.6%

180
strains

146
(Asn569Ser)
81.1%

158
(Val547Ile)
87.8%

* 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

Evolution of Ampicillin Resistance Mechanisms 2001-2008 and 2009-2011

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