IDENTIFICATION OF INVASIVE BLNAR *HAEMOPHILUS INFLUENZAE* STRAIN FROM A PATIENT WITH ACUTE BRONCHITIS IN ROMANIA

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ABSTRACT

Introduction: The most important mechanism in β-lactamase-negative-ampicillin-resistant (BLNAR) isolates of *Haemophilus influenzae* is the alteration in the transpeptidase domain of penicillin binding protein 3 (PBP_3) as a result of genetic mutation.

Objective: The objective of this study was the phenotypic and molecular identification of β-lactam resistance mechanism in one invasive *H. influenzae* strain received from Dr. Victor Babeș Hospital.

Methods: One *H. influenzae* strain, isolated from blood, sampled from a patient hospitalized, in February 2012, was sent for confirmation to the Cantacuzino Institute. After phenotypic confirmation and molecular identification of the strain, minimum inhibitory concentration for ampicillin was assessed by E-test method. The resistance phenotype was evaluated using the disk diffusion method and the algorithm proposed by the Nordic Committee on Antimicrobial Susceptibility Testing (NordicAst). To detect changes in penicillin binding protein 3 (PBP_3), we amplified by PCR followed by partial sequencing the gene encoding the transpeptidase domain of PBP_3 (*fts*I).

Results: Both phenotypic and molecular tests confirmed that the strain was *H. influenzae.* Molecular typing showed it was serotype f. Antibiotic susceptibility testing indicated the existence of a BLNAR mechanism, confirmed by the mutational analysis of *fts*I gene fragment. Based on the presence of the N526K substitution, the strain belonged to low-BLNAR group II.

Conclusion: Accurate laboratory diagnostic of BLNAR phenotype is of major importance in guiding target antibiotherapy, especially for invasive infections. NordicAst recommendations represent the compromise solution in situations when sequencing is not available.

Keywords: *Haemophilus influenzae*, BLNAR, NordicAst, PBP_3, *ftsI*.

REZUMAT

Introducere: Cel mai important mecanism la tulpinile de *Haemophilus influenzae* β-lactamază-negative-ampicilină-rezistente (BLNAR) este reprezentat de modificarea domeniului transpeptidazei proteinei 3 de legare a penicilinei (PBP3) ca rezultat al mutației genetice.

Obiective: Obiectivul acestui studiu a fost identificarea fenotipică și moleculară a mecanismului de rezistență la β-lactamice la o tulpină de *H. influenzae* invazivă, primită de la Spitalul „Dr. Victor Babeș".

Metode: O tulpină de *H. influenzae* izolată din sânge, în februarie 2012, de la un pacient internat a fost trimisă pentru confirmare la Institutul „Cantacuzino". După confirmarea fenotipică și identificarea moleculară a speciei, s-a determinat concentrația minimă inhibitorie pentru ampicilină prin metoda E-test. Fenotipul de rezistență a fost identificat utilizând metoda disc difuzimetrică și algoritmul propus de Comitetul Nordic pentru Testarea Sensibilității Antimicrobiene (NordicAst). Pentru a detecta mutațiile proteinei 3 de legare la penicilină (PBP_3), s-a efectuat amplificarea prin PCR urmată de secvențierea parțială a genei care codifică domeniul transpeptidazei PBP_3 (*ftsI*).

Rezultate: Atât testele fenotipice cât și cele moleculare au confirmat faptul că tulpina este *H. influenzae*. Prin tipizare moleculară s-a identificat tipul capsular f. Testul de susceptibilitate la antibiotice a indicat existența unui mecanism BLNAR, confirmat prin detecţia substituțiilor în fragmentul genei *ftsI* secvențiat. Pe baza prezenței substituției N526K, tulpina aparține grupului II low-BLNAR.

Concluzie: Diagnosticul de certitudine a fenotipului BLNAR este de importanță majoră în ghidarea antibioterapiei țintite, în special pentru infecțiile invazive. Recomandările NordicAst sunt soluția de compromis atunci când secvențierea nu este întotdeauna disponibilă.

Cuvinte-cheie: *Haemophilus influenzae*, BLNAR, NordicAst, PBP_3, *ftsI*.

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INTRODUCTION

Haemophilus influenzae is a major cause of respiratory tract infections and can lead to mild to severe diseases (invasive infections).

Due to inappropriate use of antimicrobial agents, *H. influenzae* strains can develop resistance to β-lactams.

The most common mechanism of resistance to β-lactams is the production of TEM-1 and ROB-1 β-lactamases. Strains displaying such resistance are called β-lactamase-positive-ampicillin-resistant (BLPAR).

Another mechanism of resistance to β-lactams is the alteration in the transpeptidase domain of penicillin binding protein 3 (PBP_3) as a result of *ftsI* gene mutation, leading to decreased affinity to penicillins and cephalosporins. Strains with such a type of resistance are called β-lactamase negative-ampicillin-resistant (BLNAR).

Strains displaying both resistance mechanisms can be isolated sporadically, and are referred to as β-lactamase-positive amoxicillin clavulanate-resistant (BLPACR) strains.

BLNAR strains have an invasive character to epithelial cells, slightly more pronounced compared to β-lactamase negative ampicillin susceptible (BLNAS) strains. This suggests that amino acid substitutions involved in the resistance mechanism may result in invasive behavior causing the destruction of epithelial cells [1].

Based on their PBP_3 mutational profile, BLNAR strains are classified into several groups (I, II, III and III like) [2]. The resistance mediated by PBP_3 is defined by the presence of mutations in well-defined positions near the conserved domain KTG (Lys-Thr-Gly) and SSN (Ser-Ser-Asn) [2, 3]. Strains containing Arg substitution with His at position 517 (R517H) or Asn with Lys at position 526 (N526K) near KTG are considered group I respectively group II low-BLNAR. Strains with additional substitution near SSN motif (S385T) in combination with R517H and N526K are considered high-BLNAR strain types (group III like and group III) [2, 3, 4, 5, 6]. Debernat *et al.* (2002) have partially modified this classification scheme, and divided group II into four subgroups (IIa to IId). In subgroup IIa the only substitution is Lys-526 for Asn-526 (K526N); in subgroup IIb, Val-502 was substituted for Ala-502 (V502A), along with several other substitutions: Asn-350 for Asp-350 (N350D), Asn-350 for Asp-350 (N350D) and Glu-490 for Gly-490 (E490G), and Asn-350 for Asp-350 (N350D) and Ser-437 for Ala-437 (S437A).

In subgroup IIc, Thr-502 was substituted for Ala-502 (A502T). In subgroup IId, Val-449 was substituted for Ile-449 (I449V) [7].

Whereas the resistance of *H. influenzae* to β-lactam antibiotics is in constant change and this requires continuous attention, the purpose of this study was to identify and characterize the mechanism of β-lactam resistance of the invasive *H. influenzae* strain received at the Romanian National Center for *Haemophilus*.

MATERIALS AND METHODS

Bacterial isolate

In February 2012, an ampicillin intermediary resistant *H. influenzae* strain from Dr. Victor Babeș Hospital was received at the National Reference Center for *Haemophilus* in Cantacuzino Institute. This strain was isolated from the blood of an immunocompromised adult with HIV/AIDS and acute bronchitis.

Phenotypic and molecular identification

The strain was confirmated by X and V factor requirements and was serotyped by a slide agglutination test using monovalent *a* to *f* antisera strains. PCR for phenotypic identification and for capsular typing were performed as described previously [8], based on specific primers [9, 10, 11]. Single PCR amplifications were used for targeting three genes. First, outer membrane protein P_2 (*omp*P_2), which confirms the isolate as *H. influenzae.* Second, *bex*A gene (Van Ketel gene), which detects the strain's ability to export capsule to the cell surface. The third was a capsule type specific gene. For molecular typing, the f1/f2 and b1/b2 primers were chosen initially, considering that the most common capsular invasive strains in Europe can be of *f* or *b* type [11, 12].

Antimicrobial susceptibility testing

The disk diffusion method with ampicillin (2 μg), amoxicillin-clavulanic acid (2/1 μg),

benzylpenicillin (1U), ceftriaxone (30 μg), meropenem (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), tetracycline (15 μg), cloramphenicol (30 μg) and trimethoprim - sulfamethoxazole (25 μg) were used to evaluate growth inhibition zones. The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (version 5.0, 2015). *H. influenzae* ATCC 49247 (BLNAR) and ATCC 49766 (BLNAS) strains were used for quality control, as recommended by EUCAST. For confirming the ampicillin susceptibility, minimum inhibitory concentration (MIC) was determined using E-test strips. For the phenotypic identification of the β-lactam resistance mechanism, the strain was tested according to the Nordic Committee on Antimicrobial Susceptibility Testing (NordicAST) guidelines [13]. An inhibition zone of < 12 mm in initial screening with benzylpenicillin (1U) and a negative result of the nitrocefin assay equated to BLNAR identification. β-lactamase production was determined by chromogenic cephalosporin test using nitrocefin as specific substrate.

PCR and gene sequencing

Alteration in PBP_3 of the strain was investigated by partially sequencing the *fts*I gene. The *fts*Ifrw primer (5'-GACGATTTGGATAACCCATA-3', corresponding to positions 1197620 to 1197639 of the *H. influenzae* strain RD KW20 sequence) and the *fts*Irev primer (5'-CTGGATAATTCTGTCTCAGA-3', corresponding to positions 1199884 to 1199865 of the *H. influenzae* strain RD KW20 sequence) were used to amplify the *fts*I gene [14]. For the partial sequencing we used the same *fts*Irev primer together with the *fts*Iinrev primer (5'TAGAAAGCGGGCGATAAACACC-3', corresponding positions 1199238 to 1199217 of the *H. influenzae* strain RD KW20 sequence) [14].

The amplification reactions were performed using 1.25U of Go - Taq DNA Polymerase (Promega, Madison, USA). PCR conditions were as follows: initial denaturation step of 95 ^{0}C for 5 minutes, 30 cycles at 95 ^{0}C for 1 minute, 52 ^{0}C for 1 minute, and 72 ^{0}C for 4 minutes, followed by the final elongation step of 72 ^{0}C for 10 minutes. The PCR products were purified using the WIZARD SV gel and PCR Clean Up System Kit. The sequencing of the *fts*I gene fragment encoding the transpeptidase region of PBP_3 was performed using the Big Dye version 3.1 Terminator Cycle Sequencing Kit with 3130 DNA Genetic Analyser.

The sequences were edited with the BioEdit version 7.2.5 software to obtain a sequence of 1547nt, corresponding to the positions 435-1833 of *H. influenzae* RD KW20 strain (gi|16271976:1197840-1199672).

RESULTS AND DISCUSSION

Phenotypic identification

Phenotypic confirmation showed that the strain is *H. influenzae.* The strain serotyped by slide agglutination did not react with any of monovalent *a* to *f* antisera.

Molecular identification

The tested strain produced one specific amplicon with *omp*P_2 primers, indicating that the isolate is *H. influenzae.* The amplicon produced with VK primers highlighted the fact that the strain is capsulated. While the strain did not react to any specific antiserum, it produced one amplicon with f1/f2 primers indicating that the strain is type *f*.

Antimicrobial susceptibility testing and identification of the β-lactam resistance mechanism with NordicAst algorithm

According to EUCAST guidelines, the studied strain was susceptible to: ampicillin (16 mm), amoxicillin-clavulanic acid (18 mm), ceftriaxone (30 mm), meropenem (36 mm), ciprofloxacin (44 mm), nalidixic acid (32 mm), tetracycline (32 mm), cloramphenicol (38 mm) and trimethoprim- sulfamethoxazole (23 mm). Meanwhile, it was resistant to benzylpenicillin (10 mm). The E-test confirmation for ampicillin showed that the strain is resistant (MIC 2.0 μg/mL).

According to NordicAst algorithm, the benzylpenicillin disc (1U) discriminates BLNAR from BLNAS and the nitrocefin test discriminates BLNAR from BLPAR and

BLPACR. The studied strain was BLNAR, resistant to the benzylpenicillin disc (1U) and non β-lactamase producing.

Results of ftsI sequencing

The partial nucleotide sequence of the *fts*I gene of the BLNAR strain was determined. The deduced amino acid sequence of the transpeptidase region of PBP_3 was compared to the corresponding sequences from *H. influenzae* strain Rd KW20. Sequence analysis revealed four amino acid substitutions in six positions: T228I, A239E, D350N, N526K (Fig.1.), A530S and N569S.

The mutation N526K is defining group II BLNAR and co-occurs frequently with D350N, as described before [2]. In contrast to the study of Ubukata *et al.* (2001) and consistent with the study of Lam TT *et al.* (2015), the substitution of Ser at position 357 by Asn was not found in the tested isolate with N526K [2, 20]. In our study, based on the presence of the D350N and N526K substitutions, this strain with ampicillin MIC 2.0 μg/mL had a modified PBP_3, and belonged to low-BLNAR group II. Four other mutations were detected in this conserved region compared to the Rd KW20 sequence: T228I, A239E, A530S and N569S.

Nucleotide sequence accession number

The sequence of the mutations of the *ftsI* gene reported in this paper were submitted to the EMBL Nucleotide Sequence Database receiving LT906661 accession number.

Recently, BLNAR *H. influenzae* infections have increased considerably and therefore the identification and characterization of this type of strains are very important. To our knowledge, this is the first partial sequencing of the *ftsI* gene of BLNAR *H. influenzae* strain in Romania. In Europe, the vast majority of low-BLNAR isolates belong to group II, similar to our study [3, 6, 15, 16, 17, 18, 19, 20].

Low-BLNAR strains compared to high-BLNAR strains present difficulties in identification. For example, in our study, because the strain was borderline susceptible to ampicillin by the disc diffusion method, we confirmed the susceptibility by determining the minimum inhibitory concentrations using E-test method to identify BLNAR phenotype.

In addition, there is no international consensus on ampicillin resistence breakpoints, for example, EUCAST MIC > 1 μg/mL while CLSI MIC ≥ 4 μg/mL.

The clinical significance of the distinction between BLNAS and low-BLNAR isolates remains unclear. The identification of the resistance mechanism of a BLNAR strain is a confirmatory test for intermediary ampicillin susceptibility as per the CLSI guidelines.

The algorithm proposed by NordicAst for the detection of resistance to β-lactam antibiotics (based on EUCAST disc diffusion

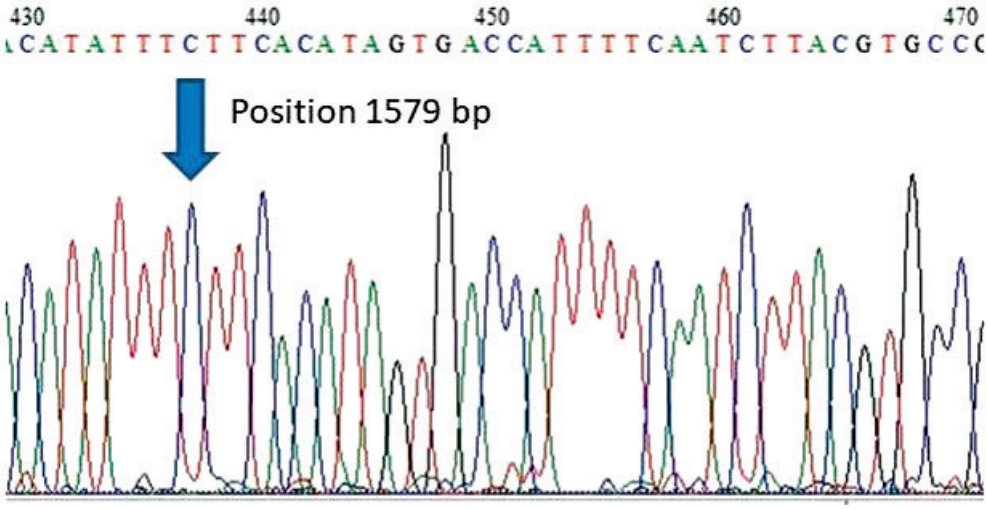

Fig. 1. Electropherogram of a 40 PCR product (reverse strand) encompassing the penicillin binding protein 3 gene (*fts*I). The arrow indicates the position corresponding to *Haemophilus influenzae* RD KW20 *fts*I gene (nc_000907, 1832pb) showing a T in G nucleotide change for N526K mutation.

breakpoints and the nitrocefin test) divides the *H. influenzae* strains into BLNAS, BLNAR, BLPAR and BLPACR. We encourage the use of the NordicAst method in routine testing when sequencing is not available.

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Conflict of interests: Authors declare that there is no conflict of interests.

REFERENCES

1. Okabe T, Yamazaki Y, Shiotani M, Suzuki T, Shiohora M, Kasuga E et al. An amino acid substitutions in PBP-3 in Haemophilus influenzae associate with the invasion to bronchial epithelial cells. Microbiol Res. 2010;165(1):11-20.
2. Ubukata K, Shibasaki Y, Yamamamoto K, Chiba N, Hasegawa K, Takeuchi Y et al. Association of Amino Acid Substitutions in Penicillin-Binding Protein 3 with β-Lactame Resistance in β-Lactamase-Negative Ampicillin-Resistent Haemophilus influenzae. Antimicrob Agents Chemother. 2001;45(6):1693-1699.
3. Skaare D, Anthonise IL, Caugant DA, Jenkins A, Steinbakk M, Strand L, et al. Multilocus sequence typing and ftsl sequencing: a powerful tool for surveillance of penicillin-binding protein 3-mediated beta-lactam resistance in nontypeable Haemophilus influenzae. BMC Microbiol. 2014;14:131.
4. Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K, et al. Rapidly increasing prevalence of beta-lactamase-nonproducing, ampicillin-resistent Haemophilus influenzae type b in patients with meningitis. Antimicrob Agents Chemother. 2004;48:1509-14.
5. Hotomi M, Fujihara K, Billal S, Suzuki K, Hishimura T, Bada S, et al. Genetic Characteristics and Clinical Dissemination of β-Lactamase-Negative-Ampicillin-Resistant Haemophilus influenzae Strains from Upper Respiratory Tract of Patients in Japan. Antimicrob Agents and Chemother. 2007;51(11):3969-3976.
6. Garcia-Cobos S, Campos J, Lazaro L, Roman F, Garcia-Rey C, Perez-Vazquez M, et al. Ampicillin-Resistant Non- β-Lactamase-Producing Haemophilus influenzae in Spain: Recent Emergence of Clonal isolates with increased Resistance to Cefotaxime and Cefixime. Antimicrob Agents Chemother. 2007;51(7)2564-2573.
7. Dabernat H, Delmas C, Seguy M, Palissier R, Faucon G, Benamanni S, et al. Diversity of β-Lactam Resistance-Conferring amino acid substitutions in Penicillin-Binding Protein 3 of Haemophilus influenzae. Antimicrob Agents Chemother. 2002;46(7):2208-2218.
8. Giucă MC, Străuț M, Surdeanu M, Nica M, Ungureanu V, Mihăescu G. Methodology Optimization and Diversification for the Investigation of Virulence Potential in Haemophilus influenzae Clinical Strains. Romanian Archives of Microbiology and Immunology. 2011;70(4):189-197.
9. Hobson RP, Williams A, Rawal K, Pennington TH, Forbeas KY. Incidence and spread of Haemophilus influenzae on an arctic base determined using the polymerase chain reaction. Epidemiol and Infect. 1995;114:93-103.
10. Van Ketel RJ, De Wever B, Van Alpher L. Detection of Haemophilus influenzae in cerebral fluids by polymerase chain reaction DNA amplification. J of Med Microbiol. 1990;33:271-276.
11. Falla TJ, Crook DWM, Brophy LM, Marskell D, Kroll JS, Moxon ER. PCR for capsular typing of Haemophilus influenzae. J of Clin Microbiol. 1994;32:2382-2386.
12. Whittaker R, Economopoulou A, Dias JG, Bancroft E, Ramliden M. Pastore Celentano L and European Centre for Disease Prevention and Control Country Experts for Invasive Haemophilus influenzae Disease. Epidemiology of Invasive Haemophilus influenzae Disease, Europa, 2007-2014. Emerg Infect Dis. 2017;23(3):396-404.
13. Sondergaard A, Petersen MT, Fuursted K, Norskov-Lauritsen N. 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. Journal of Antimicrob Chemother. 2012; 67(6):1401-1404.
14. Cerquetti M, Giufre M, Cardines R, Mastrantonio P. First Characterization of Heterogeneous Resistance to Imipenem in Invasive Nontypeable Haemophilus influenzae

Isolates. Antimicrob Agents and Chemother. 2007;51(9):3155-3161.
15. Dabernat H, Delmas C. Epidemiology and evolution of antibiotic resistance of Haemophilus influenzae in children 5 years of age or less in France, 2001-2008: a retrospective data base analysis. European J of Clin Microbiol & Infect Dis. 2012;31(10):1745-1753.
16. Barbosa AR, Giufre M, Cerquetti M, Bajanca-Lavado MP. Polymorphism in ftsI gene and β-lactam susceptibility in Portuguese Haemophilus influenzae strains clonal dissemination of β-lactamase-positive isolates with decreased susceptibility to amoxicillin/clavulanic acid. J Antimicrob Chemother. 2011;66(4):788-796.
17. Kiedrowska M, Kucha A, Zebicka D, Wasko I, Ronkiewicz P, Wasiak K, et al. β-Lactam resistance among Haemophilus influenzae isolates in Poland. J. of Global Antimicrob Resist. 2017;11:161-166.
18. Puig C, Grau I, Marti S, Tabau F, Calatayud L, Pallares L, et al. Clinical and Molecular Epidemiology of Haemophilus influenzae causing invasive Disease in Adults Patients. 2014; 9(11):e112711. PubMed PMID: 25379704.
19. Maddi S, Kolsum U, Jakson S, Barraclough R, Maschera B, Simpson KD, et al. Ampicillin resistance in Haemophilus influenzae from COPD patients in UK. Int J Chron Obstruct Pulmon Dis. 2017;12:1507-1518.
20. Lam T-T, Claus H, Elias J, Frosh M, Vogel U. Ampicillin resistance of Haemophilus influenzae isolates in Germany 2009-2012. Int Journal of Med Microbiol. 2015;305:748-755.