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Antimicrobial Susceptibility Studies

Detection of beta-lactamase-negative ampicillin resistance in *Haemophilus influenzae* in Belgium

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ABSTRACT

Haemophilus influenzae, a frequent colonizer of the respiratory tract, is the causative agent of several clinically important infections. In cases that require therapeutic intervention, laboratory susceptibility testing can detect beta-lactam antibiotic resistance and guide the best treatment course. In the absence of a beta-lactamase, beta-lactam resistance may be due to an altered form of the PBP3 protein, encoded by the *ftsI* gene. While these so-called beta-lactamase-negative ampicillin-resistant (BLNAR) strains are of serious clinical interest, identification in the clinical laboratory is not always straightforward. In the current study, the *ftsI* genes of a set of phenotypic BLNAR *H. influenzae* isolates taken from samples collected in the UZ Brussel hospital in Belgium were sequenced and re-tested at the National Reference Laboratory (NRC). Non-silent mutations in the *ftsI* gene were found in 100% of the isolates. Although 30% of the isolates were classified by the NRC as beta-lactamase-negative ampicillin-sensitive (BLNAS) strains based on the EUCAST guidelines on ampicillin minimal inhibitory concentration (MIC), all isolates showed MIC values ≥1 mg/L. These relatively high MIC values indicate a decreased susceptibility to ampicillin, and suggest that sequencing of the *ftsI* gene should be used as part of an antibiotic susceptibility testing (AST) algorithm in the clinical laboratory. This would allow clinicians to make better informed decisions regarding patient treatment.

1. Introduction

Haemophilus influenzae is a Gram-negative, facultative anaerobic bacillus known to colonize the respiratory tract. Although frequently present as a member of commensal flora, *H. influenzae* is also an opportunistic pathogen implicated in a range of diseases with varied outcomes. *H. influenzae* strains can primarily be categorized into 2 groups based on the presence or absence of a polysaccharide capsule. The encapsulated group is comprised of 6 serotypes (a–f) based on 6 different polysaccharides. The unencapsulated group is considered nontypeable and can be distinguished genetically or with protein electrophoresis. Historically, encapsulated strains were more often associated with meningitis, bacteremia, pneumonia, and epiglottitis, while unencapsulated strains were more often associated with less invasive infections such as sinusitis and otitis. However, since the introduction of the *H. influenzae* serotype b vaccination in the early 1990s, these associations have changed, and invasive infections are more often attributed to unencapsulated strains (Adam et al., 2010; Campos et al., 2004; Van Eldere et al., 2014). *H. influenzae* strains can also be differentiated based on the presence or absence of indole, urease and/or ornithine decarboxylase production. The possible combinations of these 3 products, or absence of all 3, define 8 different biotypes (I-VIII), of which biotype II is seen most frequently (40–45% of strains) in Belgium [unpublished data from the National Reference Centre for *H. influenzae*].

In cases of clinically significant infection with *H. influenzae*, antibiotic susceptibility testing is necessary to select the most appropriate course of treatment. Aminopenicillins and cephalosporins are the first choice for treatment of *H. influenzae* infections (Sanford et al., 2012), but at least 2 mechanisms of resistance against aminopenicillins have already been described in *H. influenzae*. Beta-lactamases (TEM-1 (Medeiros and O'Brien, 1975) and ROB-1 (Rubin et al., 1981)) catalyze the hydrolysis of the beta-lactam ring of aminopenicillins and thus reduce susceptibility to this class of antibiotics; however, addition of a beta-lactamase inhibitor to the prescribed treatment could potentially be used to mitigate this resistance. Reduced susceptibility to aminopenicillins can also be achieved by alteration of penicillin binding protein 3 (PBP3) (Parr and Bryan, 1984), encoded by the *ftsI* gene (Ubukata et al., 2001). Mutations

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in this gene result in amino acid substitutions in the transpeptidase domain of PBP3 and reduce the affinity of beta-lactams for their target, thus diminishing the antibiotics' efficacy. In addition, resistance to third- and fourth-generation cephalosporins has been described, but occurs less frequently (Garcia-Cobos et al., 2007; Leclercq et al., 2013). When *H. influenzae* shows resistance to aminopenicillins in the absence of the production of a beta-lactamase, the term "beta-lactamase-negative ampicillin resistance" (BLNAR) is used.

Identification of phenotypic BLNAR is based on laboratory guidelines and interpretation criteria for ampicillin susceptibility set by different committees. Both EUCAST (The European Committee on Antimicrobial Susceptibility Testing) (2018a) and CLSI (Clinical and Laboratory Standards Institute (2018)) publish guidelines for the interpretation of minimal inhibitory concentration (MIC) values. For *H. influenzae*, the EUCAST and CLSI guidelines define ampicillin breakpoints of 1 mg/L and 2 mg/L, respectively (CLSI. Clinical and Laboratory Standards Institute, 2018; EUCAST, 2018a), with an intermediate susceptibility at 2 mg/L (CLSI). Therefore, depending on the guidelines implemented in a given laboratory, sensitivity and selectivity of detecting BLNAR strains may differ (Soyletir et al., 2016).

Genotypic characterization of BLNAR strains occurs through sequencing of the transpeptidase domain of the *ftsI* gene. Depending on the amino acid substitutions present, Ubukata et al. (2001) classified BLNAR strains into different groups. Group I strains comprise isolates with an R517H substitution, and group II are those with an N526K substitution. Group III isolates also have the N526K substitution, but with 3 additional substitutions: M377I, S385 T and L389P. Later, group II was further divided into subgroups by Dabernat et al. (2002): subgroup IIa strains contain only the N526K substitution; subgroup IIb has an additional A502V substitution, sometimes in combination with other substitutions; subgroup IIc contains isolates with N526K in combination with an A502T substitution; and in subgroup IId N526K is supplemented by an I449V substitution. For all 3 major groups (I, II, III), several additional substitutions have also been observed (Lam et al., 2015).

As another level of classification, *H. influenzae* isolates exerting PBP3-mediated resistance can be divided into 2 groups based on their level of resistance (Tristram et al., 2007). The high-level resistant strains, belonging to group III as defined by Ubukata, present a major problem in Asia (Japan, South-Korea), but are rarely seen in Europe. The low-level resistant strains, belonging to Ubukata groups I and II, are predominant in the rest of the world (Skaare et al., 2014).

Currently many laboratories in Belgium and Europe have switched or are switching from CLSI to EUCAST guidelines. EUCAST proposes a protocol that includes screening with a 1 unit benzylpenicillin disc, followed by a beta-lactamase test when the zone diameter is <12 mm (EUCAST, 2018b). Irrespective of whether an isolate is beta-lactamase positive or negative, it is necessary to test sensitivity to beta-lactams and report as measured (except for ampicillin, in which case an isolate can be reported resistant when beta-lactamase is positive). In contrast, CLSI has no specific recommendation for BLNAR detection. The current study aims to evaluate the molecular basis of phenotypically determined BLNAR in samples collected in a university hospital in Belgium from October 2013 to December 2016.

2. Materials and methods

2.1. Selection of isolates

In April 2017, the database of the UZ Brussel hospital was searched retrospectively for BLNAR *H. influenzae* isolates. Due to the lack of CLSI BLNAR interpretation recommendations, the search window started from October 2013, when the switch to EUCAST guidelines had been made, and ended December 2016. Of the isolates identified in the search, those originating from normally sterile samples (*e.g.* bronchoalveolar lavages, cerebrospinal fluid), punctates of the middle ear or abscesses were still available in our collection and were used for further analysis.

2.2. Susceptibility testing of H. influenzae

At UZ Brussel, all isolates selected as described above (2.1) were tested with the chromogenic cephalosporin test using nitrocefin as a substrate (Oxoïd Thermo Scientific®, Altrincham, United Kingdom). Strains that tested positive were further tested for susceptibility to amoxicillin/clavulanic acid and sulfamethoxazol/trimethoprim. In the case of beta-lactamase-negative strains, susceptibility to ampicillin was tested. MIC's were all determined using commercially available E-tests (bioMérieux®, Marcy-l'Étoile, France). Screening for beta-lactam resistance in *H. influenzae* with an initial disc diffusion test using benzylpencillin, as proposed by EUCAST clinical breakpoint Tables v 7.1 (and Tables v 8.1), is not routine in our laboratory, but was performed retrospectively on the selected isolates for this study. In this method, an MHF plate (Mueller Hinton Fastidious Agar) is inoculated with a 0.5 McFarland suspension of *H. influenzae* in 0.85% saline, and a benzylpenicillin 1 unit disc (Sensi Disc® PEN 1 IU, BD BBL®, Franklin Lakes, NJ, USA) is applied. After 18 hours incubation at 35 °C with 5% CO_2, the diameter of the inhibition zone is measured. A zone diameter of 12 mm or more indicates susceptibility to all beta-lactam agents for which clinical breakpoints are available, precluding the presence of a beta-lactamase or other beta-lactamase-like mechanism. A zone diameter of less than 12 mm requires testing for the presence of a beta-lactamase.

2.3. Molecular analysis of BLNAR

All selected BLNAR strains were sent to the National Reference Centre for *H. influenzae* (NRC, Laboratoire de la Porte de Hal - CHU St-Pierre, Brussels, Belgium) for sequencing of the *ftsI* gene and for confirmation of their beta-lactam resistance.

2.3.1. Preliminary testing at the NRC

AST profiles were repeated before submitting the isolates to molecular analysis. Beta-lactamase production was tested using BD BBL Cefinase paper disks (Becton Dickinson, Erembodegem, Belgium) and MIC's were determined using e-tests gradient strips (bioMérieux, Marcy-l'-Etoile, France) for ciprofloxacin, tetracyclin, sulfamethoxazol/trimethoprim and the following beta-lactams: ampicillin, amoxicillin-clavulanic acid, cefotaxime and meropenem.

2.3.2. DNA extraction for PCR

Strains were grown for 24 h on chocolate agar, and 2 to 3 colonies were then picked and resuspended in 200 μL of sterile water. The bacterial suspension was transferred to a heating block and incubated at 99 °C for 10 min before being rapidly cooled to −20 °C.

2.3.3. PCR

PCR amplification of the *ftsI* gene region encoding the transpeptidase domain of PBP3 was carried out with the primers described on the University of Oxford *Haemophilus influenzae* MLST website (https://pubmlst.org/hinfluenzae/). Amplification was performed in a total reaction volume of 50 μL containing 0.4 μM of each primer, 0.2 μM deoxynucleotide triphosphates, 5 μL of 10x reaction buffer with $MgCl_2$, 0.3 μL of Taq DNA polymerase, and 5 μL of template DNA. PCR thermocycling was carried out in an Applied Biosystems GeneAmp® PCR system 2700. After initial denaturation at 94 °C for 10 min, 10 amplification cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, and primer extension at 72 °C for 40 s were carried out, followed by thirty amplification cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s, and primer extension at 72 °C

for 40 s. A final primer extension step was then run at 72 °C for 7 min. The resulting PCR products were used for sequencing.

2.3.4. DNA sequencing and analysis of sequence data

Both the sense and antisense strands of the PCR products were sequenced. Sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit with an ABI 3130xl genetic analyzer. The sequences were analyzed using Bionumerics v7.5 (Applied Maths). The PBP3 transpeptidase domain nucleotide sequence of the Rd. KW20 strain (ATCC 51907) was included as a reference in the sequence alignment. The corresponding amino acid sequences of the PBP3 transpeptidase domain (326–532) were translated from the nucleotide sequences and compared with that of *H. influenzae* Rd. KW20.

3. Results

3.1. Selection of isolates

From October 2013 to December 2016 *H. influenzae* was detected in 1481 clinical samples collected at the UZ Brussel hospital, with one sample containing 2 different isolates resulting in a total of 1482 isolates. A minority of the isolates (363 of 1482 (24.5%)) were beta-lactamase positive and were therefore not included in this study. For 3 isolates (0.2%), no beta-lactamase results were determined and these isolates were also eliminated from consideration. 1116 (75.3%) isolates were shown to be beta-lactamase negative using the nitrocefin-method. Of these, 1111 had ampicillin susceptibility testing data, and 93.2% ($n = 1036$) of these are BLNAS, using the EUCAST breakpoint of 1 mg/L, and 6.8% ($n = 75$) are BLNAR. The distribution of the MIC values for ampicillin (mg/L) of all 1111 strains is given in Fig. 1 panel A. Of the 75 EUCAST-defined BLNAR strains, isolates originating from sputum and superficial swabs were excluded, resulting in a final set of 19 isolates. This set of isolates was sent to the NRC for *H. influenzae* for genetic characterization. For 2 isolates no clinical information was available, but the other isolates ($n = 17$) originated from patients that presented with pneumonia ($n = 11$; 64.7%), otitis ($n = 5$; 29.4%), or a para-oesophageal abscess ($n = 1$; 5.9%) and were collected from the bronchi, the middle ear and the para-oesophageal abscess itself.

Applying the EUCAST screening method to each of the set of 19 isolates showed a zone diameter for benzylpencillin of less than 12 mm,

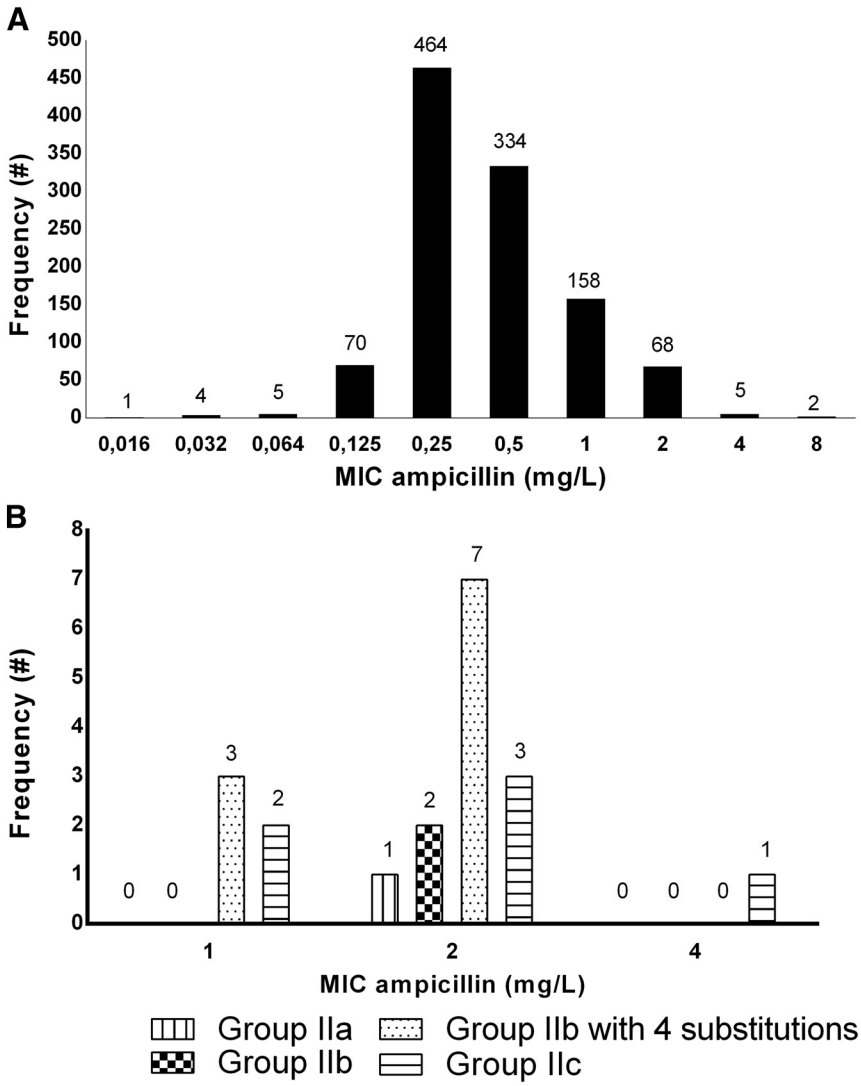

Fig. 1. The distribution of the beta-lactamase negative *H. influenzae* isolates. **A,** Distribution of the MIC values for ampicillin (mg/L) of all beta-lactamase negative *H. influenzae* strains isolated from clinical samples in the UZ Brussel hospital from October 2013 to December 2016. **B,** The isolates classified in groups as a function of the MIC value for ampicillin. *ftsI* gene sequencing of the final set of 20 *H. influenzae* isolates (19 initial isolates +1 extra from a mixed "isolate") allowed classification of the 19 isolates with an MIC > = 1 mg/L into the groups described by Ubukata et al. (2001) and later Dabernat et al. (2002). Most of the isolates belong to subgroup IIb with a total of 4 substitutions and have an MIC for ampicillin of 2 mg/L. Only one isolate has an MIC of 4 mg/L and this isolate belongs to subgroup IIc. The MIC of ampicillin does not seem to depend on the subgroup of an isolate as isolates of subgroup IIc show MIC values of 1, 2 and 4 mg/L.

Table 1
Phenotypic and genotypic analysis of selected *H. influenzae* isolates.

Isolate name	Zone diameter benzylpenicillin disc (mm)	Ampicillin MIC (mg/L)		Amoxicillin-clavulanic acid MIC (mg/L)	Cefotaxime MIC (mg/L)	Meropenem MIC (mg/L)	Amino acid substitution	Group	BLNAR/ BLNAS[c]	Biotype
	UZ Brussel	UZ Brussel	NRC	NRC	NRC	NRC				
14/1439	6	2	2	4	**0.125**	**0.250**	N526K	IIa	BLNAR	II
14/0403	6	2	2	**2**	**0.125**	**0.064**	D350N,M377I,A502V,N526K	IIb	BLNAR	II
14/0787	6	2	2	4	**0.064**	**0.125**	A502V,N526K	IIb	BLNAR	III
14/1650	6	2	2	**2**	**0.032**	**0.125**	G490E,A502V,N526K	IIb	BLNAR	II
14/1861b	6	2	2	**2**	**0.125**	**0.125**	D350N,M377I,A502V,N526K	IIb	BLNAR	II
15/0648	6	2	2	**2**	**0.125**	**0.125**	D350N,M377I,A502V,N526K	IIb	BLNAR	II
16/0446	6	2	2	**2**	**0.125**	**0.064**	D350N,M377I,A502V,N526K	IIb	BLNAR	II
16/0467	6	2	2	**2**	**0.125**	**0.064**	D350N,M377I,A502V,N526K	IIb	BLNAR	II
16/1850	6	2	2	**2**	**0.125**	**0.125**	D350N,M377I,A502V,N526K	IIb	BLNAR	II
16/1889	6	2	2	**2**	**0.125**	**0.064**	D350N,M377I,A502V,N526K	IIb	BLNAR	II
13/1474	7	2	2	**2**	**0.064**	**0.250**	D350N, A502T,N526K	IIc	BLNAR	I
14/0643	6	2	2	4	**0.064**	**0.250**	A502T,N526K	IIc	BLNAR	III
16/0075	6	2	2	4	**0.064**	**0.250**	A502T,N526K	IIc	BLNAR	III
16/0924	6	2	4	4	**0.125**	**0.125**	D350N,A502T,N526K	IIc	BLNAR	III
14/1861a	**17**	NA[a]	**0.5**[b]	**1**	**0.032**	**0.064**	D350N	/	BLNAS	III
15/0096	10	2	**1**	**2**	**0.064**	**0.125**	D350N,M377I,A502V,N526K	IIb	BLNAS	IV
16/0620	6	2	**1**	**2**	**0.125**	**0.064**	D350N,M377I,A502V,N526K	IIb	BLNAS	II
16/1721	7	2	**1**	**2**	**0.064**	**0.250**	D350N,M377I,A502V,N526K	IIb	BLNAS	II
15/0086	6	2	**1**	**2**	**0.064**	**0.250**	D350N,A502T,N526K	IIc	BLNAS	I
15/0146	7	4	**1**	**2**	**0.064**	**0.125**	D350N,A502T,N526K	IIc	BLNAS	II

[a] NA: not available.
[b] The MIC values of strains susceptible to ampicillin, amoxicillin-clavulanic acid, cefotaxime and meropenem following the EUCAST guidelines are shown in bold.
[c] The classification in BLNAR/BLNAS is based on the MIC value for ampicillin measured by the NRC following interpretation using the EUCAST guidelines.

suggesting all the isolates are resistant to beta-lactam agents (Table 1). The isolates returned diameters of 6 mm ($n = 15$), 7 mm ($n = 3$) and 10 mm ($n = 1$). With one of the isolates (14/1861) a ghost-zone of 17 mm was seen around the benzylpenicillin disc, suggesting it was actually a mixture of 2 isolates, both of which were sent to the NRC for genetic testing. As a result, the final set for analysis consisted of 20 isolates.

3.2. Phenotypic analysis of BLNAR Haemophilus influenzae at the NRC

The results of the susceptibility testing performed by the NRC and UZ Brussel, and of the EUCAST screening method performed by UZ Brussel are shown in Table 1. All of the isolates listed were resistant to ampicillin, as determined by UZ Brussel. Testing for ampicillin susceptibility at the NRC demonstrated that 70% ($n = 14$) of the isolates are ampicillin resistant (BLNAR). Of these 14 isolates, 9 were susceptible to amoxicillin-clavulanic acid and all were susceptible to meropenem and cefotaxime (breakpoint values of 2 mg/L and 0.125 mg/L) (EUCAST, 2018b). A total of 6 isolates were not confirmed to be BLNAR by the NRC, thus re-classifying them as phenotypic BLNAS instead of BLNAR.

It should be noticed that a discrepancy can be observed between the MIC values for ampicillin as determined by UZ Brussel and by the NRC. However, according to CLSI M52 guidelines, a difference of one dilution point is generally accepted and almost all of the results can therefore be considered equal (CLSI, 2015). Strain 15/0146 is one exception, with MIC values of 4 mg/L and 1 mg/L from UZ Brussel and the NRC, respectively. Retesting at UZ Brussel resulted in an MIC of 1 mg/L.

3.3. Molecular analysis of BLNAR Haemophilus influenzae at the NRC

Sequencing of the *ftsI* gene of the 20 isolates revealed that each isolate had one or more mutations in the transpeptidase domain of the *ftsI* gene. Fig. 2 shows the corresponding amino acid sequence alignment. The amino acid substitutions and the classification of the isolates following sequencing are represented in Table 1. In all isolates, the conserved capsular gene *bexA* remained undetected confirming these isolates were nontypeable *H. influenzae*.

Most of the sequenced isolates belong to subgroup IIb ($n = 12$; 63%). 32% ($n = 6$) of the isolates belong to subgroup IIc and only one isolate (5%) belongs to subgroup IIa. It should be noted that substitution N526K is present in all the isolates but one. Isolate 14/1861a has a D350N substitution instead and does not belong to any of the BLNAR groups. Furthermore, this isolate was also the isolate with the lowest ampicillin MIC 0.5 mg/L.

The distribution of the isolates in each subgroup as a function of the MIC value for ampicillin is shown in Fig. 1, panel B.

Most of the isolates belong to subgroup IIb with a total of 4 substitutions and have an MIC for ampicillin of 2 mg/L, as measured by the NRC. Only one isolate has an MIC of 4 mg/L and this isolate belongs to subgroup IIc. The MIC of ampicillin does not seem to correlate to the subgroup of an isolate as, for example, isolates of subgroup IIc show MIC values of 1, 2 and 4 mg/L.

Panel A of Fig. 1 shows the distribution of the MIC values for ampicillin of all the *H. influenzae* strains isolated from October 2013 till December 2016 for which ampicillin susceptibility was tested ($n = 1111$), as measured by UZ Brussel. The histogram shows that most of the isolates that are sensitive to ampicillin have MIC values ≤0.25 mg/L. The resistant isolates selected for further analysis in this study (MIC ≥2 mg/L) are located to the far right of the curve. The distribution is very similar to that of the wild type population, with an MIC of 0.25 mg/L as the most frequent (EUCAST, 2018c).

Susceptibility testing to cefotaxime and meropenem (MIC values shown in Table 1) revealed that none of the tested isolates are resistant to these 2 antibiotics.

In Table 2, the isolates are ordered according to susceptibility or resistance to amoxicillin-clavulanic acid as determined by EUCAST breakpoints. All strains susceptible to amoxicillin-clavulanic acid have additional amino acid substitutions.

4. Discussion

In this study, *H. influenzae* isolates from clinical samples of patients at the University Hospital Brussel in Belgium were collected and tested for the presence of a beta-lactamase. Beta-lactamase-negative strains were selected and tested for their *in vitro* susceptibility to ampicillin.

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                326         336         346         356         366         376         386
RD KW20         GSTVKPFVVL TALQRGVVKR DEIIDTTSFK LSGKEIVDVA PRAQQTLDEI LMNSSNRGVS RLALRMPPSA
14/1650         .......... .......... .......... .......... .......... .......... ..........
14/0787         .......... .......... .......... .......... .......... .......... ..........
15/0648         .......... .......... ....N..... .......... .......... .I........ ..........
16/1721         .......... .......... ....N..... .......... .......... .I........ ..........
14/1861b        .......... .......... ....N..... .......... .......... .I........ ..........
14/0403         .......... .......... ....N..... .......... .......... .I........ ..........
16/0620         .......... .......... ....N..... .......... .......... .I........ ..........
15/0096         .......... .......... ....N..... .......... .......... .I........ ..........
16/0446         .......... .......... ....N..... .......... .......... .I........ ..........
16/0467         .......... .......... ....N..... .......... .......... .I........ ..........
16/1889         .......... .......... ....N..... .......... .......... .I........ ..........
16/1850         .......... .......... ....N..... .......... .......... .I........ ..........
14/1439         .......... .......... .......... .......... .......... .......... ..........
16/0075         .......... .......... .......... .......... .......... .......... ..........
14/0643         .......... .......... .......... .......... .......... .......... ..........
13/1474         .......... .......... ....N..... .......... .......... .......... ..........
15/0086         .......... .......... ....N..... .......... .......... .......... ..........
16/0924         .......... .......... ....N..... .......... .......... .......... ..........
15/0146         .......... .......... ....N..... .......... .......... .......... ..........
14/1861a        .......... .......... ....N..... .......... .......... .......... ..........

                396         406         416         426         436         446         456
RD KW20         LMETYQNAGL SKPTDLGLIG EQVGILNANR KRWADIERAT VAYGYGITAT PLQIARAYAT LGSFGVYRPL
14/1650         .......... .......... .......... .......... .......... .......... ..........
14/0787         .......... .......... .......... .......... .......... .......... ..........
15/0648         .......... .......... .......... .......... .......... .......... ..........
16/1721         .......... .......... .......... .......... .......... .......... ..........
14/1861b        .......... .......... .......... .......... .......... .......... ..........
14/0403         .......... .......... .......... .......... .......... .......... ..........
16/0620         .......... .......... .......... .......... .......... .......... ..........
15/0096         .......... .......... .......... .......... .......... .......... ..........
16/0446         .......... .......... .......... .......... .......... .......... ..........
16/0467         .......... .......... .......... .......... .......... .......... ..........
16/1889         .......... .......... .......... .......... .......... .......... ..........
16/1850         .......... .......... .......... .......... .......... .......... ..........
14/1439         .......... .......... .......... .......... .......... .......... ..........
16/0075         .......... .......... .......... .......... .......... .......... ..........
14/0643         .......... .......... .......... .......... .......... .......... ..........
13/1474         .......... .......... .......... .......... .......... .......... ..........
15/0086         .......... .......... .......... .......... .......... .......... ..........
16/0924         .......... .......... .......... .......... .......... .......... ..........
15/0146         .......... .......... .......... .......... .......... .......... ..........
14/1861a        .......... .......... .......... .......... .......... .......... ..........

                466         476         486         496         506         516         526
RD KW20         SITKVDPPVI GKRVFSEKIT KDIVGILEKV AIKNKRAMVE GYRVGVKTGT ARKIRNGHYV NKYVAFT
14/1650         .......... .......... ....E..... ......V... .......... .......... K......
14/0787         .......... .......... .......... ......V... .......... .......... K......
15/0648         .......... .......... .......... ......V... .......... .......... K......
16/1721         .......... .......... .......... ......V... .......... .......... K......
14/1861b        .......... .......... .......... ......V... .......... .......... K......
14/0403         .......... .......... .......... ......V... .......... .......... K......
16/0620         .......... .......... .......... ......V... .......... .......... K......
15/0096         .......... .......... .......... ......V... .......... .......... K......
16/0446         .......... .......... .......... ......V... .......... .......... K......
16/0467         .......... .......... .......... ......V... .......... .......... K......
16/1889         .......... .......... .......... ......V... .......... .......... K......
16/1850         .......... .......... .......... ......V... .......... .......... K......
14/1439         .......... .......... .......... .......... .......... .......... K......
16/0075         .......... .......... .......... ......T... .......... .......... K......
14/0643         .......... .......... .......... ......T... .......... .......... K......
13/1474         .......... .......... .......... ......T... .......... .......... K......
15/0086         .......... .......... .......... ......T... .......... .......... K......
16/0924         .......... .......... .......... ......T... .......... .......... K......
15/0146         .......... .......... .......... ......T... .......... .......... K......
14/1861a        .......... .......... .......... .......... .......... .......... .......
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Fig. 2. Amino acid alignment. Multiple alignment of PBP3 amino acid sequences deduced from the sequences of the *ftsI* genes present in *H. influenzae* RD KW20 and in the 20 *H. influenzae* strains present in this study. Conserved amino acid motifs STVK, SSN and KTG are boxed. The dots represent identical amino acids.

Seventy-five isolates were found to be BLNARs of which a selection of 19 isolates was made based on sample type and preservation of the strain in the clinical laboratory. Since these selected isolates showed resistance to beta-lactam antibiotics, but lacked a beta-lactamase, we were interested in determining whether they showed any changes at the molecular level, and specifically, whether they showed any changes in the *ftsI* gene resulting in an altered PBP3. The isolates were therefore sent to the NRC for *H. influenzae* for molecular analysis.

Table 2
Overview of the amino acid substitutions found in *H. influenzae* strains susceptible or resistant to amoxicillin-clavulanic acid.

Isolate name	Ampicillin MIC (mg/L)	Amoxicillin-clavulanic acid MIC (mg/L)	Amoxicillin-clavulanic acid S or R following EUCAST	Most common substitutions	Additional substitutions
14/1861a	0.5	1	S		D350N
13/1474	2	2	S	A502T,N526K	D350N
14/1650	2	2	S	A502V,N526K	G490E
14/1861b	2	2	S	A502V,N526K	D350N,M377I
15/0086	1	2	S	A502T,N526K	D350N
15/0096	1	2	S	A502V,N526K	D350N,M377I
15/0648	2	2	S	A502V,N526K	D350N,M377I
16/0446	2	2	S	A502V,N526K	D350N,M377I
16/0467	2	2	S	A502V,N526K	D350N,M377I
16/0620	1	2	S	A502V,N526K	D350N,M377I
16/1721	1	2	S	A502V,N526K	D350N,M377I
16/1850	2	2	S	A502V,N526K	D350N,M377I
16/1889	2	2	S	A502V,N526K	D350N,M377I
15/0146	1	2	S	A502T,N526K	D350N
14/0403	2	2	S	A502V,N526K	D350N,M377I
14/0643	2	4	R	A502T,N526K	
14/0787	2	4	R	A502V,N526K	
16/0924	4	4	R	A502T,N526K	D350N
16/0075	2	4	R	A502T,N526K	
14/1439	2	4	R	N526K	

The absence of a beta-lactamase in all the tested isolates was confirmed by the NRC and all strains were found to be unencapsulated. Upon retesting by the NRC, 30% of the isolates were found to be susceptible to ampicillin (and thus classified as BLNAS). For the majority of these BLNAS isolates, this discrepancy is a consequence of the MIC differing by just one dilution point between the 2 laboratories, thus highlighting the lack of precision in the MIC value. This finding underlines the importance of not relying on a single MIC value determined in the lab, and also suggests the need to introduce tolerance values into the evaluation guidelines, particularly when it comes to evaluating MIC values that correspond to breakpoint values. This variability also emphasizes the importance of carefully choosing breakpoint values (and committee guidelines).

Sequencing of the *ftsI* gene demonstrated that none of the analyzed isolates belong to the high-level resistance group (Ubukata group III). In line with what has been previously reported in the literature, the majority (95%, $n = 19$) of the isolates belong to Ubukata group II (Skaare et al., 2014). Most (74%, $n = 14$) of these group II isolates have BLNAR phenotypes. The other 5 isolates were BLNAS with an ampicillin MIC value close to the breakpoint. This is consistent with results described by Lam et al. (2015). Using the classification of Ubukata adapted by Dabernat, 12 isolates belong to group IIb (A502V, N526K), 6 to group IIc (A502T, N526K), and one isolate to group IIa (N526K). Previous work in Norway has shown that in group IIb, strains presenting a substitution combination of D350N, M377I, A502V, N526K are most frequently observed (Skaare et al., 2010). Of the isolates tested, 50% ($n = 10$; 83% of those in group IIb) have a genotype that is most closely described by this published group IIb combination of 4 mutations. All of these isolates but one were shown to be unencapsulated biotype II. The remaining isolate is biotype IV and has an ampicillin sensitive phenotype (MIC 1 mg/L), classifying it as BLNAS.

As previously described by Lam et al. (2015) and Misawa et al. (2018), the presence of PBP3 substitutions is not exclusively associated with 'high' MIC values, and concomitantly, with BLNAR. Since PBP3 substitutions are also found in the BLNAS isolates in this study, it supports the suggestion that the current implementation of *ftsI* sequencing for identifying BLNAR is relatively non-specific. However, interestingly, the distribution of MIC values for ampicillin of all the *H. influenzae* isolates (both ampicillin susceptible and resistant) as shown in Fig. 1, panel A, demonstrates that susceptible strains tend to have lower MIC values (most frequently 0.25 mg/L) than those classified as the BLNAS in our set of evaluated isolates by using the PK/PD EUCAST breakpoint. Therefore, the strains with an MIC of 1 mg/L (as measured by the NRC) could already be considered as strains with reduced susceptibility by the PK/PD EUCAST breakpoint and *ftsI* sequencing could be proposed to differentiate these strains. Furthermore, such 'low-BLNAR strains' with MIC values of 0.5–2 mg/L were previously described by other research-groups in the early 2000s (Hasegawa et al., 2003, 2004; Ubukata et al., 2002) and have remained a research subject of interest ever since.

Potentially, amino acid substitutions are leading to decreased susceptibility to ampicillin, resulting in MIC values closer to the current EUCAST breakpoint of 1 mg/L, thereby creating a gray zone of strains that are not fully BLNAS and also not fully BLNAR. It may be clinically more relevant to include these strains into the BLNAR group; suggesting the need to decrease the breakpoint for ampicillin susceptibility to ensure a higher proportion of all BLNAR strains are intercepted. It should be noted that the ≥1 mg/L breakpoint was also used in the study by Dabernat et al. (2002) and Ubukata et al. (2001).

The benzylpenicillin-screening test suggested by EUCAST could then be used to confirm the BLNAR strains selected with a lower MIC breakpoint. In this study, the EUCAST screening test was more indicative of the presence of substitutions in the transpeptidase domain of PBP3 than the ampicillin MIC values. However, analysis of a larger set of isolates would be necessary to confirm this finding. The *ftsI* gene of *H. influenzae* strains with much lower MIC values for ampicillin would also need to be sequenced to determine if the same substitutions observed in the resistant strains are present or absent in that population.

Of the different amino acid substitutions observed, one BLNAS isolate interestingly has a single substitution (D350N) in PBP3. This isolate (14/1861a) is also the isolate with the lowest MIC value for ampicillin (0.5 mg/L), suggesting that the D350N substitution alone is insufficient to induce sufficient resistance against ampicillin to exceed the MIC breakpoint of susceptibility. This substitution is frequently (in 50% of the isolates) seen in combination with M377I, A502V and N526K, both in BLNAR and BLNAS isolates, as was also observed by Lam et al. (2015). However, when the strains are classified according to their susceptibility or resistance to ampicillin-clavulanic acid (as shown in Table 2), 4 out of the 5 resistant isolates, belonging to subgroups IIb and IIc, have substitutions at A502 (T or V) and the N526K substitution, but are lacking M377I. All of the analyzed isolates susceptible to ampicillin-clavulanic acid seem to have additional substitutions, of which D350N and M377I seem to be most frequent. More extensive sequencing is necessary to clarify which substitutions are important for predicting resistance to ampicillin and ampicillin-clavulanic acid.

A Norwegian study on non-invasive isolates of *H. influenzae* describes only a small proportion (6%) of isolates to be resistant or as having intermediate susceptibility to cefotaxime and meropenem when the breakpoint value for meningitis is used (Skaare et al., 2014). None of the isolates tested in the current study presents resistance to either of the 2 antibiotics. However, some of the group II isolates have MIC values close to the breakpoint. Since the expected percentage is small (6%), the number of isolates tested in this study may not have been sufficient to include a resistant strain.

More remarkable was the observed susceptibility to amoxicillin-clavulanic acid of 9 BLNAR strains. Both CLSI and EUCAST (with evidence grade C) recommend the reporting of resistance to amoxicillin-clavulanic acid, cefuroxime, and piperacillin-tazobactam, irrespective of *in vitro* susceptibility in BLNAR strains (Leclercq et al., 2013). Tristram et al. (2007) previously suggested the use of PK/PD breakpoints to predict the susceptibility to antibiotics. In addition, clinical studies that correlate the success of antimicrobial therapy to MIC values would be of special interest to clarify the clinical significance of the proposed MIC breakpoint values.

In conclusion, analysis of a set of *H. influenzae* isolates collected in the University Hospital UZ Brussel confirmed the presence of mutations in the *ftsI* gene and concomitant amino acid substitutions in all the analyzed isolates. Although 30% of the isolates were classified by the NRC to be BLNAS based on the EUCAST guidelines on ampicillin MIC, all isolates showed MIC values ≥1 mg/L. These relatively high MIC values suggest decreased susceptibility to ampicillin and therefore, sequencing of the *ftsI* gene should be further evaluated to determine whether or not a mutation can explain this decreased susceptibility. The analysis of a set of isolates with much lower MIC values for ampicillin will be necessary to provide more insight into the significance of the mutations observed here. Interestingly, the results also show the presence of additional substitutions (most frequently D350N and M377I) in *H. influenzae* strains that remained susceptible to ampicillin-clavulanic acid, as compared to those resistant to the latter. As an AST algorithm, we suggest the routinely use of the EUCAST algorithm based on benzylpenicillin-disk diffusion (EUCAST, 2018b) in clinical laboratories. In case of doubtful or unclear results, for example an MIC for ampicillin of 1 mg/L, an MIC for amoxicillin or amoxicillin-clavulanic acid of 2 mg/L, or unexpected resistance to second generation carbapenems…, the strain isolated from clinically relevant samples can be sent to the NRC for *ftsI*-sequencing. This would allow clinicians to make better informed decisions regarding patient treatment.

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Declarations of interest

None.
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