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Prevalence of *Haemophilus influenzae* with alteration of PBP 3 sequence over a 1-year period in a French hospital: focus on a clinical failure after ceftriaxone treatment

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ABSTRACT

Among 547 *Haemophilus influenzae* isolates recovered in our center, 45 displayed a phenotype of loss of PBP 3 affinity (8.2%). Two isolates with 6 substitutions in PBP 3 showed decreased susceptibility to third-generation cephalosporins. Clinical data revealed clinical failure after ceftriaxone treatment in a context of bronchitis in a patient with pulmonary sarcoidosis.

Haemophilus influenzae belongs to human oropharyngeal commensal flora and is incriminated in a wide variety of infections (Tristram et al., 2007). Since implementation of the *H. influenzae* type b conjugate vaccine in France in 1992, *H. influenzae* meningitis incidence has been divided by 10 (De Benoist et al., 1999). After a decrease, the bacteremia incidence is now similar to that observed in 1991, with an increase in people aged over 65 years (Réseau Epibac, 2017). As in other European countries (Whittaker et al., 2017), about 80% of invasive infections are currently caused by nontypeable strains (Gaillot, 2015), which can also cause noninvasive infections.

β-Lactams remain the main antibiotics used against *H. influenzae* (Tristram et al., 2007). Amoxicillin resistance may occur either by β-lactamase or by decreasing affinity with penicillin-binding protein 3 (PBP 3) modifications (Ubukata et al., 2001). PBP 3, encoded by the *ftsI* gene, has transpeptidase activity and is involved in the septal peptidoglycan synthesis. In 2015, 20% of the strains studied by the French National Reference Center produced β-lactamase, and 5% were β-lactamase–negative ampicillin-resistant (BLNAR) (Gaillot, 2015).

The aim of our work was to evaluate the prevalence of *H. influenzae* clinical isolates with alteration of PBP 3 sequence in our center and to study their susceptibility to third-generation cephalosporins.

Susceptibility to ampicillin (2 μg), amoxicillin–clavulanate (2–1 μg), tetracycline (30 μg), and trimethoprim–sulfamethoxazole (1.25–23.75 μg) was determined by disc diffusion method according to EUCAST 2016 recommendations. Nitrocefin test was also performed in daily practice. Minimum inhibitory concentrations (MICs) for cefotaxime, ceftriaxone, and levofloxacin were determined by Etest® on MHF medium (bioMérieux, Marcy l'Etoile, France).

Among 547 *H. influenzae* isolates recovered from 441 patients between April 1, 2016, to March 31, 2017, 45 isolates from 44 patients were amoxicillin–clavulanate resistant. Thirty-seven of 45 isolates were recovered in a context of respiratory infection, 6/45 in a context of otorhinolaryngological infection, and 2/45 in a context of genital infection. Twelve of 45 isolates produced β-lactamase. Sequencing of the *ftsI* gene could be performed as previously described (Dabernat et al., 2002) for 37 isolates and revealed PBP 3 modifications in all of them (Table 1).

Forty-three of 45 isolates were susceptible to third-generation cephalosporins with MICs for cefotaxime ranging from 0.012 to 0.094 μg/mL. Two isolates recovered from the same patient showed decreased susceptibility to third-generation cephalosporins, without producing β-lactamase. They both displayed the same MICs: 0.25 μg/mL for cefotaxime (resistant according to EUCAST), 0.094 μg/mL for ceftriaxone (10-fold that of wild strains [Hasegawa et al., 2006]), and 0.16 μg/mL for levofloxacin. They were tetracycline susceptible and trimethoprim–sulfamethoxazole resistant. The deduced amino acid sequences of PBP 3 revealed 6 substitutions for both isolates including Ser357Asn, Met377Ile, Ser385Thr, Leu389Phe, Ala502Thr, and Asn526Lys. Pulsed-field gel

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Table 1
Deduced amino acid substitutions identified in PBP 3 from 37 amoxicillin-clavulanate–resistant strains of *H. influenzae*.

Group[a]	Strain or no. of strains	Amino acid substitutions										MIC for cefotaxime (μg/mL)
		Ser-357	Ala-368	Met-377	Ser-385	Leu-389	Ala-437	Ile-449	Gly-490	Ala-502	Arg-517	Asn-526	
Control	ATCC 9007												0.012
I	1										His		0.032
II	1											Lys	0.094
	2							Val				Lys	0.023–0.032
	3									Val		Lys	0.032
	1		Thr							Thr		Lys	0.032
	1		Thr							Val		Lys	0.047
	1							Val		Val		Lys	0.047
	1						Ser			Val		Lys	0.047
	1								Glu	Val		Lys	0.047
	18			Ile						Val		Lys	0.023–0.094
	5			Ile					Glu	Val		Lys	0.023–0.047
III+	2	Asn		Ile	Thr	Phe				Thr		Lys	0.25

[a] According to Ubukata et al. (2001) and Skaare et al. (2014).

electrophoresis and relatedness analysis were performed as previously described (Dabernat et al., 2002). Macrorestriction profiles were identical for both isolates, thus belonging to the same cluster. They were recovered from sputum in a 58-year-old immunocompromised patient with digestive and pulmonary sarcoidosis. His medical history included vestibular neuritis 4 years earlier and depressive disorder. Clinical data are summarized in Fig. 1. We report here a clinical failure after an 8-day ceftriaxone treatment in a context of *H. influenzae* bronchitis. The BLNAR strain involved was isolated before and after treatment.

More than 20 different substitutions in PBP 3 have been described in BLNAR strains. The presence of Arg517His or Asn526Lys appears to be essential for loss of PBP 3 affinity (Tristram et al., 2007). Ubukata et al. demonstrated the role of 3 additional substitutions leading to increase of cefotaxime MIC: Met377Ile, Ser385Thr, and Leu389Phe (Ubukata et al., 2001). Several other Japanese studies confirmed the impact of these additional substitutions in third-generation cephalosporin's loss of activity (Hasegawa et al., 2006; Kubota et al., 2006). Such strains were also reported in Spain (Garcia-Cobos et al., 2007), South Korea (Park et al., 2013), and Norway (Skaare et al., 2014). Garcia-Cobos et al. assumed that the initial step leading to cefotaxime resistance was achieved by additional substitutions Met377Ile and Ser385Thr, and that Leu389Phe further increased resistance (Garcia-Cobos et al., 2007). Osaki et al. introduced mutations into the *ftsI* gene of a susceptible strain and found that cephalosporins resistance depended on Arg517His, Asn526Lys, Ser385Thr, and Leu389Phe but not on Met377Ile (Osaki et al., 2005).

In France, among 108 strains with PBP 3 substitutions received at the National Reference Center until 2001, the triple substitution was not encountered and no cefotaxime-resistant strain was observed (Dabernat et al., 2002). From 2002, rare cefotaxime-resistant *H. influenzae* strains exhibiting Ser385Thr were reported (Gaillot, 2015). The situation is strikingly different in Japan, where strains with Arg517His or Asn526Lys, associated with Met377Ile, Ser385Thr, and Leu389Phe, increased from 6% to 20% of clinical isolates between 1997 and 2003 (Sanbongi et al., 2006). This difference could be linked to prescribing habits, with more use of low doses of oral cephalosporins in Japan. Because of the high cephalosporins' affinity for PBP 3, mutations in *ftsI* can be selected under their selective pressure (Hasegawa et al., 2006). Such mutations may result of interspecies recombination of *ftsI* with *H. haemolyticus* (Witherden et al., 2014).

In this case, *ftsI* sequencing revealed 6 substitutions, classifying the isolates in the group III+ of the high-level PBP 3-mediated resistance strains according to Skaare classification (Skaare et al., 2014). To our knowledge, this case represents most likely the first clinical failure after ceftriaxone treatment of *H. influenzae* bronchial superinfection, with the first description of this combination of 6 substitutions in a French strain. The patient had a history of antibiotic-treated bronchial superinfections and received cefpodoxime few days before the strain isolation, which may explain mutations selection.

The strain remained susceptible to fluoroquinolones, allowing treatment with levofloxacin. Clinical failures with high-dose ceftriaxone and cefotaxime have been reported in Japan in *H. influenzae* type b BLNAR strains meningitis in children (Hasegawa et al., 2006). In this situation, meropenem could be an alternative (Fujimoto et al., 2013; Hasegawa et al., 2006). Nevertheless, nonsusceptibility in vitro to this antibiotic has been described in group III+ (Skaare et al., 2014).

Over a 1-year period in our laboratory, only 2 *H. influenzae* isolates showed decreased susceptibility to third-generation cephalosporins. However, dissemination of such strains has been described in Europe (Garcia-Cobos et al., 2007). Furthermore, a group III+ clone with β-

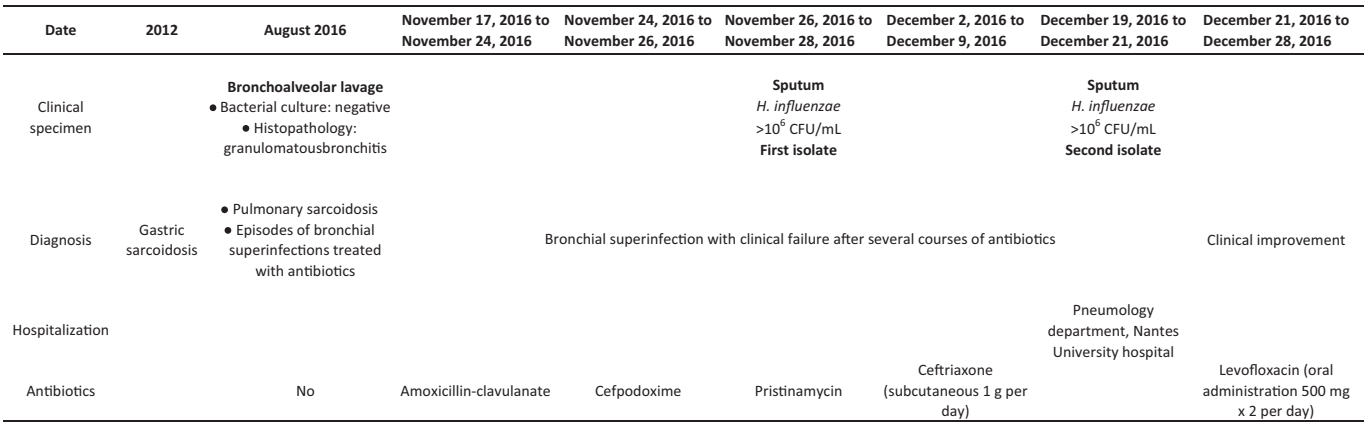

Fig. 1. Summary of the patient's clinical history.

lactamase and co-resistance to ciprofloxacin, tetracycline, chloramphenicol, and trimethoprim–sulfamethoxazole has been described in Norway (Skaare et al., 2014), highlighting the need to carefully follow the resistance of strains with loss of PBP 3 affinity. In the future, microbiologists and clinicians should be aware of the possibility of reduced susceptibility to third-generation cephalosporins in *H. influenzae*.

References

Dabernat H, Delmas C, Seguy M, Pelissier R, Faucon G, Bennamani S, et al. Diversity of beta-lactam resistance–conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. Antimicrob Agents Chemother 2002;46(7):2208–18.

De Benoist AC, Laurent E, Goulet V. Infections invasives à *Haemophilus influenzae*, *Listeria monocytogenes*, méningocoque, pneumocoque, streptocoques groupe A et groupe B en France en 1997 – évolution 1991–1997. Institut de veille sanitaire; BEH N°15; 1999.

Fujimoto K, Kanazawa K, Takemoto K, Urasaki K, Ueda Y, Ubukata K, et al. Therapeutic effect of meropenem on an experimental guinea pig model of meningitis with type b β-lactamase–nonproducing ampicillin-resistant *Haemophilus influenzae*. J Infect Chemother 2013;19(4):593–8.

Gaillot O. Capsules, résistance et infections materno-fœtales: actualités 2015 de *Haemophilus influenzae*. Communication 234, RICAI 2015, Paris; 2015.

Garcia-Cobos S, Campos J, Lazaro E, Roman F, Cercenado E, Garcia-Rey C, 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):2564–73.

Hasegawa K, Kobayashi R, Takada E, Ono A, Chiba N, Morozumi M, et al. High prevalence of type b beta-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. J Antimicrob Chemother 2006;57(6):1077–82.

Kubota T, Higa F, Kusano N, Nakasone I, Haranage S, Tateyama M, et al. Genetic analyses of beta-lactamase negative ampicillin-resistant strains of *Haemophilus influenzae* isolated in Okinawa, Japan. Jpn J Infect Dis 2006;59(1):36–41.

Osaki Y, Sanbongi Y, Ishikawa M, Kataoka H, Suzuki T, Maeda K, et al. Genetic approach to study the relationship between penicillin-binding protein 3 mutations and *Haemophilus influenzae*-lactam resistance by using site-directed mutagenesis and gene recombinants. Antimicrob Agents Chemother 2005;49(7):2834–9.

Park C, Kim K-H, Shin N-Y, Byun J-H, Kwon E-Y, Lee J-W, et al. Genetic diversity of the ftsI gene in β-lactamase–nonproducing ampicillin-resistant and β-lactamase–producing amoxicillin-/clavulanic acid–resistant nasopharyngeal *Haemophilus influenzae* strains isolated from children in South Korea. Microb Drug Resist 2013;19(3):224–30.

Réseau Epibac. Bulletin du réseau de surveillance des infections invasives bactériennes. France: Santé Publique; 2017.

Sanbongi Y, Suzuki T, Osaki Y, Senju N, Ida T, Ubukata K. Molecular evolution of -lactam-resistant *Haemophilus influenzae*: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. Antimicrob Agents Chemother 2006;50(7):2487–92.

Skaare D, Anthonisen IL, Kahlmeter G, Matuschek E, Natås OB, Steinbakk M, et al. Emergence of clonally related multidrug resistant *Haemophilus influenzae* with penicillin-binding protein 3–mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013. Euro Surveill 2014;19(49). (pii: 20986).

Tristram S, Jacobs MR, Appelbaum PC. Antimicrobial resistance in *Haemophilus influenzae*. Clin Microbiol Rev 2007;20(2):368–89.

Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, et al. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase–negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother 2001;45(6):1693–9.

Whittaker R, Economopoulou A, Dias JG, Bancroft E, Ramliden M, Celentano LP, et al. Epidemiology of invasive *Haemophilus influenzae* disease, Europe, 2007–2014. Emerg Infect Dis 2017;23(3):396–404.

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A. Role of inter-species recombination of the ftsI gene in the dissemination of altered penicillin-binding-protein-3–mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. J Antimicrob Chemother 2014;69(6):1501–9.