Imipenem Heteroresistance in Nontypeable Haemophilus influenzae

Cherkaoui, Abdessalam

UNIVERSITE DE GENEVE

Département de biologie moléculaire — FACULTE DES SCIENCES

Professeur Robbie LOEWITH

Département de médecine — FACULTE DE MEDECINE

Professeur Jacques SCHRENZEL

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Par

Abdessalam CHERKAOUI

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Eur J Clin Microbiol Infect Dis. 2018 Nov;37(11):2201-2210. doi: 10.1007/s10096-018-3362-z. Epub 2018 Aug 25.

Transcriptional Modulation of Penicillin-Binding Protein 1b, Outer Membrane Protein P2 and Efflux Pump (AcrAB-TolC) during Heat Stress Is Correlated to Enhanced Bactericidal Action of Imipenem on Non-typeable *Haemophilus influenzae*.
Cherkaoui A, Diene SM, Fischer A, Leo S, François P, Schrenzel J.
Front Microbiol. 2018 Jan 12;8:2676. doi: 10.3389/fmicb.2017.02676. eCollection 2017.

Imipenem heteroresistance in nontypeable *Haemophilus influenzae* is linked to a combination of altered PBP3, slow drug influx and direct efflux regulation.
Cherkaoui A, Diene SM, Renzoni A, Emonet S, Renzi G, François P, Schrenzel J.
Clin Microbiol Infect. 2017 Feb;23(2):118.e9-118.e19. doi: 10.1016/j.cmi.2016.10.009. Epub 2016 Oct 15.

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Eur J Clin Microbiol Infect Dis. 2015 Oct;34(10):1937-45. doi: 10.1007/s10096-015-2435-5. Epub 2015 Jul 18.

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1. Abbreviations

Hib: *Haemophilus influenzae* type b

NTHi: nontypeable (i.e. nonencapsulated) *Haemophilus influenzae*

IMI^{hR}: Imipenem heteroresistance

PBP3: penicillin-binding protein 3

PBP1b: penicillin-binding protein 1b

AcrAB-TolC: multidrug efflux pump

OmpP2: outer membrane protein P2

PRP: polyribosyl-ribitol phosphate

COPD: chronic obstructive pulmonary disease

NGS: Next-Generation Sequencing

NAD: nicotinamide-adenine-dinucleotide

NMN: nicotinamide mononucleotide

NR: nicotinamide riboside

sBHI: XV-supplemented brain-heart infusion broth

EUCAST: European Committee for Antimicrobial Susceptibility Testing

CLSI: Clinical and Laboratory Standards Institute

MH-F: Müller-Hinton agar + 5% horse blood + 20 mg/L β-NAD

MIC: minimum inhibitory concentration

PBPs: penicillin-binding proteins

BLNAR: β-lactamase-negative, ampicillin-resistant

BLPACR: β-lactamase positive, amoxicillin/clavulanic acid-resistant

gBLNAR: genomic BLNAR (Isolates with key mutations in PBP-3 regardless of resistance phenotype)

gBLPACR: genomic BLPACR (Isolates with key mutations in PBP-3 regardless of resistance phenotype)

ESBL: extended-spectrum β-lactamase

SCVs: small colony variants

Hsps: heat-shock proteins

CCCP: Carbonyl cyanide *m*-chlorophenylhydrazone

IC_{50} : 50% inhibitory concentration

DDDs : defined daily doses

QRDRs : quinolone resistance-determining regions

2. Résumé

Haemophilus influenzae est un bacille pléomorphique à Gram négatif. Les souches peuvent exister sous forme non-encapsulée ou sous forme encapsulée qui se caractérisent par la présence d'une capsule polysaccharidique composée de polyribosyl-ribitolphosphate (sérotypes a à f) (1).

H. influenzae sérotype b (Hib) a longtemps été considéré comme la principale cause d'infection invasive chez les jeunes enfants (<5 ans). Ainsi avant la mise en place des programmes de vaccination contre Hib, l'incidence des infections invasives à *H. influenzae* était de 20 à 50 pour 100'000 dans les pays industrialisés et jusqu'à dix fois plus élevée encore dans les pays en voie de développement (2).

Entre 1988-1990 et 2011 en Suisse, l'incidence des infections invasives à *H. influenzae* chez les enfants de moins de 5 ans avait chuté drastiquement en passant de 40 à 1 pour 100'000. Cependant durant la même période, il était constaté que chez les personnes âgées (>64 ans), l'incidence des infections invasives à *H. influenzae* avait augmenté de 1 à 5 pour 100'000 (3, 4). Cette tendance, relevée dans plusieurs pays, a été associée à des souches d'*H. influenzae* non-encapsulées.

De manière générale, la réduction drastique et constante de l'incidence des infections invasives à *H. influenzae* dans différents pays a été associée à l'introduction du vaccin conjugué Hib dans les programmes nationaux de vaccination infantile. Malheureusement, de nos jours, les infections invasives à *H. influenzae* sont principalement causées par les souches non-encapsulées, suivies par les sérotypes encapsulés non-b (5-7), donc pas couverts par les vaccins conjugués contre Hib.

H. influenzae est classiquement décrit dans deux types d'infections, différentes selon leur épidémiologie et leur gravité. L'infection non-invasive affecte généralement les voies respiratoires supérieures, alors que l'infection invasive englobe la bactériémie, la méningite, ainsi que l'infection d'autres sites normalement stériles. La colonisation

asymptomatique du nasopharynx humain a été considérée comme la première étape de la pathogenèse de ce microorganisme. Chez les patients colonisés, les infections non-invasives, comme la sinusite et l'otite moyenne, sont la conséquence de la diffusion contiguë des bactéries vers les sinus paranasaux ou l'oreille moyenne. Cependant, la méningite et les autres infections invasives graves, y compris l'arthrite septique, l'ostéomyélite, et la péricardite, sont la conséquence du passage de la bactérie dans le sang et de sa diffusion secondaire vers les autres sites (8, 9). Les souches non-encapsulées sont responsables d'un large éventail de maladies. La pneumonie et la bactériémie demeurent cependant les infections les plus fréquentes chez les personnes âgées présentant des facteurs de risques (5).

Ces dernières années, le traitement des infections à *H. influenzae* a été sérieusement affecté par la résistance aux aminopénicillines et à certaines céphalosporines. Pendant longtemps, l'ampicilline (seule ou en combinaison avec le chloramphénicol) a été considérée comme l'antibiotique de première intention dans le traitement des infections invasives à *H. influenzae* (10). De nos jours, la résistance à l'ampicilline chez *H. influenzae* est globalement répandue, avec des taux allant de 8 à 30% dans différents pays européens et en Amérique du Nord, jusqu'à plus de 50% dans certains pays d'Asie de l'Est (11). La résistance aux carbapénèmes semble, par contre, très faible chez *H. influenzae.* Cependant, avec la découverte du phénomène d'hétéro-résistance à l'imipénème chez *H. influenzae,* on pourrait supposer que cette faible résistance est due en grand partie à un sous-diagnostic par les laboratoires de microbiologie clinique. Quelques études ont montré que certaines substitutions spécifiques d'acides aminés au sein de PBP3, telles que Asn526Lys, Gly490Glu Ala502Thr, et Met377Ile, semblent être impliquées dans la résistance à l'imipénème (12, 13). Par contre, ces substitutions ne permettent pas, à elles seules, d'expliquer les valeurs très élevées des concentrations minimales inhibitrices de l'imipénème

mesurées chez certains souches (CMI >32 mg/L), suggérant ainsi que les mécanismes menant à l'hétéro-résistance à l'imipénème chez *H. influenzae* n'ont pas été totalement clarifiés.

Dans ce travail de thèse, nous avons démontré qu'une modification de la protéine de liaison à la pénicilline 3 (PBP3), le ralentissement de l'influx, ainsi que la régulation négative de l'efflux direct de l'imipénème contribuent au développement de l'hétéro-résistance à l'imipénème chez *H. inflenzae*.

Nous avons ensuite établi que l'amélioration de la susceptibilité à l'imipénème dans des conditions de stress thermique dépend en grande partie des niveaux d'expression de PBP1b, AcrAB-TolC et OmpP2.

La 3ème partie de cette thèse a été consacrée à l'analyse des mutations au niveau des QRDRs (quinolone resistance-determining regions) et la confirmation du rôle de AcrAB-TolC dans la résistance de *H. influenzae* à l'imipénème.

Enfin, nos travaux mettent l'accent sur la nécessité d'établir des méthodologies et des critères standardisés pour détecter l'hétéro-résistance à l'imipénème voire à d'autres antibiotiques dans les laboratoires de microbiologie clinique.

3. Abstract

Several lines of evidence demonstrate that the impact of the pleomorphic Gram-negative bacterium, *Haemophilus influenzae* on human health has significantly changed over the past decades. Prior to the introduction of the *Haemophilus influenzae* type b (Hib) conjugate vaccine into childhood immunization programs, Hib was a leading cause of invasive bacterial infections in children younger than 5 years. In the post-vaccine era, the nontypeable (i.e. nonencapsulated) *H. influenzae* (NTHi) revealed as an opportunistic pathogen causing and exacerbating multiple upper and lower respiratory tract diseases. Worryingly, several studies in various post-vaccine populations have observed a steadily increase of NTHi invasive incidence rates and antibiotic resistance, highlighting the importance to investigate its resistance mechanisms and to better understand its stress response pathways. In this thesis work, we provide evidence indicating that altered penicillin-binding protein 3 (PBP3), slowed drug influx and direct efflux regulation contribute to the development of imipenem hetero-resistance in NTHi. We then established that the enhancement of imipenem-heteroresistant NTHi susceptibility to imipenem under heat stress conditions depends largely on the expression levels of PBP1b, AcrAB-TolC, and OmpP2, indicating again the role of the same pathways. Finally, we characterized the mechanisms of resistance to fluoroquinolones and macrolides in *H. influenzae*; assessed the extent of the AcrAB-TolC-mediated imipenem resistance; and defined a core genome multilocus sequence typing (cgMLST) scheme for *H*. *influenzae* by using whole-genome sequencing.

Furthermore, our work underlines the need to establish standardized methodologies and criteria to detect imipenem heteroresistance in clinical microbiology laboratories.

4. Introduction

Haemophilus influenzae are pleomorphic, small Gram-negative rods or coccobacilli with occasional longer filamentous forms. The history of this microorganism has been marked by some interesting and notable events. On the need to identify the etiologic agent responsible for the overwhelming 1889-1892 pandemic of influenza, Richard Friedrich Johannes Pfeiffer (27 March 1858 – 15 September 1945) noted the constant presence of large numbers of small rod-shaped bacteria in the sputum of patients. Thereby, this microorganism was wrongly considered to be the causative agent (14). The real agent responsible of influenza was identified in 1933 as a virus (*Myxovirus influenzae*). However it remains more than likely that bacterial superinfection has been a significant cause of the high mortality rates seen during the 1889-1892 and 1918-1919 pandemics. *H. influenzae* type b (Hib) has long been seen as a leading cause of invasive infection in young children (less than 5 years old). The incidence of a child contracting an invasive *H. influenzae* infection, before the introduction of Hib immunization, was 20 to 50 per 100’000 in industrialized countries and up to ten times higher in developing countries (2). In the 1970s, the polyribosyl-ribitol phosphate (PRP) capsular polysaccharide of Hib was used to develop the first vaccines for the prevention against Hib disease (15). An important trial conducted in 1974 in Finland showed that among children aged 18 to 71 months, the efficacy of one dose of PRP vaccine was about 90%. It shoud be noted, however, that the infants aged 3 to 17 months were not protected by these vaccines (15, 16). In the 1980s a new generation of Hib vaccines was developed. The better immunogenicity of these new vaccines was achieved by conjugating PRP with different protein antigens.

Three conjugate vaccines were licensed by the US Food and Drug Administration (15):

- December 1987 : PRP-D
- December 1988 : HbOC
- December 1989 : PRP-OMP

Date		Vaccine type	Age (month) at First Dose
April	1985	PRP	24*
December	1987	Conjugate	18
April	1990	Conjugate	15
October	1990	Conjugate	2

Table-1 (source (15)): Chronology of Hib vaccines licensure in the United States related to the recommended age for the first dose.

PRP: Hib polysaccharide vaccine (polyribosyl-ribitol phosphate).
*Eighteen months for children enrolled in day care.

The most spectacular achievements in the deployment of huge Hib vaccine programs were a rapid and important decline in the incidence of children contracting invasive *H. influenzae* infection (17, 18). These epidemiological trends could also be linked to the real impact of these vaccines to decrease oropharyngeal colonization by Hib (19).

The Hib vaccination of infants was recommended in Switzerland in 1991 (Table-2).

	Age			
Vaccine type	**2 months**	**4 months**	**6 months**	**15 months**
PRP–D		dose 1	dose 2	booster dose
PRP–OMP	dose 1	dose 2		booster dose
HbOC	dose 1	dose 2	dose 3	booster dose

Table-2 (source (4)): Hib vaccination schedule recommended by Swiss Federal Office of Public Health in 1991.

In Switzerland the incidence of invasive *H. influenzae* infection in children younger than 5 years fell from 40 to 1 per 100'000 in the period between 1988-1990 and 2011 (3, 20). However, in the same period, the incidence of invasive *H. influenzae* infection in elderly persons (>64 years old) increased from 1 to 5 per 100'000. The same phenomenon was witnessed in different countries. The nonencapsulated *H. influenzae* (NTHi) was identified as the principle cause of increased invasive *H. influenzae* infection in elderly persons (21-23).

Nowadays, fewer than 10 cases of invasive *H. influenzae* infection are reported annually in children younger than 5 years in Switzerland (Figure-1). Most of them are not -or incompletey- vaccinated (3).

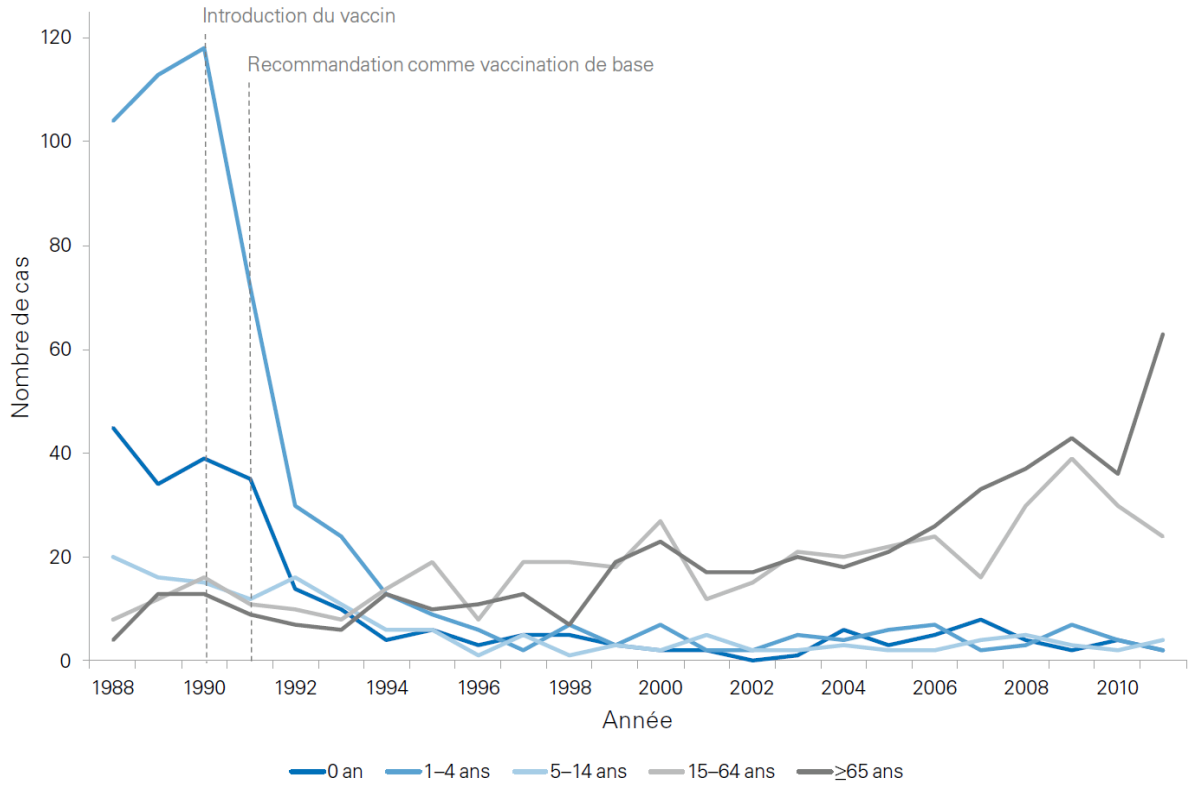

Figure-1 (source (3)): Number of cases of invasive *H. influenzae* infection, by patient age group, in Switzerland in the period between 1988 and 2010.

Comparable figures are observed when analyzing *H. influenzae* strains isolated in Geneva University Hospitals (HUG) in the period between January 2009 and December 2016 (Figure-2, and Figure-3).

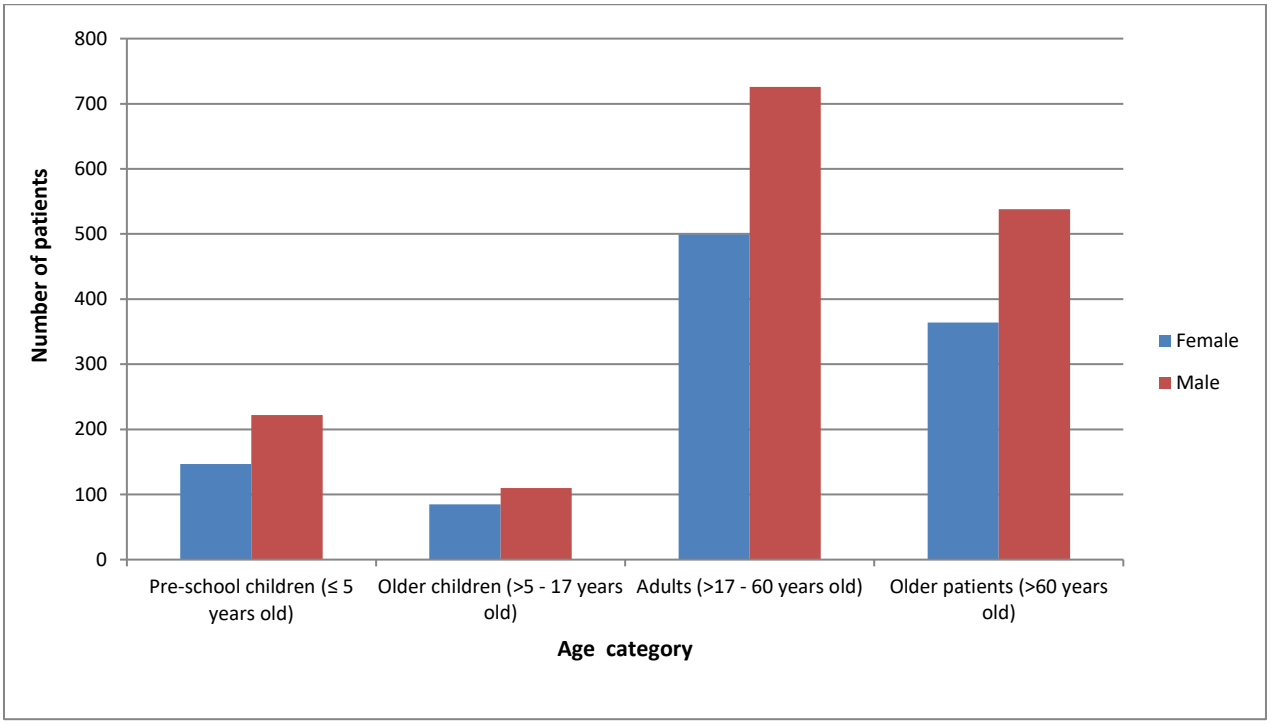

Figure-2: Patients with any type of *H. influenzae* infection according to the age category and gender in Geneva University Hospitals (HUG) in the period between January 2009 and December 2016. (A. Cherkaoui, unpublished data).

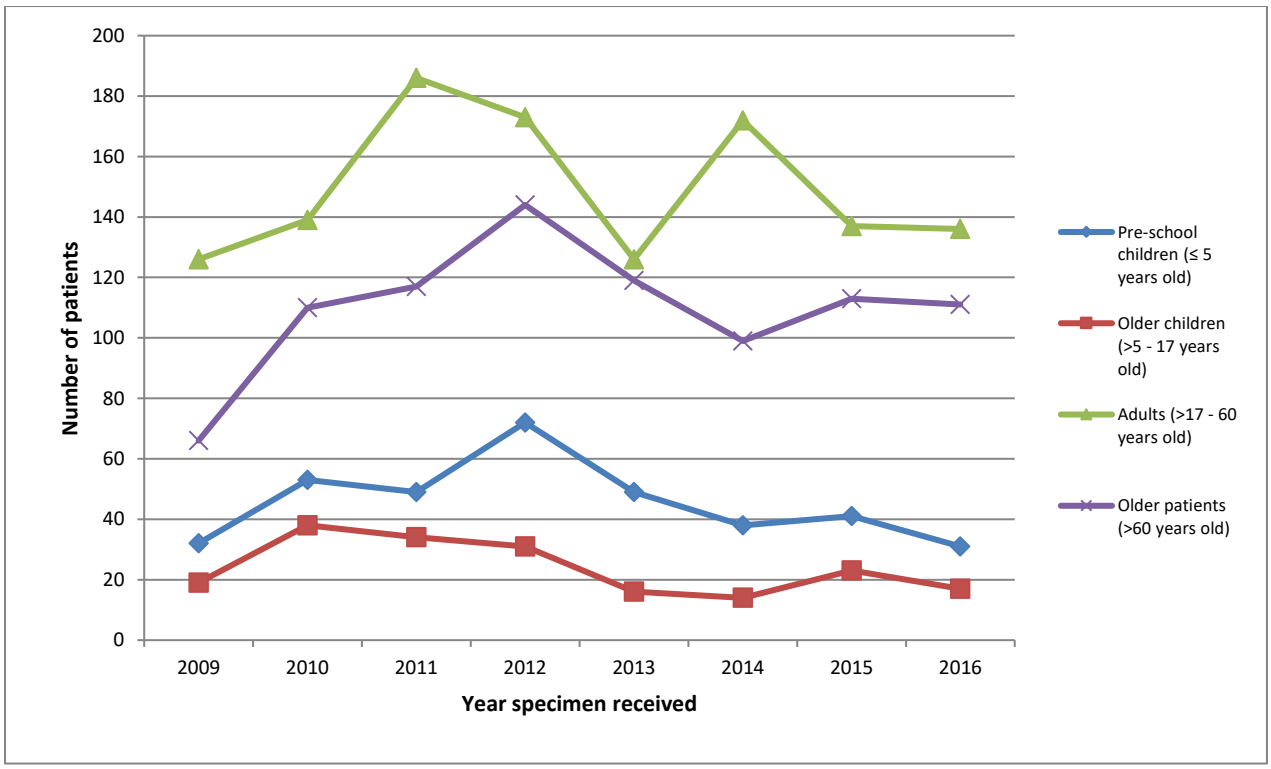

Figure-3: Temporal trends for any type of *H. influenzae* infection according to the age category in Geneva University Hospitals (HUG) in the period between January 2009 and December 2016. (A. Cherkaoui, unpublished data).

To further analyze bacterial trends, one needs to classify the organism according to specific features, using typing methods. Regarding *H. influenzae,* strains are described as either typeable (i.e. encapsulated) or nontypeable (i.e. nonencapsulated) (Figure-4).

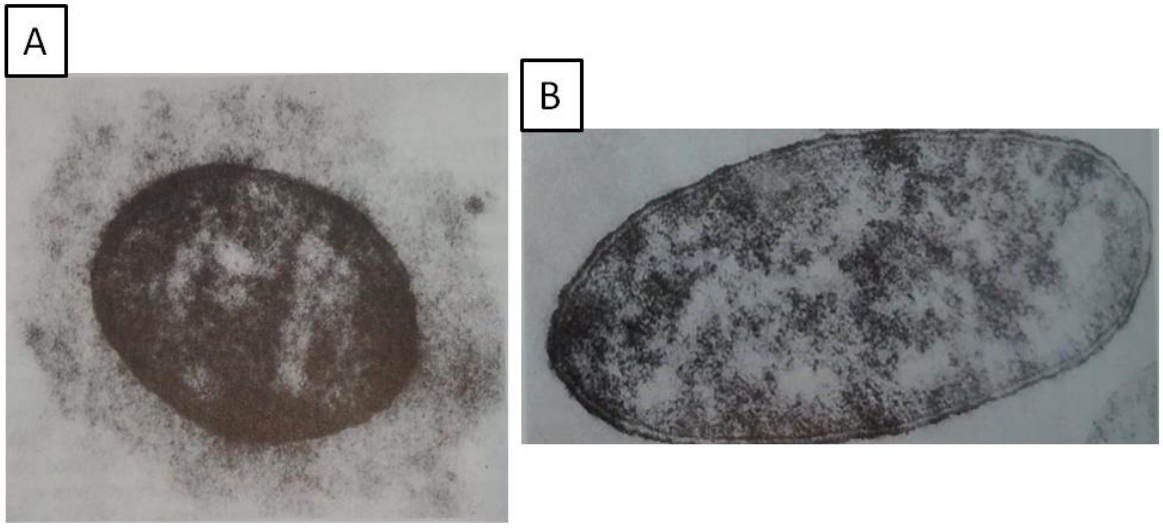

Figure-4 (source (1)): Electron micrographs depicting an encapsulated type b strain (**A**) and a nonencapsulated (i.e. nontypeable) strain (**B**) of *H. influenzae.*

There are currently six encapsulated serotypes (designated a–f) that possess distinct capsular polysaccharides (5). Importantly, Hoiseth and Gilsdorf reported in 1988 that some Hib strains may control and switch their capsular expression *in vivo* and *in vitro* (24). This suggests that further typing methods might be required to provide more robust genotyping information.

Both, typeable and nontypeable *H. inflIuenzae* strains can be classified into eight biotypes based upon the presence or absence of indole, urease, and ornithine decarboxylase activities (Table-3).

Biotype	Production of :		
	Indole	Ornithine decarboxylase	Urease
I	+	+	+
II	+	+	-
III	-	+	-
IV	-	+	+
V	+	-	+
VI	-	-	+
VII	+	-	-
VIII	-	-	-

Table-3 (source (1)) : Biotypes of *Haemophilus inflluenzae.*

It is reported that some biotypes are associated to certain specific clinical syndromes. *H. influenzae* biotype *aegyptius* (also known as biotype III) were shown to be associated with the syndrome of Brazilian purpuric fever. This syndrome was first reported in the town of Promissao, Sao Paulo State, Brazil in late 1984 (25). The disease was characterized by purulent conjunctivitis, vomiting, abdominal pain and onset of high fever followed by purpura, and vascular collapse (25, 26). The biotype IV was shown associated with urogenital, neonatal and mother-infant infections (27).

Nontypeable *H. influenzae* (NTHi) are commonly identified within the nasopharyngeal flora. The prevalence of nasopharyngeal carriage varies with age, geographic location, and vaccine coverage (28-30). Schumacher *et* al. reported that the prevalence of NTHi varied from 14% in infants younger than than 6 months to 32% in infants aged 19-26 months (28). The colonization rate of NTHi in the first year of life was estimated to about 20% (29, 30). In addition, NTHi can also be transmitted directly between humans (31-33). The analysis of the genetic diversity of NTHi revealed that this group is much

more diverse, with less evidence of clonal clustering than that observed in encapsulated strains (34, 35).

5. Pathogenesis

H. influenzae is classically described in two types of infections, differing in their epidemiology and severity. The non-invasive infection generally affects the upper respiratory tract, however, the invasive infection encompasses bacteremia, meningitis and the infection of other normally sterile tissue sites (5, 29). The colonization of the human nasopharynx asymptomatically is recognized as the first step in the pathogenesis of this microorganism. In colonized patients, non-invasive infections such as sinusitis, otitis media are caused by the contiguous spread of the bacteria to the paranasal sinuses or the middle ear. However, meningitis and other serious infections including septic arthritis, osteomyelitis, pericarditis, cellulitis and epiglottitis are initiated by the invasion of the bloodstream with the secondary spread to other sites (8, 9).

Before Hib vaccine era, Hib was the most common cause of invasive bacterial infection in children, with more than 20% of patients surviving Hib meningitis suffering long-term sequelae, ranging from mild hearing loss to mental retardation (9). A considerable and constant reduction in the incidence of invasive *H. influenzae* infection in different countries has been linked to the introduction of the Hib conjugate vaccine into national childhood immunization programs. Unfortunately the invasive *H. influenzae* infection appears nowadays primarily associated with NTHi, followed by non-b encapsulated serotypes with more cases reported for serotype f (5-7) (Figure-5 and Figure-6).

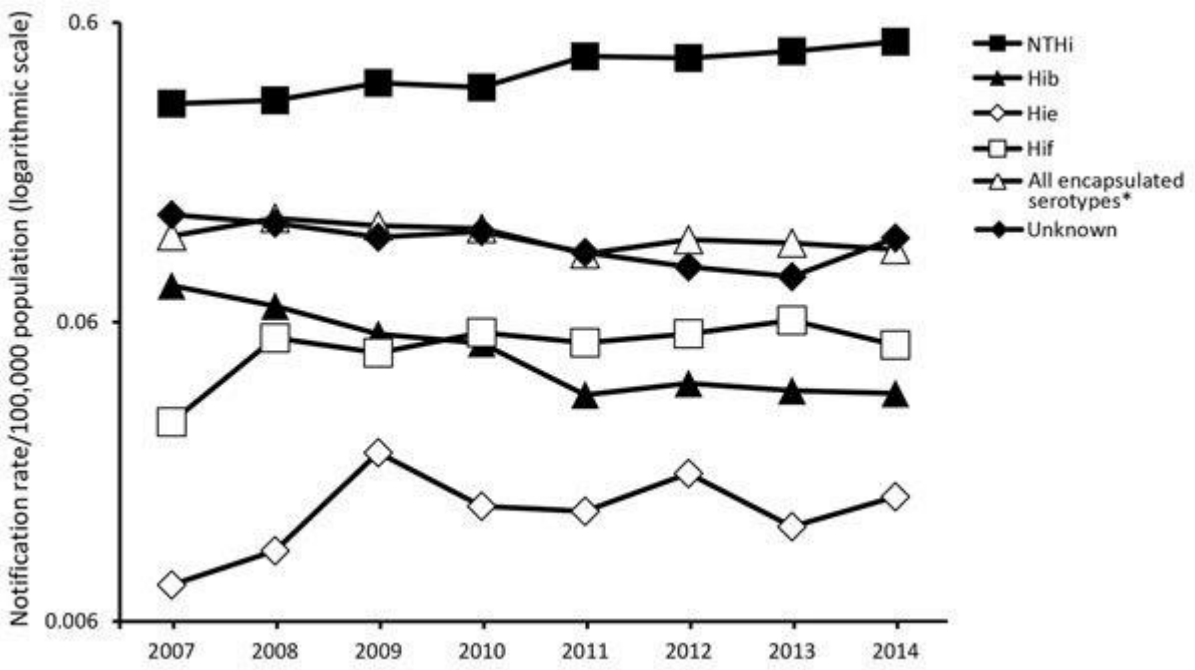

Figure-5 (source (5)): Temporal trends for invasive *H. influenzae* infections according to the serotype and year of notification, in 12 European countries (Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and the United Kingdom), in the period between 2007 and 2014.

In total 8'781 cases were reported.

*Refers to all cases reported as encapsulated *H. influenzae* (serotypes a, b, c, d, e, and f).

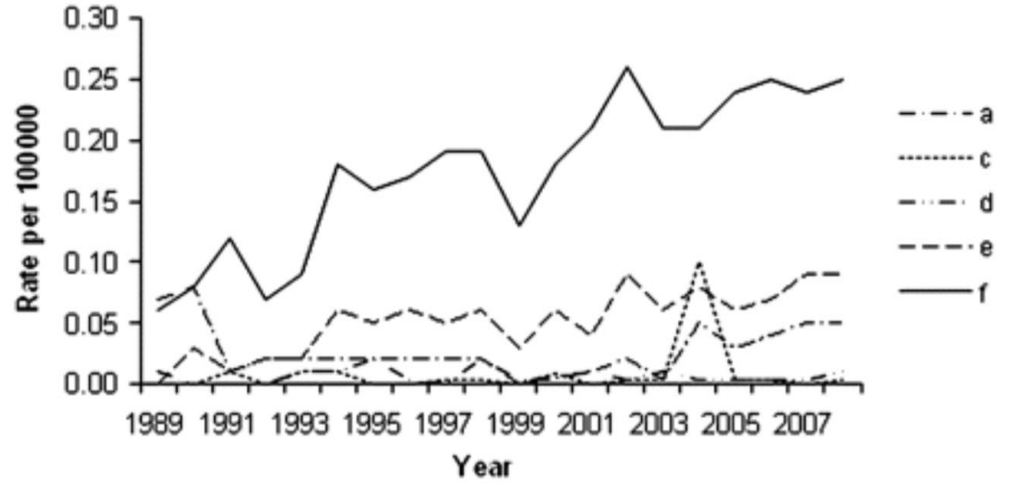

Figure-6 (source (7)): Trends in incidence of invasive infections engendered by non-b encapsulated *H. influenzae* in the United States between 1989 and 2008.

NTHi is responsible for a wide range of diseases. Pneumonia and bacteraemia remain the most frequent infections, often among elderly persons who suffer predisposing medical conditions (Figure-7). Acute sinusitis, otitis media, and acute exacerbations of chronic obstructive airways disease are generally triggred by a viral infection, which predisposes the patients to bacterial superinfection (5, 36-38). NTHi is capable to form biofilms *in vitro* and in otitis media (39-41).

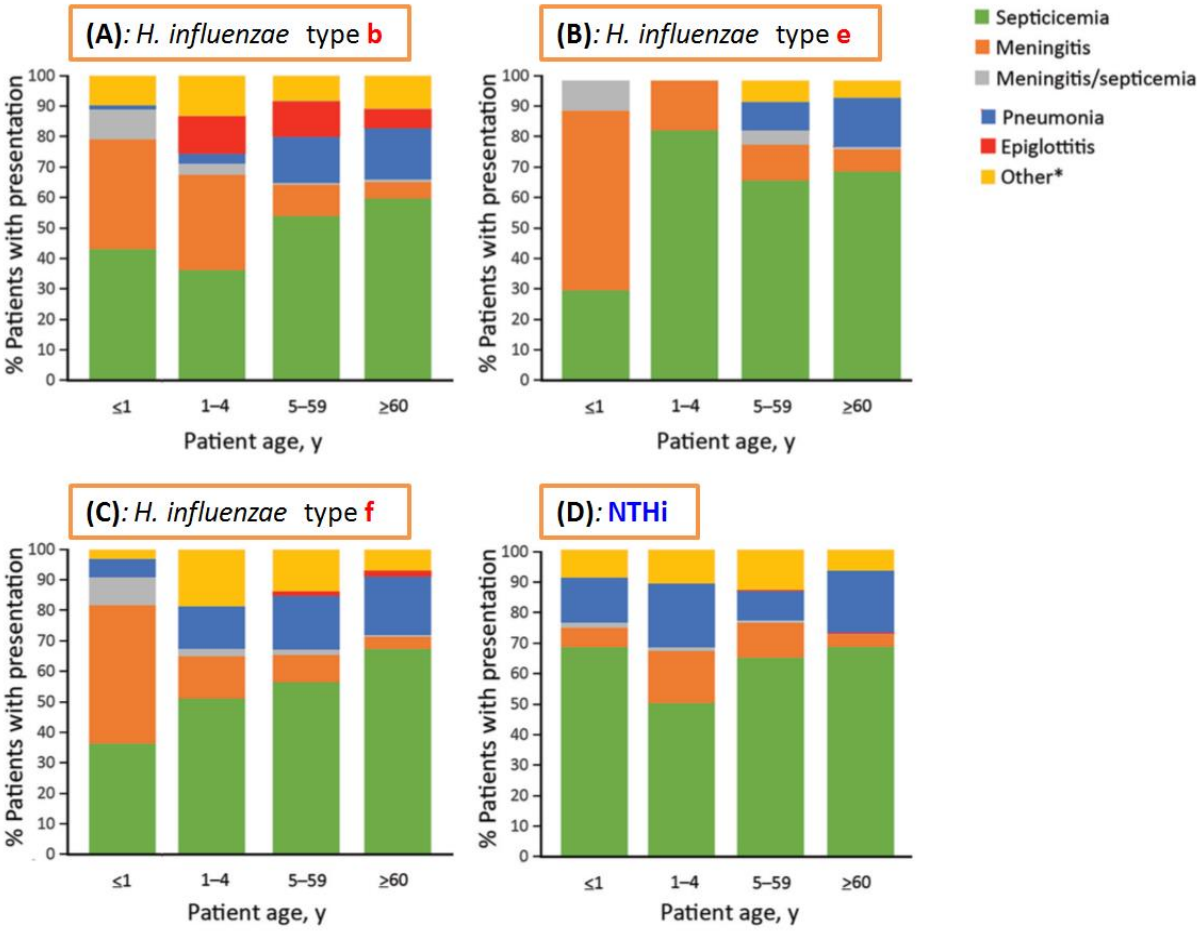

Figure-7 (source (5))**:** *H. influenzae* infections vary according to the strain types.

Percentage of cases, stratified by age groups, caused by *H. influenzae* serotype **b** (**A**), serotype **e** (**B**), serotype **f** (**C**) and by nontypeable *H. influenzae,* **NTHi** (**D**), in 12 countries in Europe (Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and the United Kingdom), in the period between 2007 and 2014.

*Other refers to cases such as cellulitis, septic arthritis, or osteomyelitis.

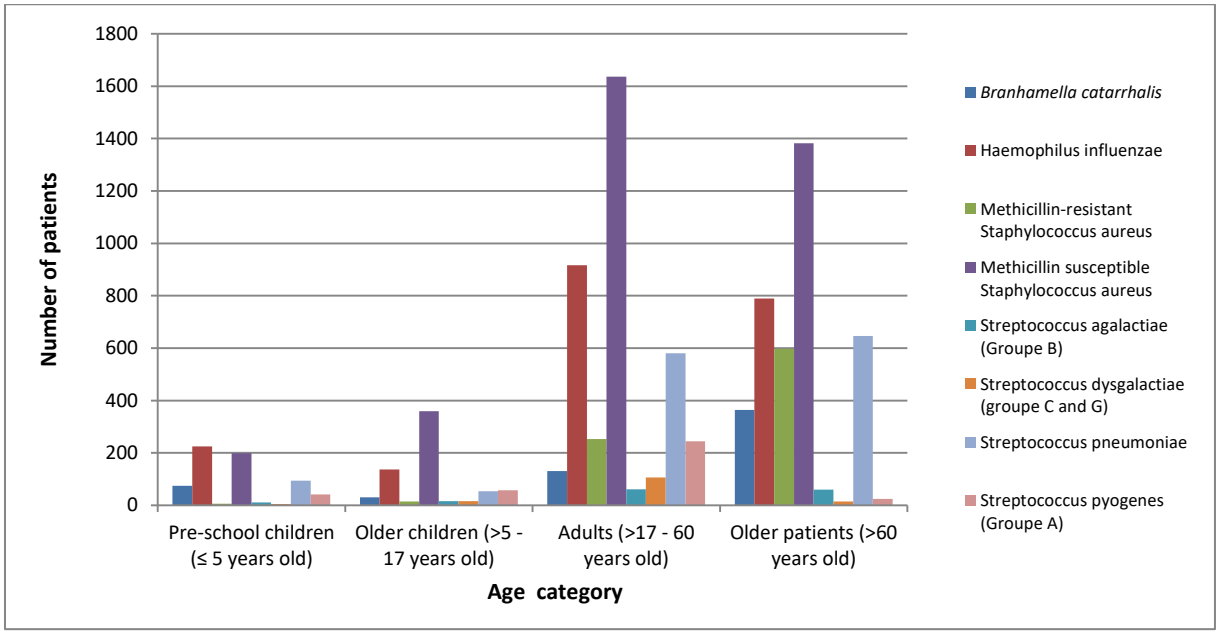

Figure-8: Distribution of the different respiratory tract pathogens according to age category in Geneva University Hospitals (HUG) between January 2009 and December 2016.

(A. Cherkaoui, unpublished data).

NTHi can also colonize the female urogenital tract and this colonization might be the origin of locally severe infections such as endometritis, amnionitis, or Bartholin's gland abscess. Moreover among pregnant women an association was clearly identified between invasive NTHi infection and preterm birth or fetal loss (42, 43). As depicted in Figure-9, more than 3% of the *H. influenzae* strains identified in Geneva University Hospitals (HUG) in the period between January 2009 and December 2016 were isolated from the urogenital tract specimens.

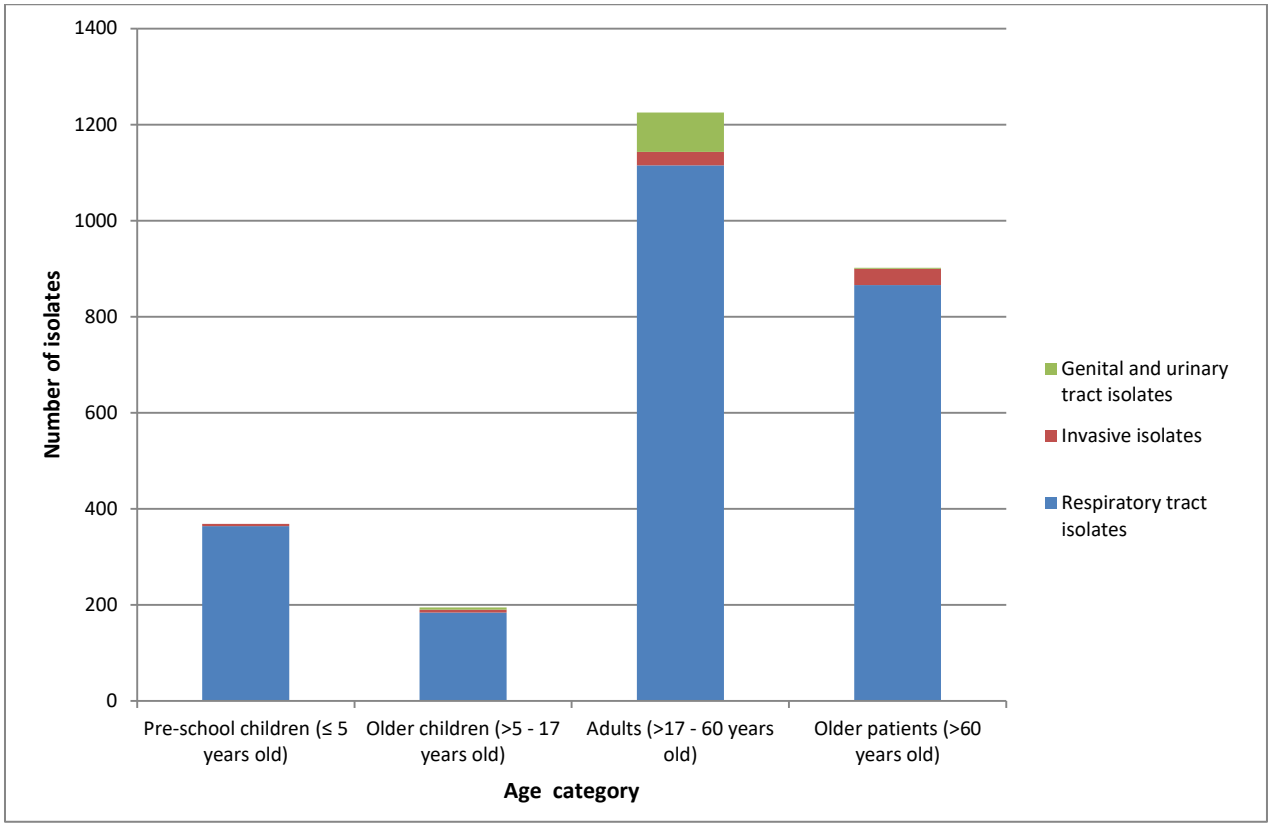

Figure-9: Recovery of *H. influenzae* according to specimen types in Geneva University Hospitals (HUG) in the period between January 2009 and December 2016.

(A. Cherkaoui, unpublished data).

The link between some bacteria and acute exacerbations of chronic obstructive pulmonary disease (COPD) was already established. Nonetheless, only recently NTHi was considered to play an important role in maintaining significant levels of inflammation in COPD patients, thereby increasing the risk of complications (44). NTHi is able to invade local tissue and survive intracellularly in the respiratory tract. The macrophages and epithelial cells appear to be the cell targets of NTHi (45). *In vitro* the ability of NTHi to survive inside these cells was documented in different studies (45, 46). In addition, the ability of NTHi strains to resist killing by complement proteins correlates with the severity of pulmonary and invasive diseases (47).

Possible explanations for the emergence of NTHi, as a pathogen causing also invasive diseases, were proposed in the litterature (e.g. improvement of bacterial detection and typing, and increased virulence of NTHi strains) (48). However the underlying mechanisms for the NTHi increase remain yet to be precisely determined. High-quality epidemiological data using accurate molecular methods such as whole-genome sequencing (WGS) appear therefore very important for monitoring and understanding the changes related to NTHi isolates.

6. Antimicrobial susceptibility testing of *H. influenzae*

H. influenzae lacks genes encoding enzymes for the *de novo* biosynthesis of nicotinamide-adenine-dinucleotide (NAD, also known as the V factor); and therefore it has an absolute requirement for an exogenous source of this factor. Most of the V factor uptake pathway has been characterized in *H. influenzae*. The organism *in vivo* can utilize NAD (NAD^+), nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR) as V factor sources, but not their precursor (nicotinamide) (49). To cultivate *H. influenzae*, a complex medium is therefore required; and if not blood-based, it must contain two growth factors: NAD and hemin (X factor). The standard medium used for growing *H. influenzae* is a chocolate-agar medium, which can be prepared with heat-lysed sheep or horse blood (Figure-10).

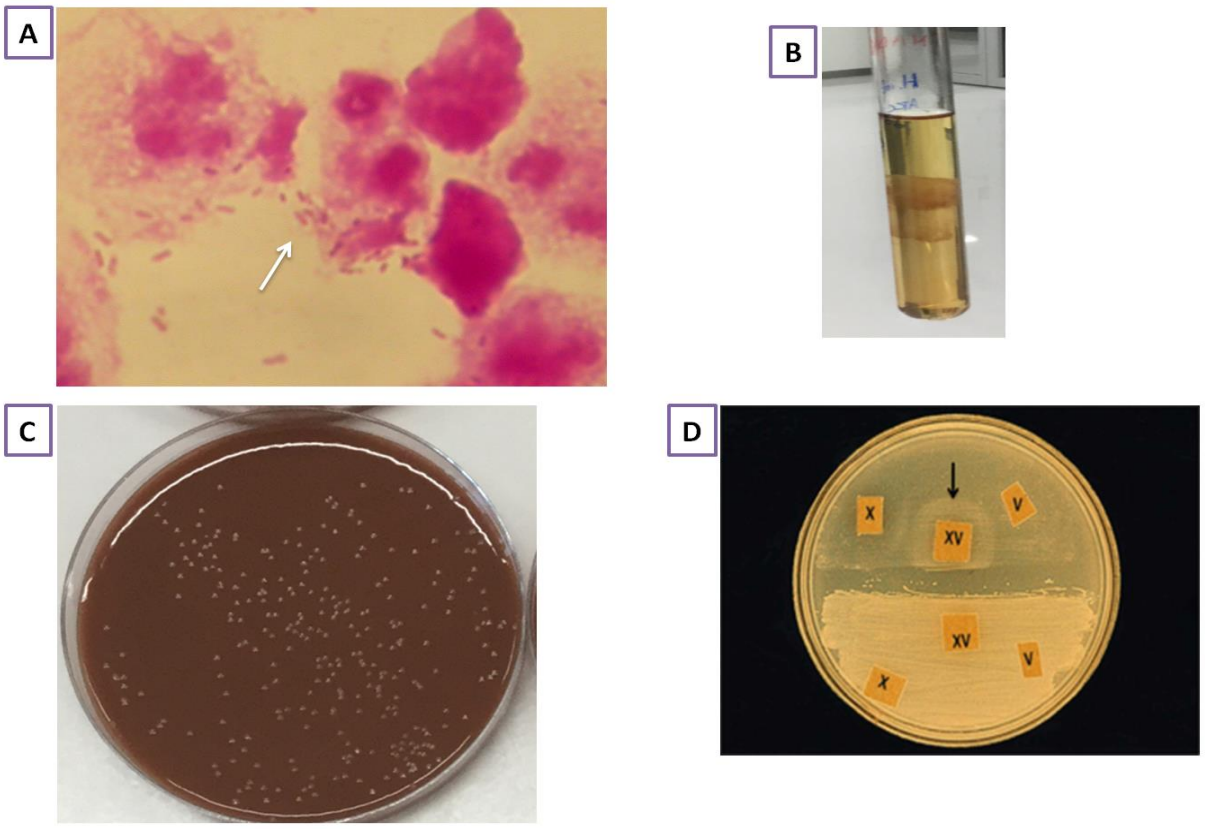

Figure-10: Phenotypic characteristics of *H. influenzae* isolates

(A) Microscopy from cerebrospinal fluid (Gram stain, magnification ×100). *H. influenzae* appears as a Gram-negative, rod-shaped bacterium (white arrow).

(B) *H. influenzae* grown at 37°C in XV-supplemented brain-heart infusion broth (sBHI).

(C) *H. influenzae* colonies on a chocolate agar medium.

(D) Identification of X factor and V factor as growth requirements using paper disks. The black arrow indicate the strain which grows only arrond the disk containing both hemin and NAD. This strain is identified as *H. influenzae.*

A, B and C (source Bacteriology Laboratory, Geneva University Hospitals)
D (source https://www.cdc.gov)

The results of qualitative and quantitative susceptibility testing methods for *H. influenzae* are clearly influenced by the composition of the growth medium as well as the breakpoints for interpreting antimicrobial susceptibility testing results (50). Various susceptibility testing media providing the required V and X factors have been developed. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) has specifically recommended the use of a Mueller Hinton agar + 5% horse blood + 20 mg/L β-NAD (MHF) for performing antibiotic disk diffusion susceptibility assays.

Various parameters are considered to define the clinical breakpoints (e.g. pharmacodynamics, pharmacokinetic, drug dosing, and administration mode). Different breakpoints for antimicrobial susceptibility testing were available many years ago. However, the important variation in these breakpoints has long been identifed as an issue because different breakpoints lead to different reports of susceptibility for the same isolate. In addition, this heterogeneity makes the comparaison of the resistance rates across surveillance studies poorly accurate. For these reasons, EUCAST has harmonized minimum inhibitory concentration (MIC) breakpoints across European clinical microbiology laboratories.

7. Mechanisms of resistance to β-lactams

The bacterial cell-wall synthesis is the target of β-lactam antibiotics. Their effects can be bactericidal (antibiotics kill the bacteria) or merely inhibit cell growth (bacteriostatic) (51). As a general rule, the phenotypic expression (i.e. susceptible or resistant) to β-lactam antibiotics is the result of a complex interaction between i) the presence, spectrum of activity, and levels of bacterial production of β-lactamases which act by hydrolyzing the β-lactam ring of penicillins; ii) the bacterial outer membrane permeability (porins); iii) the presence and efficiency of drug efflux systems; and iv) the affinity of the antibiotic to the penicillin-binding proteins (PBPs) (51, 52). It is commonly established that in *H. influenzae*, the resistance to ampicillin and narrow-spectrum cephalosporins is linked to either the production of a β-lactamase and/or the overexpression of a low-affinity PBP. According to the EUCAST clinical breakpoints, the disk diffusion test with benzylpenicillin (1 unit) can reliably predict the susceptibility of *H. influenzae* to the other β-lactam antibiotics. Thus, the clinical microbiology laboratories can report that the tested *H. influenzae* strain is susceptible to all β-lactam agents for which clinical breakpoints exist, whenever the penicillin zone diameter is ≥ 12 mm. This disk diffusion test appears able to rule out both, the presence of a β-lactamase production and other β-lactam resistance mechanisms.

In the literature, the acronym BLNAR (β-lactamase-negative ampicillin-resistant) is used to define *H. influenzae* strains that are tested resistant to ampicillin, but that have neither phenotypic nor genotypic evidence for the presence of a β-lactamase. In such strains the resistance to ampicillin is conferred by the decreased ampicillin affinity to penicillin-binding protein 3 (PBP3). Likewise, BLPACR (β-lactamase positive, amoxicillin/clavulanic acid-resistant) is used to define *H. influenzae* strains expressing a β-lactamase but that remain resistant to ampicillin despite β-lactamase inhibition by clavulanic acid. In that case, the resistance is based on the combined effects of the

two following mechanisms: a β-lactamase production and a decreased affinity to PBP3 (53, 54). While these concepts are routinely used, *H. influenzae* resistance to β-lactam antibiotics seems to be more complicated and cannot be reduced to a set of only two mechanisms. For exemple, in many *H. influenzae* strains, different key mutations in the *ftsI* gene (encoding PBP3) were identified but the strains remained phenotypically susceptible to ampicillin, yet displaying different MIC values (55). Reaching significant levels of resistance (i.e. to be labeled as resistant by the EUCAST breakpoints) is the result of different molecular mechanisms acting in combination, typically implicating efflux pumps and porins.

Resistance rates to ampicillin show wide variations according to the geographical area. From 1999 to 2000, an important international surveillance study was conducted on antimicrobial susceptibility of *H. influenzae* and *Moraxella catarrhalis* in community-acquired respiratory tract infections (11). This study showed major variations in the prevalence of β-lactamase production in *H. influenzae.* Among all the isolates analysed 16.6% (489/2948) were β-lactamase-positive, but the highest percentage of β-lactamase-positive isolates was identified in South Korea (65%) (11).

Nowadays several elements can explain the low prevalence of BLNAR and BLPACR strains in different countries. For instance, the lack of a consensus-defining breakpoint between the two major regulatory agencies (EUCAST *vs* the Clinical and Laboratory Standards Institute (CLSI)), and the different performances of antimicrobial susceptibility testing methods used for *H. influenzae* in clinical microbiology laboratories.

7.1. β-lactamase mediated resistance in *H. influenzae*

The early 1970s witnessed the first detection of β-lactamase-mediated ampicillin resistance in *H. influenzae*, and in 1975 that β-lactamase was identified as a TEM type (56). In 1981, Rubin *et al*. revealed the presence of a novel β-lactamase in *H. influenzae*, later called ROB-1 (57). Both enzymes are plasmid-mediated class A serine β-lactamases that confer resistance to ampicillin and are efficientely inhibited by β-lactamase inhibitors such as clavulanic acid. However, they display clear differences in their isoelectric points (ROB-1 = 8.1, TEM-1 = 5.4) (57). In clinical microbiology laboratories, the detection of β-lactamase-mediated ampicillin resistance is generally easy. Among 14'870 *H. influenzae* strains isolated in different contries from patients with community-acquired respiratory tract infection included in the PROTEKT study (1999 - 2003), 15% were β-lactamase positive. Among the β-lactamase positive strains, 93.7% were positive for TEM-1, 4.6% for ROB-1, and only 0.04% for both genes. Twenty seven β-lactamase-positive *H. influenzae* isolates were negative for ROB-1 and TEM-1 genes. The authors explained the absence of both genes in these strains by the possible mutation that occurred in ROB-1 and/or TEM-1 genes, or the presence of other enzymes (58). The prevalence of ROB-1 shows substantial variations according to the studied geographical area, as exemplified by the following rates: Mexico (30%), United States (13.2%) and Canada (9.4%) (58). The isolates positive for either enzyme (TEM type or ROB-1) display ampicillin MICs well above the resistant breakpoint (i.e. MIC_{90} ≥32 µg/mL), and both enzymes are detected by nitrocefin hydrolysis (58), enabling reliable routine detection. However, only molecular methods can determine which of the two β-lactamases is present in a given isolate.

7.1.1. TEM-type β-lactamases

In the early 1960s the first β-lactamase was identified in Gram-negative bacteria in Greece. Thus, it was named TEM after the Greek patient name (Temoniera) from whom an *E. coli* strain carring this enzyme has been isolated (59). Afterwards, TEM-2 was discovered. TEM-1 and TEM-2 have identical biochemical properties in spite of the fact that TEM-2 has an amino acid substitution at position 37 (Gln37Lys) corresponding to a single base pair difference (C317A) in the coding region and a single base pair difference (C32T) in the promoter region of the *bla*$_{\text{TEM-2}}$ gene (60). These β-lactamases are frequently encountered in Gram-negative bacteria, including *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *H. influenzae*, and *Neisseria gonorrhoeae*. TEM-1 and TEM-2 hydrolyze penicillins and the first-generation cephalosporins (e.g. cephalothin and cefazolin). However, they are not effective against broad-spectrum cephalosporins, such as ceftriaxone, ceftazidime, or cefepime (61). Large-scale analysis of plasmids identified in *H. influenzae* revealed that the *bla*$_{\text{TEM}}$ originating from *Enterobacteriaceae* was transposed through Tn*2* or Tn*3* onto cryptic plasmids already present in other *Haemophilus* species (50, 62).

Importantly, *bla*$_{\text{TEM}}$ genes have four differents promoters (*P3, Pa/Pb, Prpt*, and *Pdel*). The promoter of the *bla*$_{\text{TEM-1A}}$ gene which is situated on the first widely used *E. coli* cloning vectors (plasmid pBR322) and on a Tn*3* transposon, is corresponding to the promoter *P3* (63). The promoter *Pa/Pb* is the result of the overlapping of two promoters *Pa* and *Pb* deriving from the mutation C32T according to the Sutcliffe numbering system (Figure-11) (63).

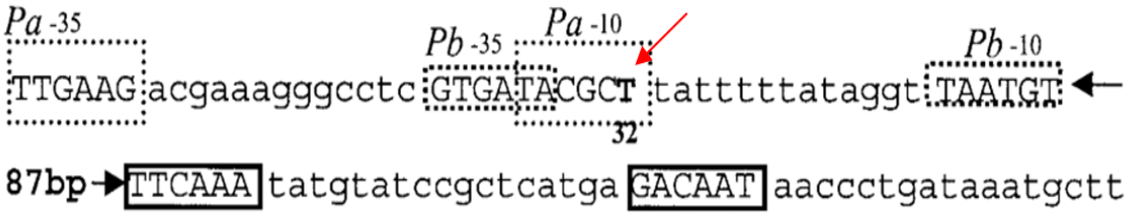

Figure-11 (source (63))**: Nucleotide sequence of *Pa/Pb* promoter**

Red arrow indicates the mutation C32T which conducted to the generation of promoter *Pa/Pb.*

Prpt promoter is characterized by the insertion of 54 base pairs. This insertion is composed by a repeat of bp 145T to 198A inclusive, inserted directly after bp 198. *Pdel* promoter is defined by the deletion of 135 base pairs from bp G23 through to C157 inclusive, and one substitution (G162T) (50). Among the β-lactamase-positive *H. influenzae* strains, the distribution of these four promoters shows substantial variations. Using a collection of 104 β-lactamase-positive *H. influenzae* strains, Tristram *et al*. identified *Pa/Pb* promoter in about 60% of the strains. *Pdel* and *Prpt* were present in 20% of the strains (64). The TEM β-lactamase-specifying plasmids in *H. influenzae* represent two groups, non-conjugative (less than10kb) and conjugative (about 40kb). Interestingly, the *bla*$_{TEM}$ is the only resistance determinant carried by the non-conjugative plasmids (50, 65, 66).

Over the last decades, the *bla*$_{TEM}$ genes have undergone important variations. Different types of *bla*$_{TEM}$ genes were reported in association with extended-spectrum β-lactamases (ESBLs) in Gram-negative bacteria. These ESBLs are effective against broad-spectrum cephalosporins such as ceftriaxone, or ceftazidime. In contrast, carbapenems (e.g. imipenem) are generally active against ESBL-producing strains. In the study conducted by Tristram et al., TEM-type ESBL genes (*bla*$_{TEM-3}$, *bla*$_{TEM-4}$ and *bla*$_{TEM-5}$) were cloned into the reference strain *H. influenzae* Rd KW20. The analysis of the recombinant strains showed that in spite of the fact that cefotaxime MICs increased

up to 0.5 µg/mL (as compared to 0.03 µg/mL for the strain control which carry only *bla*$_{TEM-1}$), these recombinant strains would still not be classified as resistant according to the current clinical breakpoints (67). Thus, such ESBLs would be missed by the phenotypic screening methods commonly used in clinical microbiology laboratories. The relative contribution of PBP3 alterations and TEM-1, TEM-15, or ROB-1 β-lactamases on cefotaxime resistance using an isogenic environment of *H. influenzae* was then reported by A. Søndergaard and N. Nørskov-Lauritsen (68). In 2002 two *Haemophilus parainfluenzae* isolates collected from two South African patients were shown to produce a TEM-15 ESBL, conferring cefotaxime MICs of >16 µg/mL (69). *H. parainfluenzae* and *H. influenzae* can exchange plasmids carring β-lactamase (70). It is therefore surprising that TEM-type ESBLs have not yet been detected in *H. influenzae.*

7.1.2. ROB β-lactamase

The first report of the ROB-1 β-lactamase was in a Hib causing meningitis (57). Nowdays this enzyme is detected in different related species including *Pasteurella multocida* and *Haemophilus ducreyi* (71, 72). It is supposed that ROB-1 β-lactamase has an animal reservoir given the fact that the porcine pathogen *Haemophilus pleuropneumoniae* also produces this enzyme (73). Thus, its prevalence in animal strains may contribute to the spread of resistance to human pathogens.

7.2. β-lactamase-negative ampicillin-resistant (BLNAR) and altered penicillin binding proteins

In the context of the evaluation of a new rapid test for β-lactamase, Thornsberry and Kirven identified in 1974 the first ampicillin resistance in β-lactamase-negative strain (74). The second report of this phenotype was published by Markowitz in 1980. This

strain was isolated during an invasive Hib infection (endocarditis and meningitis) (75). The use of genomic DNA from a BLNAR Hib clinical isolate to transform a susceptible *H. influenzae* Rd KW20 strain to a BLNAR phenotype was performed by Parr and Bryan in 1984 (76). Thus, they demonstrated the association between BLNAR phenotype and the alterations in penicillin-binding proteins 3A and 3B. Penicillin-binding protein 3 (PBP3) is a relatively large size protein constituted by a short cytoplasmic domain, and a periplasmic domain enclosing the transpeptidase activity which is implicated in the septal peptidoglycan synthesis. In *H. influenzae* PBP3, three highly conserved amino acid motifs are reported (STVK (Ser327-Thr-Val-Lys), SSN (Ser379-Ser-Asn), and KTG (Lys-512-Thr-Gly)) (77). These motifs play an essential role in the function of PBP3.

Using the sequence of the *ftsI* gene that encodes the transpeptidase region of PBP3, other groups reported that the amino acid substitutions in PBP3 appear to be close to the conserved PBP3 motifs (78, 79). BLNAR strains were then classified into differents groups based on the patterns of amino acid substitutions present in the transpeptidase region of PBP3. The most common substitutions were Asp350Asn, Met377Ile, Ser385Thr, Leu389Phe, Ala502Val, Ala502Thr, Arg517His, and Asn526Lys (77, 78, 80, 81). To set a relationship between various PBP3 substitutions and resistance, Osaki *et al*. used site-directed mutagenesis to examine single and several amino acid substitutions at positions 377, 385, 389, 517, and 526 (13). The authors reported that the introduction of Ser385Thr or Ser385Thr and Leu389Phe mutations to a background of either Asn526Lys or Arg517His accentuated the level of cephalosporin resistance.

It is now accepted that *H. influenzae* strains with altered PBP3 that would normally be classified as BLNAR are classified as BLPACR (β-lactamase positive, amoxicillin/clavulanic acid-resistant) if they also produce a β-lactamase. In the study conducted by Kaczmarek *et al*. the reference strain *H. influenzae* Rd KW20 was

transformed with PCR-amplified *ftsI* genes from BLNAR strains expressing high ampicillin MICs (range 4 to 16 µg/mL). The ampicillin MICs for the recombinant strains were only in the range of 1 to 4 µg/mL, suggesting that the different amino acid substitutions induced in the transpeptidase region of PBP3 could not explain *per se* the high ampicillin MICs (82). In the same study, the *acrR* gene that negatively regulates the AcrAB efflux pump was sequenced. The totality of the high-level ampicillin-resistant strains showed an early termination of the *acrR* gene reading frame, which was induced by a single nucleotide insertion (82). This suggested that the AcrAB efflux pump should be considered among the mechanisms underlying ampicillin resistance.

Ampicillin resistance in *H. influenzae* is now globally widespread, with incidence rates ranging from 8 to 30 % in different European countries and North America to more than 50 % in some Eastern Asian countries (11).

As shown in Figure-12, the rates of ampicillin and amoxicillin/clavulanic acid resistance in *H. influenzae* strains isolated in Geneva University Hospitals (HUG) have been increasing steadily since 2011. Thereby, the continuous monitoring of β-lactam susceptibility seems to be mandatory to detect a future shift from low-level to high-level β-lactam resistance.

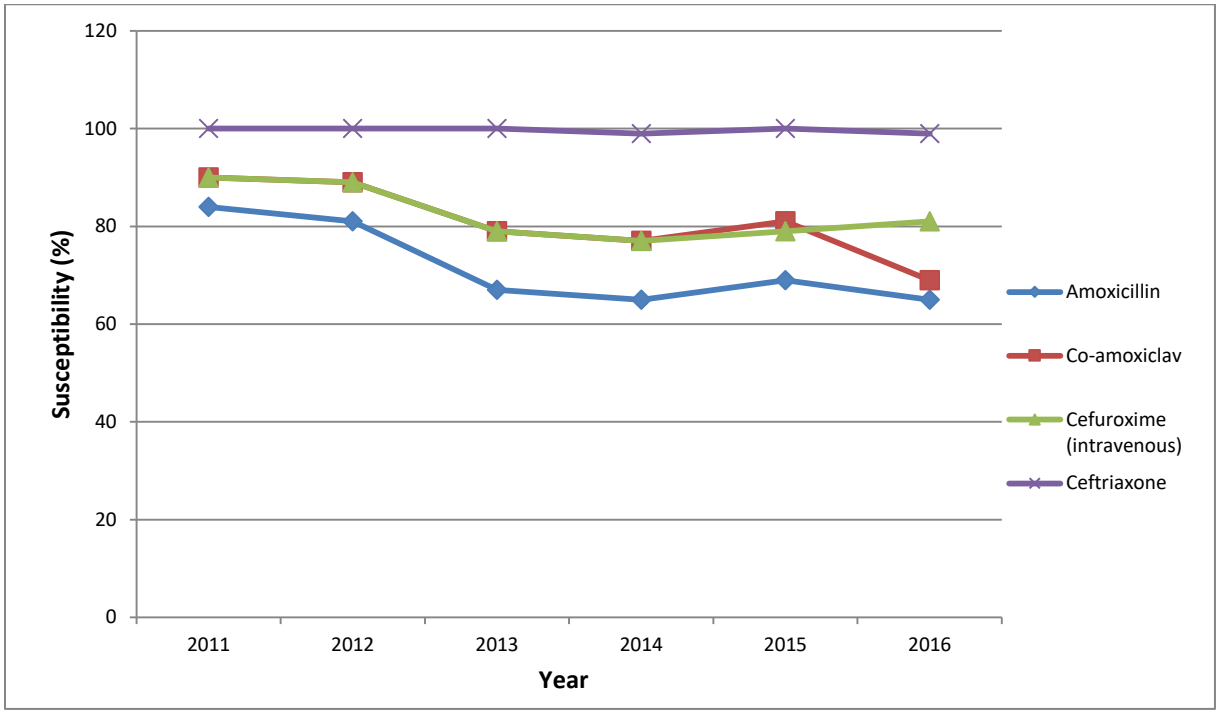

Figure-12: Percentage of susceptible *H. influenzae* strains isolated in Geneva University Hospitals (HUG) in the period between 2011 and 2016.

(A. Cherkaoui, unpublished data).

8. Antimicrobial heteroresistance

Antimicrobial heteroresistance depicts a phenomenon characterized by the presence of subpopulations of bacterial cells with higher MICs than that of the rest of the population within the same clonal culture (83). Antibiotic heteroresistance was first described in 1947 by Hattie E. Alexader and Grace Leidy for *H. influenzae* in the presence of streptomycin (84). In 1964, R. Sutherland and G.N. Rolinson studied this phenomenon in *S. aureus* in response to methicillin (85). Thereafter this phenomenon was generalized to other β-lactams. The concept of “heteroresistance” was coined in 1970 (86). Heteroresistance to glycopeptides was first observed in Japanese vancomycin-resistant *S. aureus* strains (87) and several reports have described heteroresistance in Gram-negative bacteria (e.g. *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Acinetobacter* spp., *Burkholderia cenocepacia, Bacteroides fragilis, and Clostridium perfringens)* (88-92).

Heteroresistance constitutes a relevant cause of infectious complications, in recurrent or chronic infections. Thus, the prescription of drugs without taking into account the presence of a highly resistant subpopulation increases the risk of treatment failure by selecting the more resistant subpopulation. This risk is maximal when a very small subset of the population displays resistance to the administered antibiotic. For instance it was reported that under prolonged exposure to imipenem, the phenotype of certain *A. baumannii* strains switches from susceptible to heteroresistant (93). Moreover, in *Acinetobacter baumannii,* the meropenem pressure can induce meropenem-heteroresistant subpopulations (90).

The population analysis profiling (PAP) method is commonly considered as the gold standard for detecting and quantifying heteroresistance (94-96). In many studies, a modified PAP assay (PAP-AUC) was performed to establish the heteroresistance of

S. aureus to vancomycin. In this assay the area under the curve of a given strain is correlated to that of a reference heteroresistant strain (97).

Disc diffusion (Kirby-Bauer test) and the gradient strip (e.g., E-test) methods are commonly used in routine clinical microbiology laboratories for phenotypic antimicrobial susceptibility testing. These assays have also been employed to detect heteroresistance as evidenced by the presence of distinct colonies growing within the inhibition zone (i.e. satellite colonies) Figure-13 (98, 99).

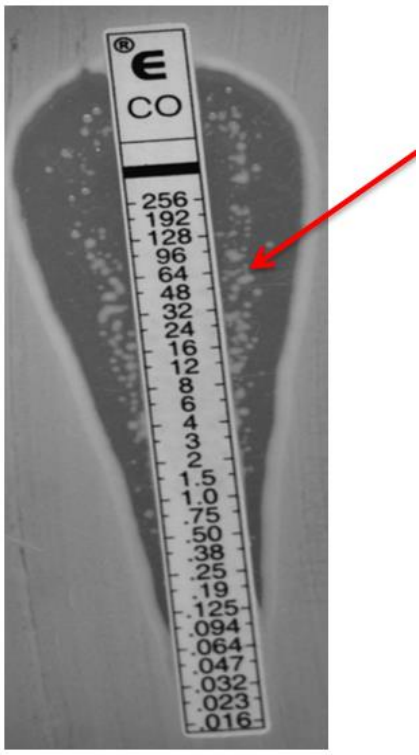

Figure-13 (source (100)): Minimum inhibitory concentration (MIC) determination using a colistin E-test strip for *Enterobacter cloacae* strain isolated from a bronchoalveolar lavage specimen from a kidney transplant patient. The red arrow indicates the colistin-resistant subpopulation.

When the frequency of heteroresistance is very low (e.g. 10^{-7}), only PAP can detect the phenomenon. Yet, the PAP method is labor-intensive and time-consuming, but the lack of standard guidelines as well as their limited analytical sensitivities prevents the

use of E-test and disk diffusion methods to reliably detect heteroresistance in clinical microbiology laboratories.

So far, rare imipenem or meropenem-resistant *H. influenzae* isolates have been reported among BLNAR isolates. The reference strain *H. influenzae* Rd KW20, which displayed an imipenem MIC ranging between 0.38 and 1 µg/mL, was transformed with PCR-amplified *ftsI* gene from a BLNAR strain expressing high imipenem MIC (>32µg/mL). The imipenem MICs for the recombinant strains were only in the range of 4 to 8 µg/mL (12). Some specific amino acid substitutions in PBP3, such as Asn526Lys Gly490Glu Ala502Thr Met377Ile, seem to be involved in imipenem hetreroresistance, but could not explain alone the high imipenem MIC (12, 13). This suggests that the particular pattern of substitutions in PBP 3 transferred to the transformant strains was unable to confer *per se* the high level of resistance, underlining that the mechanisms leading to the heterogeneous expression of resistance in imipenem-resistant *H. influenzae* have not been fully determined.

In Gram-negative bacteria other than *H. influenzae*, the molecular basis of resistance to carbapenems includes the production of enzymes such as serine-β-lactamases or metallo-β-lactamases (e.g., *Klebsiella pneumoniae* carbapenemase (KPC), the New Dehlhi metallo-β-lactamase (NDM), Verona Integron encoded Metallo-β-lactamase (VIM), Imipenemase (IMP) and OXA-48) (101); alterations in the outer membrane permeability (102); increased expression of the efflux pumps (103); and/or modifications in the contents of PBPs (104).

9. Thesis objectives

Chapter-1: Heteroresistance represents an important cause of infectious complications, in recurrent or chronic infections. Although heteroresistance is common in a wide range of microorganisms, heteroresistance in *H. influenzae* has rarely been reported in clinical isolates. The poor performance of the broth microdilution method for detecting these microorganisms might have resulted in an underestimation of the prevalence of such heteroresistance in *H. influenzae*. Currently, information regarding the resistance of *H. influenzae* to imipenem remains scarce, particularly because clinical isolates with imipenem minimum inhibitory concentration (MIC) values above the susceptible breakpoint seem to be rare and might be related to an heterogeneous expression of that resistance.

Objective: to investigate the potential roles of altered PBP3 (Asp350Asn, Ala502val, Ala502Thr, Asn526Lys, combined with other mutations), slow drug influx and the overexpression of efflux pumps in *H. influenzae* imipenem heteroresistance.

Methodology: (a) we will investigate whether amino acid substitutions in PBPs might contribute to imipenem heteroresistance using Bocillin FL and whole-genome sequencing, (b) the affinities of the PBPs to imipenem will be determined (steady-state concentration-response experiments will be carried out using imipenem in a competition assay with Bocillin-FL), (c) we will use carbonyl cyanide m-chlorophenylhydrazone (CCCP) and sequencing of the *acrR* regulatory gene to assess the implication of the AcrAB-TolC efflux pump in imipenem heteroresistance, (d) we will assess the relationship between imipenem heteroresistance and variations in the major outer membrane protein P2 (OmpP2) and (e) we will explore, using whole-genome sequencing, whether other mechanisms involving proteins EnvC and NlpD (LytM proteins) and the dcw cluster of genes implicated in division and cell wall

biosynthesis could trigger the emergence of imipenem heteroresistance in an apparently susceptible strain.

Chapter-2: This part of the thesis project originated with the unexpected observation that highly imipenem resistant *H. influenzae* strains, although viable when exposed at 37°C to high concentrations of imipenem, revealed more susceptible to lower concentrations of this antibiotic when the cells were grown at 42°C. This raised the question on how heat stress does contribute to the active enhancement of imipenem susceptibility.

***Objective*:** To gain insights into the effect of heat stress on imipenem resistance in *H. influenzae.*

Methodology: We will measure the interaction between controlled heat stress and imipenem resistance in *H. influenzae* by using four mutually supportive approaches: (a) we will simultaneously measure growth and cell viability at either 37 or 42°C in *H. influenzae* cells exposed to increasing concentrations of imipenem; (b) we will investigate binding affinity to PBPs by using Bocillin-FL and bacterial cells growth at either 37 or 42°C; (c) we will monitor transcriptome changes by RNA-seq after pre-incubation of bacterial cells at either 37 or 42°C; and (d) we will confirme by real-time quantitative reverse transcription-PCR (qRT-PCR) the transcriptional changes of different key genes (*ponA, ponB, pbp2, ftsI, acrR, acrB, ompP2*) that can be implicated in the resistance of *H. influenzae* to β-lactam antibiotics.

Chapter-3: This part of the thesis project originated with the steady increase of *H. influenzae* clinical strains resistant to fluoroquinolones and imipenem. In addition to the recent report indicating the clonal emergence of high level ciprofloxacin-monoresistant *H. influenzae* in the region of southern Denmark implicating both children and elderly in different hospitals and wards. *H. influenzae* clinical strains with the same MLST profile and mutation in quinolone resistance-determining regions compared to Danish strains were reported recently in Spain.

Objectives: My project is aimed at understanding better this worrisome observation, by analysing the underlying mechanisms and epidemiology.

Methodology: (a) we will determine fluoroquinolones, macrolides, and β-lactams susceptibility profiles of six fluoroquinolone-resistant *H. influenzae* clinical isolates identified at Geneva University Hospitals; (b) we will analyse the whole-genome of these isolates; (c) we will characterize their mechanisms of resistance to fluoroquinolones, macrolides, and imipenem; (d) we will assess the extent of the efflux pump-mediated imipenem, erythromycin, and levofloxacin resistance by using carbonyl cyanide m-chlorophenylhydrazone (CCCP); and (e) we will define and assess a core genome multilocus sequence typing (cgMLST) scheme for the isolates analysed in this study in comparison to other available closed genomes, including 4 Danish *H. influenzae* strains monoresistant to ciprofloxacin, and 8 *H. influenzae* strains heteroresistant to imipenem isolated at Geneva University Hospitals.

Research article

Imipenem heteroresistance in nontypeable *Haemophilus influenzae* is linked to a combination of altered PBP3, slow drug influx and direct efflux regulation

A. Cherkaoui, S.M. Diene, A. Renzoni, S. Emonet, G. Renzi , P. François , J. Schrenzel

Authors' contribution statements

Abdessalam CHERKAOUI designed the study and all the experiments. He carried out the following elements:

- Selection of the 59 clinical bacterial strains included in this study
- Antimicrobial susceptibility testing (disk diffusion and E-test assays)
- Determination of biotypes and serotypes
- Effect of CCCP on imipenem susceptibility
- Determination of PBPs affinity to imipenem and IC_{50s} determination
- Population analysis profile assays
- He analysed all the data and wrote the manuscript

S.M. Diene carried out the sequencing analysis

A. Renzoni provided help and support for the population analysis profile assays

S. Emonet provided input for the clinical aspects

G. Renzi provided help and support for Rep-PCR DNA fingerprinting

P. François helped in interpreting the results and revised the manuscript

J. Schrenzel supervised the research and revised the manuscript

Objective: The purpose of this study was to investigate the potential roles of PBPs, AcrAB-TolC efflux pump and outer membrane protein P2 (OmpP2) for imipenem heteroresistance in nontypeable *Haemophilus influenzae* (NTHi).

Methods: All clinical bacterial strains included in this study were collected at Geneva University Hospitals. Antimicrobial susceptibility testing was performed by disk diffusion and E-test assays. Imipenem heteroresistance was confirmed by population analysis profile assays. Genetic diversity among the 46 clinical isolates was assessed by Rep-PCR DNA fingerprinting. Alterations in PBPs were investigated by sequencing *ftsI, dacB* and *dacA* genes. To assess the affinities of the PBPs to imipenem, steady-state concentration-response experiments were carried out using imipenem in a competition assay with Bocillin-FL. The effect of the carbonyl cyanide m-chlorophenylhydrazone (CCCP) on imipenem susceptibility was defined using broth dilution and viable cell counting. To determine the potential roles of OmpP2, LytM proteins and the dcw cluster in imipenem heteroresistance, whole-genome sequencing was used.

Results: Among the 46 imipenem heteroresistant clinical strains, 78% (36/46) had imipenem MICs of ≥32 mg/mL, 2% (1/46) had imipenem MICs of 4 mg/mL and were categorized as susceptible (CLSI) or resistant (EUCAST), 4% (2/46) had imipenem MICs of 6 mg/mL, 11% (5/46) had imipenem MICs of 8 mg/mL, 2% (1/46) had imipenem MICs of 12 mg/mL and 2% (1/46) had imipenem MICs of 24 mg/mL. All 46 imipenem heteroresistant clinical strains harboured amino acid substitutions in the *ftsI* gene, which encodes PBP3, corresponding to 25 different mutation patterns that varied from the *ftsI* gene mutation patterns found in the 15 imipenem-susceptible isolates. Among all PBPs, the highest affinity to imipenem was documented for PBP3 (IC_{50}, 0.004 mg/mL). Different amino acid substitutions and insertions were noted in OmpP2, suggesting a relationship with imipenem heteroresistance. The heteroresistant clinical strains displayed a higher percentage of killing by imipenem in CCCP treated cells at concentrations ranging between 0.5 and 8 mg/mL.

Conclusions: This study established that altered PBP3, slowed drug influx and enhanced efflux due to the loss of regulation led to the development of imipenem heteroresistance in NTHi.

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Original article

Imipenem heteroresistance in nontypeable *Haemophilus influenzae* is linked to a combination of altered PBP3, slow drug influx and direct efflux regulation

A. Cherkaoui [1, *], S.M. Diene [2], A. Renzoni [1], S. Emonet [1], G. Renzi [1], P. François [2], J. Schrenzel [1, 2]

[1)] *Bacteriology Laboratory, Division of Laboratory Medicine, Geneva University Hospitals, Geneva, Switzerland*
[2)] *Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, Geneva, Switzerland*

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ABSTRACT

Objective: To investigate the potential roles of PBPs, efflux pumps and slow drug influx for imipenem heteroresistance in nontypeable *Haemophilus influenzae* (NTHi).
Methods: Fifty-nine NTHi clinical isolates examined in this study were collected at Geneva University Hospitals between 2009 and 2014. Alterations in PBPs were investigated by gene sequencing. To evaluate the affinities of the PBPs to imipenem, steady-state concentration–response experiments were carried out using imipenem in a competition assay with Bocillin-FL. The effect of the carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on imipenem susceptibility was assessed using broth dilution and viable cell counting. Using whole-genome sequencing, we explored the potential roles of outer membrane protein P2 (OmpP2), LytM proteins and the *dcw* gene cluster in imipenem heteroresistance.
Results: All 46 imipenem-heteroresistant isolates (IMI^{hR}) harboured amino acid substitutions in the *ftsI* gene, which encodes PBP3, corresponding to 25 different mutation patterns that varied from the *ftsI* gene mutation patterns found in imipenem-susceptible isolates. Among all PBPs, the highest affinity to imipenem was documented for PBP3 (IC_{50}, 0.004 μg/mL). Different amino acid substitutions and insertions were noted in OmpP2, suggesting a relationship with imipenem heteroresistance. The IMI^{hR} isolates were affected by CCCP differently and displayed a higher percentage of killing by imipenem in CCCP-treated cells at concentrations ranging between 0.5 and 8 μg/mL.
Conclusions: The present study provides robust evidence indicating that in combination with the altered PBP3, the slowed drug influx and its enhanced efflux due to the loss of regulation led to the development of imipenem heteroresistance in NTHi. **A. Cherkaoui, CMI 2017;23:118.e9–118.e19**

Introduction

Haemophilus influenzae is an opportunistic bacterial pathogen that colonizes humans exclusively and is associated with both acute infections and chronic disease[1]. The plasmid-mediated β-lactamases (TEM-1 type [2] or, rarely, ROB-1 type [3]) and the alteration of penicillin-binding protein 3 (PBP3) [4] are the most important causes of resistance to β-lactams in *H. influenzae*. Although heteroresistance is common in a wide range of microorganisms, heteroresistance in *H. influenzae* clinical isolates has rarely been reported. The poor performance of the broth microdilution method for detecting these microorganisms might have resulted in an underestimation of the extent of heteroresistance in *H. influenzae*. Currently, a large amount of data are available regarding the resistance of *H. influenzae* to aminopenicillins and cephalosporins. However, information regarding the resistance of *H. influenzae* to imipenem remains scarce, particularly because isolates with imipenem minimum inhibitory concentration (MIC) values above the susceptible breakpoint seem to be rare and might be related to the heterogeneous expression of resistance.

According to previously published studies using *H. influenzae* Rd KW20, which carries a mutated *ftsI* gene [5], or by site-directed mutagenesis [6], specific amino acid substitutions in PBP3 appear

* Corresponding author. A. Cherkaoui, Bacteriology Laboratory, Division of Laboratory Medicine, Department of Genetics and Laboratory Medicine, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland.
E-mail address: Abdessalam.Cherkaoui@hcuge.ch (A. Cherkaoui).

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to be involved in imipenem resistance. However, many aspects of imipenem heteroresistance mechanisms remain to be elucidated. Notably, reaching significant levels of resistance usually requires synergistic cooperation among different molecular mechanisms. A comparison of the *in vitro* susceptibility testing methods showed that the Etest and agar dilution methods are reliable for detecting resistant subpopulations [5,7,8]; nonetheless, for *H. influenzae,* the broth microdilution remains the reference method for determining imipenem MICs (https://www.escmid.org/escmid_publications/escmid_elibrary/material/?mid=31996).

The objective of the present work was to investigate the potential roles of *H. influenzae* PBPs, slow drug influx and the overexpression of efflux pumps in imipenem heteroresistance. For this purpose, (a) we investigated whether amino acid substitutions in PBPs might contribute to imipenem heteroresistance, (b) we determined the 50% inhibitory concentrations (IC_{50}s) of imipenem, (c) we used carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and sequencing of the *acrR* regulatory gene to assess the implication of the AcrAB-TolC efflux pump in imipenem heteroresistance, (d) we investigated the relationship between imipenem heteroresistance and variations in the major outer membrane protein P2 (OmpP2) and (e) we explored, using whole-genome sequencing, whether other mechanisms involving proteins EnvC and NlpD (LytM proteins) and the *dcw* cluster of genes implicated in division and cell wall biosynthesis could trigger the emergence of imipenem heteroresistance in an apparently susceptible strain.

Materials and Methods

Bacterial isolates and patients

The 59 nontypeable *H. influenzae* (NTHi) isolates examined in this study were taken from a collection of 124 nonconsecutive clinical isolates identified and stored in the bacteriology laboratory at Geneva University Hospitals between 2009 and 2014. Among the 124 isolates, 37% (46/124) had imipenem MICs >2 μg/mL using the Etest method and were included in the present study as resistant group (IMI^{hR}). Among the isolates with imipenem MICs ≤2 μg/mL using the Etest method, 13 isolates were randomly chosen and used as control group (IMI^{S}). The sources of the 46 isolates (resistant group) were as follows: six blood cultures, one cerebrospinal fluid, five bronchoalveolar lavages, one minibronchoalveolar lavage, two tracheal aspirates, ten bronchial aspirates, two nasopharyngeal aspirates, fourteen sputa, two eye swabs, one throat swab, one superficial wound swab and one from the lung tissues of a patient at autopsy. The patients ranged in age from 1 month to 93 years, with 29 male and 17 female subjects. Five isolates were from preschool-aged children (<5 years old), 16 from adults (18–60 years old) and 25 from older patients (>60 years old).

All isolates were stored at −80°C in skim milk with 15% glycerol. *H. influenzae* isolates were cultured on chocolate agar supplemented with PolyViteX (bioMérieux, la Balme-les-Grottes, France) and incubated at 35°C for 18 to 24 hours in a humid atmosphere containing 5% CO_2. The identification of the *H. influenzae* isolates was confirmed using matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Maldi Biotyper 2.0, Bruker Daltonics, Bremen, Germany) [9,10] according to the manufacturer's instructions. The MALDI-TOF MS score values ranged between 2.3 and 2.5.

Antimicrobial susceptibility testing

The inoculum suspension was prepared by selecting several colonies from overnight growth (16–24 hours of incubation) on chocolate agar plates with a cotton swab and suspending the colonies in sterile saline (0.85% NaCl w/v in water) to the density of a 0.5 McFarland standard, corresponding to $3–4 \times 10^8$ CFU/mL. The density of the suspension was measured with a photometric device calibrated using a McFarland standard according to the manufacturer's instructions. The final density was regularly controlled by viable cell counting on chocolate agar after overnight incubation. The inoculum was spread over the entire surface of the Mueller-Hinton agar plate (supplemented with 5% defibrinated horse blood and 20 μg/mL β-NAD) by swabbing in three directions, and the plates were incubated in a humid atmosphere containing 5% CO_2 at 35 ± 1°C for 18 ± 2 hours. The following antibiotics were tested using the Etest method: ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, cefotaxime, ceftriaxone, cefixime, ceftobiprole, imipenem, ertapenem, meropenem, levofloxacin, clarithromycin and cotrimoxazole (all Etests were from bioMérieux except the Etest for ceftobiprole, which was a gift from Basilea Pharmaceutica, Basel, Switzerland). Disk diffusion (1 unit benzylpenicillin, 2 μg ampicillin and 10 μg imipenem) was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods.

Given that isolates with imipenem MIC values above the susceptible breakpoint are very rare, the susceptibility tests were run three times (from 5 to 30 separate days apart) for all 46 isolates (resistant group) using different lots of media, Etests and diffusion disks. The MICs and the sizes of the inhibition zone diameters were independently read by the first author and at least two experienced lab assistants. *H. influenzae* ATCC (American Type Culture Collection (ATCC), Manassas, VA, USA) 49247 and *H. influenzae* ATCC 49766 were used as quality controls. Isolates that displayed an imipenem MICs >2 μg/mL according to the Etest method were further analysed using the population analysis method according to previously described procedures [5,7,11]. Briefly, overnight growth cultures of the 46 IMI^{hR} clinical isolates, the 13 IMI^{S} clinical isolates and *H. influenzae* ATCC 49766 were suspended in saline solution. The suspensions were adjusted to the modified starting inoculum dose of 10^{10} CFU/mL. Cultures were serially diluted from 0 to 10^{-6}. The microdilution plate count was performed by plating 10 μL droplets and 100 μL of each dilution onto a set of *Haemophilus* Test Medium (HTM) agar plates containing increasing concentrations of imipenem (concentration range 4–256 μg/mL) and onto antibiotic-free HTM agar plates. After incubation at 37°C with 5% CO_2 for 48 hours, bacterial colonies were counted.

β-Lactamase production was screened by the chromogenic cephalosporin assay using a nitrocefin disk (Becton Dickinson, Allschwil, Switzerland) and the hydrolysis of penicillin G in the API *Neisseria–Haemophilus* system (bioMérieux). The presence of the β-lactamase-encoding *bla*TEM-1 gene was investigated in all isolates examined in this study by PCR using the primers and probe previously described [12].

Determination of biotypes and serotypes

Biotypes were determined using the reactions for indole, urea and ornithine decarboxylase in the API *Neisseria–Haemophilus* system. The capsular status was determined by PCR using the primers and conditions previously described [13].

Sequencing the genes encoding penicillin-binding proteins (PBPs)

Alterations in PBPs were investigated by sequencing *ftsI, dacB* and *dacA* using the primers and conditions previously described [5,12]. Primer pairs for these genes were designed from sequences in the GenBank nucleotide sequence database (Supplementary Table S1). The sequenced amplicons were analysed and corrected using CodonCode aligner software (http://www.codoncode.com/

aligner). The amino acid substitutions in the protein sequences were investigated using ClustalX 2.0. The sequences were compared against that of *H. influenzae* Rd KW20.

Sequencing of the acrR gene

The *acrR* regulatory gene of the AcrAB-TolC efflux pump was amplified using the primers listed in Supplementary Table S1 and analysed as described above.

Effect of CCCP on imipenem susceptibility

The effect of the broad-spectrum efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on imipenem susceptibility was assessed for 3 IMI^{hR} NTHi isolates (GE49, GE65 and GE129). These strains were chosen because they did not have any amino acid substitutions in the *ftsI* gene close to the KTG, SSN or STVK motifs. Moreover, the GE65 and GE129 strains possessed a deletion in the *acrR* regulatory gene controlling the AcrAB-TolC efflux pump, leading to early termination of the *acrR* reading frame. These analyses were conducted using broth dilution and viable cell counting. Briefly, CCCP (Sigma-Aldrich Chimie, Schnelldorf, Germany) was diluted in dimethyl sulfoxide at a concentration of 100 mM and added to XV-supplemented brain–heart infusion broth to final concentrations of 0.195, 0.390, 0.781, 1.56 and 3.12 μM CCCP. The highest concentration of CCCP that did not affect cell growth was 0.78 μM (Supplementary Fig. S1), which was used in all experiments.

The difference between CCCP-treated and nontreated cells was tested three times independently with the same isolates and assessed by a paired Student's *t* test. A *p* value of <0.05 was considered statistically significant. The analysis was performed by GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA).

Rep-PCR DNA fingerprinting

Genetic diversity among the 46 IMI^{hR} isolates was assessed using the DiversiLab *Haemophilus* kit (bioMérieux) according to the manufacturer's instructions.

Determination of PBP affinity to imipenem and IC$_{50}$s

The detection of PBPs in *H. influenzae* was carried out using a nonradioactive method with the commercially available Bocillin-FL (Thermo Fisher, Waltham, MA, USA), a fluorescent penicillin, as a labelling reagent for the detection and study of PBPs [14,15]. *H. influenza*e Rd KW20 was used for the preparation of bacterial membranes. To evaluate the affinities of the PBPs to imipenem and to determine the IC$_{50}$s, steady-state dose–response experiments were carried out using imipenem in a competition assay with Bocillin-FL as previously described [15–17].

Whole-genome sequencing

To investigate whether other mechanisms were involved in imipenem heteroresistance (e.g. outer membrane protein P2, penicillin-binding proteins 1a, 1b and 2, the division cell wall (*dcw*) gene cluster or LytM proteins), we sequenced eight IMI^{hR} NTHi isolates exhibiting different *ftsI* gene mutation patterns (IIa (GE68), IIb (GE3, GE6, GE117), IId (GE71), III-like (GE146) and miscellaneous group (GE42, GE49)). The genomic DNA was sequenced using Illumina MiSeq (150 bp paired-end reads) technology (Illumina, San Diego CA, USA). The Illumina sequence quality was evaluated using the Fastqc program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and filtered using the Fastq-mcf programs (Ea-utils: http://code.google.com/p/ea-utils/). The genome sequences were assembled using the Edena v3 assembler [18]. The resulting coverage ranged from 393× to 665×. The genome annotation was performed using the National Center for Biotechnology Information annotation pipeline. The results of the genome sequencing are summarized in Supplementary Table S2.

Statement of ethics

This laboratory study was performed on bacterial strains without any access to patient data (e.g. treatment received or final clinical diagnosis). Patient's consent was therefore not needed.

Results

Biotyping, serotyping and antimicrobial susceptibility testing

Table 1 shows the MIC$_{50}$, MIC$_{90}$, MIC range and susceptibility categories for 14 antibiotics according to the EUCAST and Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints for the 46 IMI^{hR} NTHi isolates. They are assigned to the following two groups: 80% (37/46) genomic β-lactamase-negative, ampicillin-resistant (gBLNAR) strains and 20% (9/46) genomic β-lactamase-positive, amoxicillin/clavulanic acid–resistant (gBLPACR) strains. All β-lactamase-positive isolates were of the TEM-1 type. Among the 46 IMI^{hR} isolates, 78% (36/46) had imipenem MICs of ≥32 μg/mL (Supplementary Fig. S2), 2% (1/46) had imipenem MICs of 4 μg/mL and were categorized as susceptible (CLSI) or resistant (EUCAST), 4% (2/46) had imipenem MICs of 6 μg/mL, 11% (5/46) had imipenem MICs of 8 μg/mL, 2% (1/46) had imipenem MICs of 12 μg/mL and 2% (1/46) had imipenem MICs of 24 μg/mL. By the Etest method, 46% (21/46) of the isolates had ampicillin MICs of ≤1 μg/mL and were categorized as susceptible according to both guidelines. By disk diffusion (AMP2, 2 μg ampicillin), 28% (13/46) of the isolates had an ampicillin inhibition diameter ≥16 mm and were categorized as susceptible (EUCAST). With the minor zone breakpoint adjustment (+2 mm) for AMP2 proposed by Skaare *et al.* [19], only 2% (1/46) of the isolates were susceptible. Using the 1-unit benzylpenicillin screening disk (PG1), 28% (13/46) had a PG1 zone of ≥12 mm and were categorized as susceptible (EUCAST) (Supplementary Fig. S3). By disk diffusion (IMP10, 10 μg), 100% of the isolates had an imipenem zone <20 mm and were categorized as resistant (EUCAST). When the MIC of imipenem for IMI^{hR} isolates was measured using Etest strips, several subpopulations were identified close to the Etest strip (Supplementary Fig. S4). Isolates displaying a heterogeneous imipenem resistance profile by the Etest method were further analysed by population analysis (Supplementary Fig. S5).

To investigate whether colonies of cells growing in the presence of high concentrations of imipenem were composed of subpopulations of cells differing from each other in their imipenem MICs, a colony of 7 IMI^{hR} strains was picked from HTM agar containing 16 or 32 μg of imipenem/mL and passaged in imipenem-free HTM agar. After overnight growth, these cultures, named strainID/16 or 32, were used as a starting cell suspension for further population analysis. Cultures of strainID/16 or 32 remained heterogeneously resistant but enriched in subpopulations resistant to 128 μg/mL and 256 μg/mL of imipenem (Supplementary Fig. S6). We checked the sequence of the *ftsI, dacB, dacA* and *acrR* genes from the subpopulations capable of growth in the presence of 16 and 32 μg/mL of imipenem, and they were 100% identical to that of the original clinical isolates. No subpopulations of imipenem-resistant cells were detected in the culture of strain ATCC 49766 or the 13 IMI^{S} clinical isolates.

Using the reactions for indole, urea and ornithine decarboxylase in the API *Neisseria–Haemophilus* system, the 46 IMI^{hR} isolates fell

Table 1
Susceptibilities of 46 imipenem heteroresistant *Haemophilus influenzae* isolates to 14 antibiotics according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints

Antibiotic	Genotype	Strain (n)	MIC µg/ml			EUCAST clinical breakpoints (% isolates)			CLSI clinical breakpoints (% isolates)		
			MIC50	MIC90	MIC range	S	I	R	S	I	R
Imipenem	gBLNAR	37	>32	>32	4 - >32	0	0	100	3	0	97
	gBLPACR (TEM-1)	9	>32	>32	6 - >32	0	0	100	0	0	100
Ampicillin	gBLNAR	37	1	2	0.19 - 4	57	0	43	57	40	3
	gBLPACR (TEM-1)	9	32	256	8 - 256	0	0	100	0	0	100
Amoxicillin / clavulanic acid	gBLNAR	37	1.5	3	0.125 - 4	78	0	22	100	0	0
	gBLPACR (TEM-1)	9	1.5	2	0.5 - 12	89	0	11	89	0	11
Piperacillin/ tazobactam	gBLNAR	37	0.032	0.125	0.016 - 0.25				100	0	0
	gBLPACR (TEM-1)	9	0.064	0.125	0.023 - 0.25				100	0	0
* Cefuroxime	gBLNAR	37	3	12	0.25 - 64	24	24	52	65	22	13
	gBLPACR (TEM-1)	9	2	6	0.75 - 24	22	33	45	67	22	11
Cefotaxime	gBLNAR	37	0.047	0.094	0.023 - 3	89	0	11	95	0	5
	gBLPACR (TEM-1)	9	0.032	0.094	0.008 - 0.125	100	0	0	100	0	0
Ceftriaxone	gBLNAR	37	0.023	0.094	0.012 - 0.125	100	0	0	100	0	0
	gBLPACR (TEM-1)	9	0.032	0.094	0.012 - 0.25	89	0	11	100	0	0
Cefixime	gBLNAR	37	0.047	0.094	0.023 - 6	89	0	11	89	0	11
	gBLPACR (TEM-1)	9	0.032	0.064	0.012 - 2	89	0	11	89	0	11
Ceftobiprole	gBLNAR	37	0.094	0.19	0.032 - 0.38						
	gBLPACR (TEM-1)	9	0.094	0.094	0.032 - 0.25						
Ertapenem	gBLNAR	37	0.125	0.25	0.032 - 1.5	95	0	5	95	0	5
	gBLPACR (TEM-1)	9	0.094	0.094	0.016 - 0.19	100	0	0	100	0	0
** Meropenem	gBLNAR	37	0.19	0.5	0.094 - 1.5	100	0	0	95	0	5
	gBLPACR (TEM-1)	9	0.19	0.19	0.032 - 0.25	100	0	0	100	0	0
Levofloxacin	gBLNAR	37	0.023	0.047	0.023 - 0.064	100	0	0	100	0	0
	gBLPACR (TEM-1)	9	0.032	0.047	0.023 - 0.047	100	0	0	100	0	0
Co-trimoxazol	gBLNAR	37	0.125	32	0.006 - >32	61	3	36	61	11	28
	gBLPACR (TEM-1)	9	0.19	16	0.002 - >32	67	0	33	67	0	33
Clarithromycine	gBLNAR	37	16	32	8 - 64	0	94	6	17	66	17
	gBLPACR (TEM-1)	9	24	64	16 - 64	0	78	22	0	67	33
Benzylpenicillin 1unit (screening)	gBLNAR	37	Zone diameter range		6 - 16 (mm)	35	0	65			
	gBLPACR (TEM-1)	9			6 (mm)	0	0	100			
Ampicillin (2µg)	gBLNAR	37	Zone diameter range		6 - 19 (mm)	35	0	65			
	gBLPACR (TEM-1)	9			6 - 10 (mm)	0	0	100			
Imipenem (10µg)	gBLNAR	37	Zone diameter range		6 - 19 (mm)	0	0	100	32	0	68
	gBLPACR (TEM-1)	9			6 - 19 (mm)	0	0	100	67	0	33

gBLNAR: Genomic β-lactamase-negative, ampicillin resistant (amino acid substitution in the PBP3)
gBLPACR: Genomic β-lactamase-positive, amoxicillin/clavulanic acid-resistant (β-lactamase-producing with amino acid substitution in the PBP3)
* EUCAST clinical breakpoints for intravenous (iv) ** EUCAST clinical breakpoints for infections other than meningitis

into biotype I (2%, 1/46), IV (2%, 1/46), V (46%, 21/46) and VI (50%, 23/46). Different results were obtained according to carbohydrate fermentation. Among the IMI^{hR} isolates, 28% (13/46) were not able to ferment fructose (one invasive and 12 respiratory tract isolates). None of these biochemical activities has been shown to be strongly associated with the heteroresistance to imipenem. By PCR, all *H. influenzae* isolates analysed herein were nontypeable.

Rep-PCR DNA fingerprinting

A 98% similarity threshold was chosen to discriminate the genotypes of the tested isolates. The 46 IMI^{hR} isolates were successfully genotyped with the DiversiLab system. High genetic diversity was observed among the 46 IMI^{hR} NTHi isolates. The dendrogram showed that nine isolates clustered and were classified into four subgroups (Supplementary Fig. S7).

Mutation patterns in the ftsI gene encoding PBP3

All 46 IMI^{hR} isolates had amino acid substitutions in the *ftsI* gene corresponding to 25 different mutation patterns, which were different than the *ftsI* gene mutation patterns found in the IMI^{S} isolates (Table 2). Of the 46 isolates with mutations in the *ftsI* gene, 89% (41/46) had amino acid substitutions surrounding the KTG motif (Lys512-Thr-Gly), the four most common being Asn526Lys (85%, 39/46), Ala502val (50%, 23/46), Ala502Thr (9%, 4/46) and Arg517His (4%, 2/46). Subgroup IIa (9%, 4/46) was characterized by the substitution Asn526Lys combined with other mutations. Subgroup IIb (50%, 23/46) was characterized by the substitution Ala502Val, usually combined with the substitutions Asp350Asn and/or Gly490Glu. One isolate (GE117) was characterized by the substitution Ala502Val combined with the substitution Asp350Ser. The substitution Asp350Ser had not been

Table 2
Amino acid substitutions identified in transpeptidase domain of *ftsI* gene from 61 strains of *Haemophilus influenzae* strains according to their imipenem minimum inhibitory concentrations

Group*	Strain (n)	Ampicillin MIC range (μg/ml)	Imipenem MIC range (μg/ml)	TEM-1	Amino acid substitution for :																															
						Near STVK motif					Close to SSN motif						Surrounding KTG motif																			
	Rd KW20	0.064 - 0.094	0.25 - 0.38		Glu-141	Ser-273	Glu-274	Ser-311	Asp-350	Ser-357	Met-377	Ser-385	Leu-389	Ala-437	Ile-449	Gly-490	Ala-502	Val-511	Arg-517	Asn-526	Ala-530	Thr-532	Val-547	Asp-551	Ala-554	Tyr-557	Ala-561	Val-562	Asp-569	Ala-586	Ala-587	Asp-589	Thr-591	Ser-594	Ala-595	Ile-601	Glu-603
IIa	**1**	256	>32	+	.	.	.	.	Asn	.	.	.	.	.	.	.	.	.	.	Lys	.	.	.	.	.	.	.	Leu	.	Ser	.	.	.	Thr	Thr	.	Asp
GE68	**1**	2	8		.	.	.	.	.	.	.	.	.	.	.	Glu	.	Ala	.	Lys	.	.	Ile	.	.	.	.	.	.	Ser	.	.	.	Thr	Thr	.	Asp
	2	0.5 - 0.75	>32		.	.	.	.	Asn	.	.	.	.	.	.	Glu	.	.	.	Lys	Ser	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
IIb	**1**	1.5	>32		.	Ala	.	.	.	.	.	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Pro	.	Lys	Ala	.	.	Val	Asp
	1	24	>32	+	.	.	.	.	.	.	.	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	.
	1	1	>32		Lys	.	.	.	Asn	.	Ile	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	.
	4	0.38 - 1	8 - >32		.	.	.	.	Asn	.	Ile	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	.
	6	1.5 - 2	4 - >32		.	.	.	.	Asn	.	Ile	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	.
	2	0.75	6 - >32		Lys	.	.	.	.	.	.	.	.	.	.	Glu	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Pro	.	Lys	Ala	.	.	Val	Asp
	1	1	>32		.	.	.	.	Asn	.	.	.	.	Ser	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
	1	256	>32	+	Lys	.	.	.	.	.	Ile	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	.
	3	0.75 - 1	8 - >32		.	.	.	.	Asn	.	Ile	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	.	.	.	.
GE117	**1**	1	>32		Lys	.	.	.	Ser	.	Ile	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	.
	1	12	>32	+	.	.	.	.	Asn	.	Ile	.	.	.	.	.	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	.	.	.	.
	1	2	>32		.	.	.	.	Asn	.	.	.	.	.	.	.	Val	.	.	Lys	.	.		.	.	.	.	.	.	Pro	Val	Lys	Ala	.	.	Val	Asp
IIc	**1**	0.75	>32		.	.	.	.	Asn	.	.	.	.	.	.	.	Thr	.	.	Lys	.	.	Ile	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1	3	>32		Lys	.	.	.	Asn	.	.	.	.	.	.	.	Thr	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	.	.	.	.	.	.	.	Asp
	1	0.19	>32		Lys	.	Asp	.	Asn	.	.	.	.	.	.	.	Thr	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	.	.	.	.	.	.	.	Asn
IId	**1**	1.5	12		.	.	.	.	Asn	.	.	.	.	.	Val	.	.	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
	3	0.75 - 1	>32		Lys	.	.	.	.	.	.	.	.	.	Val	.	.	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
	3	1.5 - 3	>32		Lys	.	.	.	.	.	.	.	.	.	Val	.	.	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
	1	32	8	+	Lys	.	.	.	.	.	.	.	.	.	Val	.	.	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
IIc + IId	**1**	1.5	8		Lys	.	.	.	.	.	.	.	.	.	Val	.	Thr	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
III-like	**1**	256	>32	+	.	.	.	.	Asn	Asn	Ile	Thr	.	.	.	.	.	.	His	.	.	Ser	Ile	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1	4	>32		.	.	.	.	.	Asn	Ile	Thr	Phe	.	.	.	.	.	His	.	.	Ser	Ile	.	.	His	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
M (GE42)	**1**	1	>32		.	.	.	.	Asn	.	.	.	.	.	.	.	.	.	.	.	.	.	Ile	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GE111	**1**	12	>32	+	.	.	.	.	Asn	.	.	.	.	.	.	.	.	.	.	.	.	.	Ile	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GE65	**1**	8	6	+	Lys	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.
GE129	**1**	256	>32	+	Lys	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.
GE49	**1**	1	>32		Lys	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Asp
Control group (imipenem susceptible isolates)																																				
ATCC 49247		3	2		Lys	Ala	.	.	.	.	.	.	.	.	.	.	.	.	.	Lys	.	.	Ile	.	Thr	.	Glu	.	Ser	.	.	.	.	.	.	.	Asn
ATCC 49766		0.125 - 0.19	0.75 - 1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Asp
IIb	**2**	0.5 - 0.75	1.5 - 2		.	Ala	.	.	Asn	.	Ile	.	.	.	.	Glu	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Pro	.	Lys	Ala	.	.	Val	Asp
	1	0.5	2		.	.	.	.	.	.	.	.	.	.	.	.	Val	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Asp
	1	1.5	2		.	.	.	.	Asn	.	Ile	.	.	.	.	Glu	Val	.	.	Lys	.	.	Ile	.	.	.	.	.	Ser	Pro	.	Lys	Ala	.	.	Val	Asp
GE119	**1**	1.5	1		.	.	.	.	.	.	.	.	.	.	.	Glu	Val	.	.	Lys	.	.	Ile	Tyr	.	.	.	.	.	.	.	.	.	.	.	.	.
M	1	0.38	0.38		.				Asn														Ile						Ser	Ser				Thr	Thr	.	Asn
	1	0.75	0.38		.	.	.	.	Asn	.	.	.	.	.	.	.	.	.	.	.	.	.	Ile	.	.	.	.	.	Ser	Ser	.	.	.	Thr	Thr	.	Asp
	1	8	1.5	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Asp
	1	24	0.38	+	.	.	.	.	Asn	.	.	.	.	.	.	.	.	.	.	.	.	.	Ile	.	.	.	.	.	Ser	.	Thr	.	.	.	.	.	Asp
	1	0.75	2		.	Ala	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ile	.	.	.	.	.	Ser	.	Thr	.	.	.	.	.	Asp
	1	1	1		.	.	.	Pro	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ile	.	.	.	.	.	.	.	.	.	.	.	.	.	Asp
	2	0.094 - 1	0.38 - 2		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

*The strains with *ftsI* mutations were classified into six groups : II (a, b, c and d) according to Dabernat et al.; and III-like according to Garcia-Cobos et al.; and the Miscellaneous (M) group, according to the data from this study.

"." indicates no amino acid substitution

[shaded] shows strains with profil : ampicillin susceptible by Etest and imipenem resistant

Three conserved motifs in the active site: **#STVK motif** (Ser327-Thr-Val-Lys) # shows the position of the catalytic serine residue
SSN motif (Ser379-Ser-Asn)
KTG motif (Lys512-Thr-Gly)

previously described. Subgroup IIc (7%, 3/46) was characterized by the Ala502Thr substitution. Finally, subgroup IId (17%, 8/46) was defined by the Ile449Val substitution. Group IIc + IId (2%, 1/46) presented the characteristics of both IIc and IId, which included the following amino acid substitutions: Ile449Val and Ala502Thr. We observed an additional mutation pattern in 4% (2/46) of the isolates, characterized by the following amino acid substitutions: Met377Ile, Ser385Thr and/or Leu389Phe close to the SSN motif (Ser379-Ser-Asn); Arg517His and Thr532Ser surrounding the KTG motif; Asp350Asn near the STVK motif (Ser327-Thr-Val-Lys); Ser357Asn and Tyr557His. These isolates were classified into group III—like as described by Garcia-Cobos *et al.* [20]. The Asn526Lys and the Arg517His appear to be central to phenotypic resistance, and 89% (41/46) of the IMI^{hR} isolates characterized so far had one of these substitutions, although no strain had both. An additional miscellaneous group, consisting of three patterns not previously described, was characterized by the following amino acid substitutions: Asp350Asn and Val547Ile (GE42 and GE111); Glu141Lys and Ala586Ser (GE65 and GE129); and Glu141Lys and Glu603Asp (GE49). The amino acid substitutions Asp350Asn, Ser357Asn, Met377Ile, Ser385Thr, Leu389Phe, Ala502Val, Ala502Thr, Val511Ala, Arg517His, Asn526Lys, Val547Ile and Asp569Ser, which are known to be closely associated with aminopenicillin and cephalosporin resistance [21,22], may contribute to imipenem resistance when present with other substitutions. Nonetheless, three IMI^{hR} isolates (GE49, GE65 and GE129) did not have any of these mutations, suggesting that imipenem heteroresistance was conferred by other mechanisms.

Mutation patterns in the dacB gene encoding PBP4

An analysis of the deduced amino acid sequences of the *dac*B gene encoding PBP4 from the 46 IMI^{hR} isolates and control groups revealed considerable polymorphisms in comparison with *H. influenzae* strain Rd KW20, irrespective of the imipenem susceptibility phenotype (Table 3). All 46 IMI^{hR} isolates had amino acid substitutions in the *dacB* gene, corresponding to 13 different mutation patterns. One strain (GE119) also had a 7 bp deletion in *dacB*. This deletion inserted a stop codon after the C terminus of the 21st amino acid residue from the SDN motif (Ser310-Asp-Asn). However, this deletion does not appear to correlate with elevated imipenem MICs.

Mutation patterns in the dacA gene encoding PBP5

Thirty-five IMI^{hR} isolates had amino acid substitutions in the *dac*A gene, corresponding to different mutation patterns

Table 3
Amino acid substitutions identified in transpeptidase domain of *dacB* gene from 61 strains of *Haemophilus influenzae* strains according to their imipenem minimum inhibitory concentrations

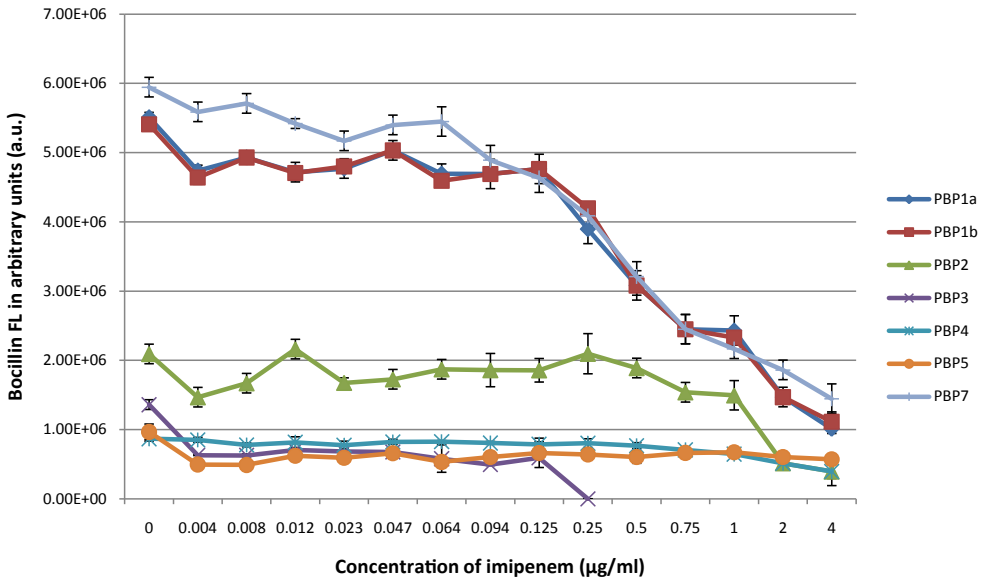
Group	Strain (n)	Ampicillin MIC range (µg/ml)	Imipenem MIC range (µg/ml)	TEM-1	Amino acid substitution for :																																							
					Close to STQK																												Near SDN				Near KTG								
	Rd KW20	0.064 - 0.094	0.25 - 0.38		Val-43	Phe-45	Ile-55	Asp-58	Ser-67	Thr-75	Ala-78	Ala-82	Asp-84	Gln-100	Asn-101	Asn-107	Arg-111	Gly-134	Ile-159	Ala-172	Lys-189	Gly-192	Ile-194	Val-213	Ala-214	Ala-219	Gln-223	Tyr-244	Ser-250	Asn-255	Glu-262	Gln-270	Val-279	Leu-280	Val-340	Thr-372	Ala-437	Leu-476								
I	1	1.5	>32		Ala	Val	.	Asn	Pro	.	.	.	Gly	Lys	.	.	.	.	.	Ser	.	.	.	.	.	Val	.	.	.	.	Ala	Lys	.	.	.	.	.	.							
II	1	12	>32	+	Ala	Val	.	Asn	Pro	.	.	.	Gly	.	Ser	His	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	Ile							
	3	0.25 - 3	>32		Ala	Val	.	Asn	Pro	.	.	.	Gly	.	Ser	His	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	Ile							
III	3	0.75 - 1	8 - >32		Ala	Val	.	Asn	Pro	.	.	.	Gly	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	Ile							
	1	12	6	+	Ala	Val	.	Asn	Pro	.	.	.	Gly	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	Ile							
IV	3	24 - 256	8 - >32	+	.	.	.	.	.	.	.	.	Gly	.	Ser	His	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
	20	0.19 - 4	4 - >32		.	.	.	.	.	.	.	.	Gly	.	Ser	His	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
V	1	0.75	>32		Ala	Val	Met	.	Pro	.	.	.	Gly	.	.	.	.	Ser	.	.				.	Val			.		Ala	.	.	.	.	Met	.	.							
VI	2	1 - 1.5	12 - >32		Ala	Val	.	Asn	Pro	.	Val	.	Gly	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
VII	2	1 - 2	8 - >32		Ala	Val	.	Asn	Pro	Ile	.	.	Gly	.	.	.	.	.	Val	.	.	.	.	Val	.	.	.	.	.	.	Ala	Lys	.	.	.	.	Val	.							
VIII	1	0.5	>32		Ala	Val	.	Asn	Pro	.	.	.	Gly	.	Ser	His	.	.	.	.	.	.	.	.	.	.	.	.	Asn	.	Ala	Lys	.	.	.	.	.	.							
IX	2	256	>32	+	Ala	Val	.	.	Pro	.	.	.	Gly	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	.							
	2	0.75 - 1	8 - >32		Ala	Val	.	.	Pro	.	.	.	Gly	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	.							
X	1	2	>32		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	.							
XI	1	1.5	>32		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Gln	Arg	Thr	Ile	.	.	.	Ser	.	Asp	Ala	.	.	.	.	.	.	.							
XII	1	8	6	+	.	.	.	.	Cys	.	.	.	Gly	.	Ser	His	.	.	.	.	Gln	Arg	Thr	Ile	.	.	.	Ser	.	Asp	Ala	.	.	.	.	.	.	.							
XIII	1	256	>32	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
Control group (imipenem susceptible isolates)																																													
ATCC 49766		0.125 - 0.19	0.75 - 1		.	.	.	.	.	.	.	Thr	Gly	.	Ser	His	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
	1	0.094	0.38		Ala	Val	.	Asn	Pro	.	.	.	Gly	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
	1	1.5	2		Ala	Val	.	.	Pro	.	.	.	Gly	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	.							
	1	0.38	2		.	.	.	.	.	.	.	Thr	Gly	.	Ser	His	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
	1	8	1.5	+	.	.	.	.	.	.	.	Thr	Gly	.	Ser	His	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
	1	24	0.38	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Gln	.	Thr	.	.	.	.	Ser	.	Asp	Ala	.	Ile	.	Ile	.	.	.							
	1	0.19	0.38		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ala	.	.	.	.	.	.	.							
	2	0.38-0.75	0.38		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Gln	Arg	Thr		Val	.	His	.	.	Asp	Ala	.	.	.	.	.	.	.							
	1	0.75	2		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Gln	Arg	Thr	.	.	.	.	Ser	.	Asp	Ala	.	.	Met	.	.	.	.							
	2	0.5- 0.75	1.5 - 2		Ala	Val	.	Asn	Pro	.	.	.	Gly	.	Ser	His	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	Ile							
	1	0.25	2		Ala	Val	.	Asn	Pro	.	.	.	Gly	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.	.	.	.	Ala	Lys	.	.	.	.	.	Ile							
ATCC 49247		3	2		Deletion of 1 base (A) behind nucleotide 915 / Genetic consequence : Early stop codon																																									
GE119		1.5	1		Deletion of 7 bases (GCCGATT) behind nucleotide 942 / Genetic consequence : Early stop codon																																									

Table-3. Amino acid substitutions identified in the transpeptidase domain of the *dacB* gene from 61 strains of *H. influenzae* strains according to their imipenem MICs

Three conserved amino acid sequences:

STQK (Ser69-Thr-Gln-Lys)
SDN (Ser310-Asp-Asn)
KTG (Lys420-Thr-Gly

"." indicates no amino acid substitution



H. influenzae Rd KW20	PBP1a	PBP1b	PBP2	PBP3	PBP4	PBP5	PBP7
50% inhibitory concentrations (IC_{50}s) of imipenem (µg/ml)[a]	0.75	0.75	1.5	**0.004**	2	>4	0.5

Fig. 1. Inhibition of Bocillin-FL and *Haemophilus influenzae* Rd KW20 PBPs by increasing amounts of imipenem. Assays contained variable concentrations of imipenem with fixed concentrations of membrane preparation and Bocillin-FL. Maximal Bocillin-FL values indicate 0% competition, and no Bocillin-FL signal indicates 100% competition. Values represent means ± SD from two independent experiments. [a]Imipenem concentration producing 50% reduction in Bocillin-FL binding for each individual PBP.

(Supplementary Table S3). For 11 IMI^{hR} and 7 IMI^{S} isolates, no products were obtained using primer sets targeting the full *dac*A gene (~1487 bp), but products of approximately 678 bp were amplified. The reason for this is closely related to single nucleotide polymorphisms. Thus, a direct connection between altered PBP5 and imipenem heteroresistance was not evident here.

Affinity of imipenem binding to H. influenzae Rd KW20 PBPs

To determine whether imipenem heteroresistance is linked to decreased antibiotic affinity to penicillin-binding proteins, we measured imipenem affinity to PBPs by a competition assay using penicillin-labelled Bocillin-FL. As depicted in Fig. 1, imipenem shows higher affinity for PBP3 (IC_{50}, 0.004 µg/mL) than for PBP1a (IC_{50}, 0.75 µg/mL), PBP1b (IC_{50}, 0.75 µg/mL), PBP2 (IC_{50}, 1.5 µg/mL), PBP4 (IC_{50}, 2 µg/mL), PBP5 (IC_{50}, >4 µg/mL), or PBP7 (IC_{50}, 0.5 µg/mL).

Sequence analysis of the acrR regulatory gene controlling the AcrAB-TolC efflux pump

To determine whether increased efflux of imipenem played a role in conferring higher imipenem MICs in IMI^{hR} isolates, the *acrR* coding sequence was determined and compared to that of *H. influenzae* Rd KW20. All 46 IMI^{hR} and 15 IMI^{S} isolates (including control strains ATCC 49247 and ATCC 49766) had amino acid substitutions in the *acrR* gene, corresponding to different mutation patterns (Supplementary Table S4). Strikingly, among the miscellaneous group, which was characterized by three mutation patterns in the *ftsI* gene not previously described, one base pair (T) behind nucleotide 482 and one base pair (A) behind nucleotide 65 were deleted from the *acrR* gene of GE65 and GE129, respectively. In both cases, these changes led to the early termination of the *acrR* reading frame, yielding a constitutive increase in the transcription of the target gene.

Inhibition of the AcrAB-TolC efflux pump is correlated with increased imipenem susceptibility

Fig. 2 shows that the 3 IMI^{hR} isolates were affected differently by CCCP. GE65, GE129 and GE49 displayed a higher percentage of CCCP-treated cells killed by imipenem at concentrations of 1.0, 0.5 and 8.0 µg/mL, respectively. These data indicate that treatment with CCCP enhanced *H. influenzae* susceptibility to imipenem. Taken together, these results demonstrate that drug efflux is one of the major mechanisms accounting for imipenem heteroresistance in NTHi.

OmpP2 protein patterns

The sequence analysis of the *ompP2* gene was compared between the eight IMI^{hR} isolates and the control strain Rd KW20 (Supplementary Table S5). The eight IMI^{hR} isolates had amino acid substitutions and insertions in the OmpP2 protein. While size variations of the OmpP2 protein were evident and have been observed previously with NTHi [23,24], there were obvious changes in this protein between the imipenem-susceptible control and the imipenem-heteroresistant isolates.

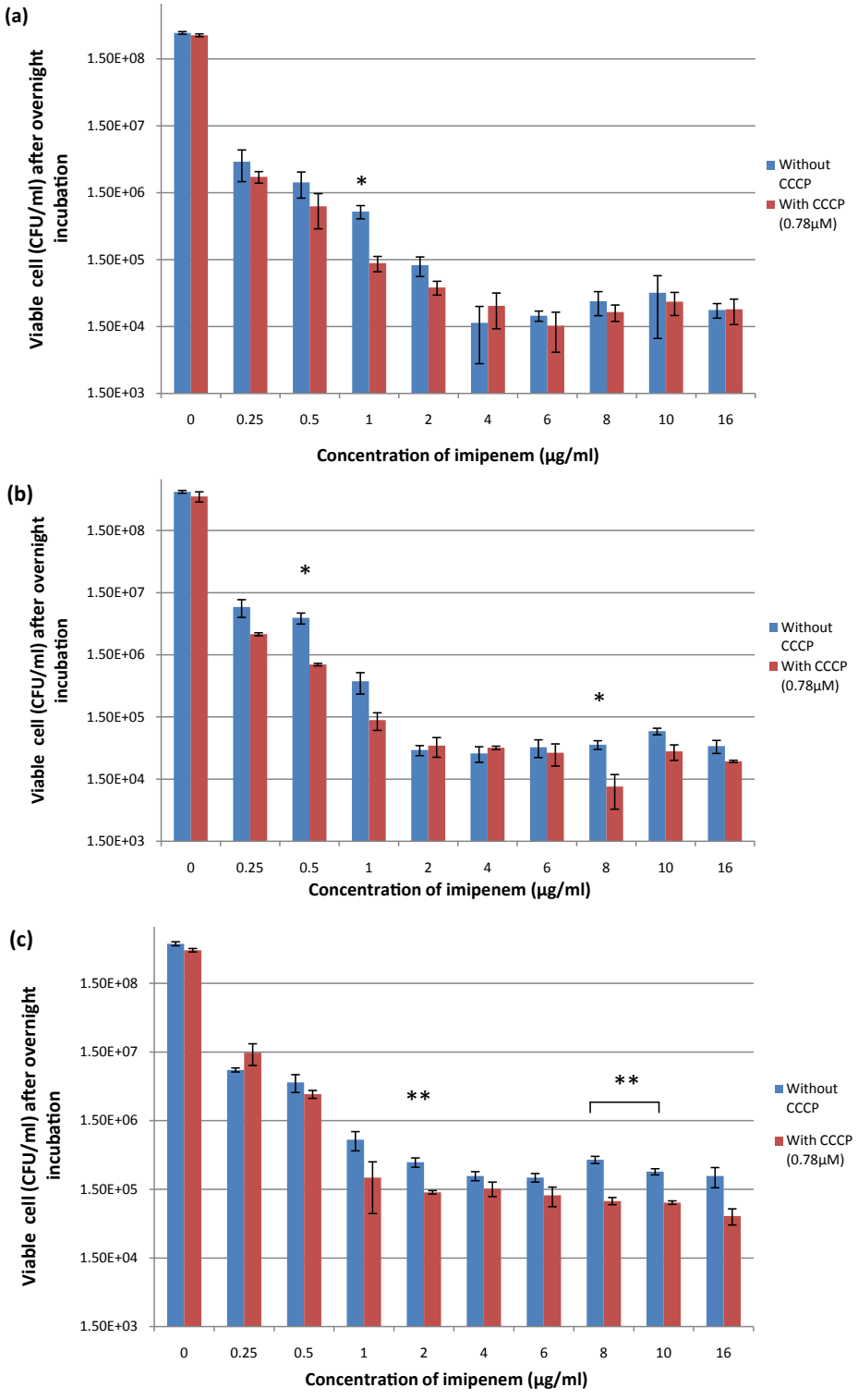


Fig. 2. Effect of broad-spectrum efflux pump inhibitor CCCP on imipenem susceptibility. We used highest concentration of CCCP (0.78 µM, Supplementary Fig. S1) that did not affect cell growth. (a) Strain GE129 (imipenem MIC >32 µg/mL). (b) Strain GE65 (imipenem MIC = 6 µg/mL). (c) Strain GE49 (imipenem MIC >32 µg/mL). Values represent means ± SD from three independent experiments. *p <0.05, **p <0.01 (paired Student's *t* test). CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MIC, minimum inhibitory concentration.

Whole-genome sequencing

To explore the potential involvement of other mechanisms in imipenem heteroresistance, the genes encoding EnvC and NlpD (LytM proteins) and a cluster of genes implicated in division and cell wall (*dcw*) biosynthesis were investigated in eight IMI^{hR} NTHi isolates exhibiting different *ftsI* gene mutation patterns. The gene organization of the *dcw* cluster revealed similar results to those observed in *Escherichia coli and Neisseria gonorrhoeae* [25]. Compared with *H. influenzae* strain Rd KW20, the eight IMI^{hR} NTHi isolates had different amino acid substitutions in *envC* and *nlpD* (LytM proteins), as well as in *mraZ, rsmH, ftsL, murE, murF, mraY, murD, ftsW, murG, murC, ddlB, ftsQ, ftsA* and *ftsZ* (*dcw* gene cluster), as depicted in Supplementary Table S6. Importantly, no pattern consistently correlated with imipenem heteroresistance.

Discussion

The carbapenems (e.g. imipenem) are a group of antibiotics that belong to the β-lactam class. Low toxicity and a low prevalence of resistance make carbapenems a valuable alternative for initial empirical treatment of severe infections before a definitive microbiological diagnosis is made available [26]. In 2015, the total number of defined daily doses (DDDs) of imipenem-cilastatin (ATC group J01DH51) consumed in Geneva University Hospitals was 7913. Many of these DDDs were used in the intensive care and visceral surgery wards (870 DDDs, 11% and 778 DDDs, 10%, respectively) (unpublished data from Geneva University Hospitals). One aspect in the use of the 1-unit benzylpenicillin screening disk (PG1) to detect β-lactam resistance in *H. influenzae* that has not received consideration is the accuracy of this screening disk to detect imipenem heteroresistance. Of the 46 IMI^{hR} isolates examined in this study, 28% (13/46) had a PG1 zone ≥12 mm and were categorized as susceptible (EUCAST). These 13 strains were β-lactamase negative. The identification of isolates with imipenem MIC values above the susceptible breakpoint is rare, and this low frequency may be related to the heterogeneous expression of resistance and the small number of studies that have examined this phenomenon. Additionally, standardized methodologies and criteria to detect imipenem heteroresistance in clinical microbiology laboratories are not yet established. Because of the heterogeneous resistance of imipenem, a number of clinical NTHi may contain resistant subpopulations that are not detected by broth microdilution [5]. Importantly, by disk diffusion (IMP10, 10 μg), all 46 IMI^{hR} isolates were categorized as imipenem resistant according to EUCAST.

Given the ability of amino acid substitutions in the transpeptidase region of PBP3 to drive β-lactam resistance, we expected that IMI^{hR} isolates would adopt a consistent pattern correlated with imipenem heteroresistance. However, sequencing of the *ftsI* genes encoding PBP3 revealed 25 different mutation patterns of amino acid substitutions associated with imipenem heteroresistance. These observations strongly suggest that an altered PBP3 protein is linked to imipenem heteroresistance. Similarly, our results show also that PBP3 has the highest affinity for imipenem in *H. influenzae* Rd KW20 (IC_{50}, 0.004 μg/mL). Our findings are in agreement with a previous publication examining the recombinants of the *H. influenzae* Rd KW20 strain carrying a mutated *ftsI* gene [5]. The presence of identical *ftsI* genes in diverse IMI^{hR} isolates may have arisen from recombination events involving horizontal gene transfer. The uptake signal sequence ACCGCACTT was indeed found in the *ftsI* gene of all IMI^{hR} isolates analysed herein. Considering the role of the uptake signal sequence in efficient species-specific DNA uptake in *Haemophilus* spp. [27], it is reasonable to speculate that the transfer of complete *ftsI* alleles is probably less common than the exchange of shorter sequences, causing mosaicism. When considering PBP4, *dacB* revealed an unexpectedly high number of sequence changes compared to the prototypic *H. influenzae* strain

Table 4
Amino acid substitutions identified in transpeptidase domain of *ponA, ponB* and *pbp2* genes from eight imipenem-heteroresistant *Haemophilus influenzae* isolates collected at Geneva University Hospitals between 2009 and 2014

	Amino acid substitutions identified in the transpeptidase domain of the *pbp2* gene (encoding for PBP2)																					
H. influenzae Rd KW20	Gln-59	Asp-126	Arg-132	Glu-165	Ala-205	Thr-229	His-259	Arg-268	Leu-321	Glu-428	Ile-437	Glu-496	Ala-498	Leu-508	Asn-513	Ala-518	Ala-523	Ala-534	Thr-569	Ala-570	Asp-589	Lys-592
GE3	.	.	.	.	.	.	.	.	.	.	.	.	Glu	.	.	Thr	Val	.	Ala	.	.	.
GE6	Arg	His	Leu	.	Val	.	.	.	Pro	.	Met	.	.	Ile	Ser	Thr	.	.	Ala	Val	Glu	Arg
GE42	.	.	.	.	.	.	Tyr	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GE49	Arg	His	Leu	.	.	.	.	.	.	.	.	.	Glu	.	.	Thr	Val	.	Ala	.	.	.
GE68	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GE71	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GE117	Arg	His	.	Asp	.	Ile	.	His	Pro	Asp	Met	Asp	.	Ile	Ser	Thr	.	Glu	Ala	Val	Glu	Arg
GE146	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	Amino acid substitutions identified in the transpeptidase domain of the *ponA* gene (encoding for PBP1a)																														
H. influenzae Rd KW20	Asn-9	His-28	Lys-40	Ser-88	Asp-92	Ser-133	Ser-187	Ala-298	Gly-356	Val-358	Asp-363	His-397	Ala-411	Met-487	Ile-513	Ala-518	Ala-564	Asp-588	Ile-591	Gly-624	Asn-626	Met-657	Thr-782	Ser-793	Asn-802	Ala-822	Thr-829	855	856	Thr-860	Gln-863
GE3	.	.	Arg	.	.	.	.	.	.	.	Glu	Arg	Ser	.	Met	.	.	.	.	.	Ser	Phe	.	Pro	Ser	Val	.	-	-	.	.
GE6	Ser	Tyr	.	.	Glu	Pro	Ala	Asp	.	.	.	Arg	Ser	Leu	Met	.	Ser	Glu	Val	Ser	Ser	Phe	.	The	Ser	Val	Ser	The	Pro	Ala	Glu
GE42	.	.	.	.	.	.	.	.	.	.	Glu	Arg	Ser	.	Met	Val	.	.	.	.	Ser	Phe	.	Pro	Ser	Val	.	-	-	.	.
GE49	.	.	.	.	.	.	.	.	.	.	Glu	Arg	Ser	.	Met	.	.	.	.	.	Ser	Phe	.	Pro	.	Val	.	-	-	.	.
GE68	.	.	Arg	Asn	.	Pro	Ala	.	Glu	Ala	.	Arg	.	.	Met	.	.	.	.	.	Ser	Phe	Met	Pro	.	Val	.	-	-	.	.
GE71	.	.	.	.	.	.	.	.	.	.	Glu	Arg	Ser	.	Met	Val	.	.	.	.	Ser	Phe	.	Pro	Ser	Val	.	-	-	.	.
GE117	.	.	Arg	.	Glu	Pro	Ala	Asp	.	.	.	Arg	Ser	Leu	Met	.	Ser	Glu	Val	Ser	Ser	Phe	.	The	Ser	Val	Ser	The	Pro	Ala	Glu
GE146	.	.	Arg	Asn	.	Pro	Ala	.	Glu	Ala	.	Arg	.	.	Met	.	.	.	.	.	Ser	Phe	Met	Pro	.	Val	.	-	-	.	.

	Amino acid substitutions identified in the transpeptidase domain of the *ponB* gene (encoding for PBP1b)																														
H. influenzae Rd KW20	Ser-6	Gly-23	Ala-32	Val-110	Leu-111	Ser-132	His-133	Arg-333	Lys-380	Ile-386	Gly-411	Met-421	Gly-428	Arg-436	Pro-488	Val-498	Asp-515	The-569	Leu-582	Ala-590	Ala-598	Ala-612	Ser-659	Ala-710	Thr-715	Gly-726	Asp-727	Ile-738	Thr-750	Ser-751	leu-755	Thr-759	760	Glu-763
GE3	.	.	The	.	.	.	Lys	Glu	Met	Val	Thr	.	.	.	.	.	Ser	.	.	.	.	.	The	.	.	.	Val	.	.	.	.	-	.
GE6	.	.	The	.	.	.	Lys	Glu	Met	Val	Thr	Ser	.	Leu	.	Gly	.	Ile	Ser	The	Val	Ala	.	Ala	Ser	.	Val	.	.	.	.	-	.
GE42	.	.	Val	.	.	.	.	Glu	.	.	.	.	His	.	.	.	Ser	.	.	.	.	.	The	.	.	.	Val	.	.	.	.	-	.
GE49	.	.	.	.	Phe	Asn	Asn	Lys	Glu	.	Val	.	.	.	.	.	Ser	.	.	.	.	.	The	.	.	.	Val	.	.	.	.	-	.
GE68	.	.	.	.	.	.	.	Lys	Glu	.	Val	Thr	.	.	.	Asp	Gly	Ser	.	.	.	.	The	.	.	.	Val	.	.	.	.	-	.
GE71	.	.	The	.	.	.	.	Lys	Glu	Met	Val	Thr	Ser	.	Leu	.	Gly	.	Ile	Ser	The	Val	Ala	.	Ala	.	Gly	Val	Ile	Ala	Phe	Glu	Ala	The
GE117	.	.	.	Ile	.	.	.	.	Glu	.	Val	.	.	.	.	.	.	Ser	.	.	.	.	.	The	.	.	.	Val	.	.	.	.	-	.
GE146	Ala	arg	.	Ile	.	.	.	.	Glu	.	Val	.	.	.	.	.	.	Ser	.	.	.	.	.	.	.	.	.	Val	.	.	.	.	-	.

"." indicates no amino acid substitution
Red box indicates amino acid insertion

Rd KW20. However, no further mutations were detected in that gene within the resistant heteropopulation. Such genetic diversity will have to be considered for future sequencing-based assays aimed at predicting antimicrobial resistance.

The deduced amino acid sequences of the *ponA, ponB* and *pbp2* genes from eight IMI^{hR} isolates revealed amino acid substitutions in all these genes corresponding to different mutation patterns (Table 4). Accordingly, the competition assays using Bocillin-FL showed low imipenem affinity for the PBP1a, PBP1b and PBP2 proteins. Using the hydrolysis of penicillin G and a Nitrocefin disk, no enzyme-mediated mechanism was found to be involved in imipenem heteroresistance. Additional analysis of the IMI^{hR} isolates revealed a partial deletion in *acrR* leading to the loss of regulation of the AcrAB-TolC efflux pump in two isolates. Furthermore, our analyses revealed that in IMI^{hR} isolates, imipenem susceptibility was enhanced by the presence of the efflux pump inhibitor CCCP. These results document the requirement for an efflux system in the appropriate development and expression of imipenem heteroresistance in NTHi isolates.

As described previously [28], porins isolated from the *H. influenzae*–resistant strains present a lower conductibility than wild-type porins. This might explain the nonsusceptibility to β-lactams and to other drugs of low molecular weight. It seems therefore likely that the changes in the amino acid sequence of OmpP2 found in IMI^{hR} isolates were related to an increase in imipenem heteroresistance.

The analysis of the deduced amino acid sequences of *envC, nlpD* and the *dcw* gene cluster revealed different patterns of amino acid substitutions. However, we did not find any specific pattern correlated with imipenem heteroresistance. The small number of isolates explored herein does not allow any final conclusion to be made, and we cannot exclude the possibility that some of these genes are related to imipenem heteroresistance in NTHi.

The present study has the following limitations: we did not have access to clinical data, and we cannot assess the potential role of imipenem preexposure; the transcription levels of *acrR* (regulatory gene of the AcrAB-TolC efflux pump) and *acrB* (encoding protein AcrB) were not investigated; the effect of the broad-spectrum efflux pump inhibitor CCCP on imipenem susceptibility was not assessed on all 46 IMI^{hR} isolates; and the sequence of the *ompP2* gene was not determined for all 46 IMI^{hR} isolates.

Conclusions

To our knowledge, this is the first study to provide evidence indicating that altered PBP3, slowed drug influx and direct efflux regulation contribute to the development of imipenem heteroresistance in NTHi. Furthermore, this study points towards the need to consider the potential impact of using carbapenems on lower respiratory tract infections, knowing that NTHi frequently colonize the human airway. Importantly, all 46 IMI^{hR} isolates were susceptible to meropenem (MIC $\leq$2 μg/mL) according to the EUCAST clinical breakpoints for infections other than meningitis. Further work is required to determine the best method to detect imipenem-heteroresistant *H. influenzae* isolates in clinical laboratories.

Transparency Declaration

All authors report no conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2016.10.009.

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Imipenem Heteroresistance in Nontypeable *Haemophilus influenzae* is linked to a combination of altered PBP3, slow drug influx and direct efflux regulation

A. Cherkaoui[1], S. M. Diene[2], A. Renzoni[1], S. Emonet[1], G. Renzi[1], P. Francois[2], J. Schrenzel[1,2]

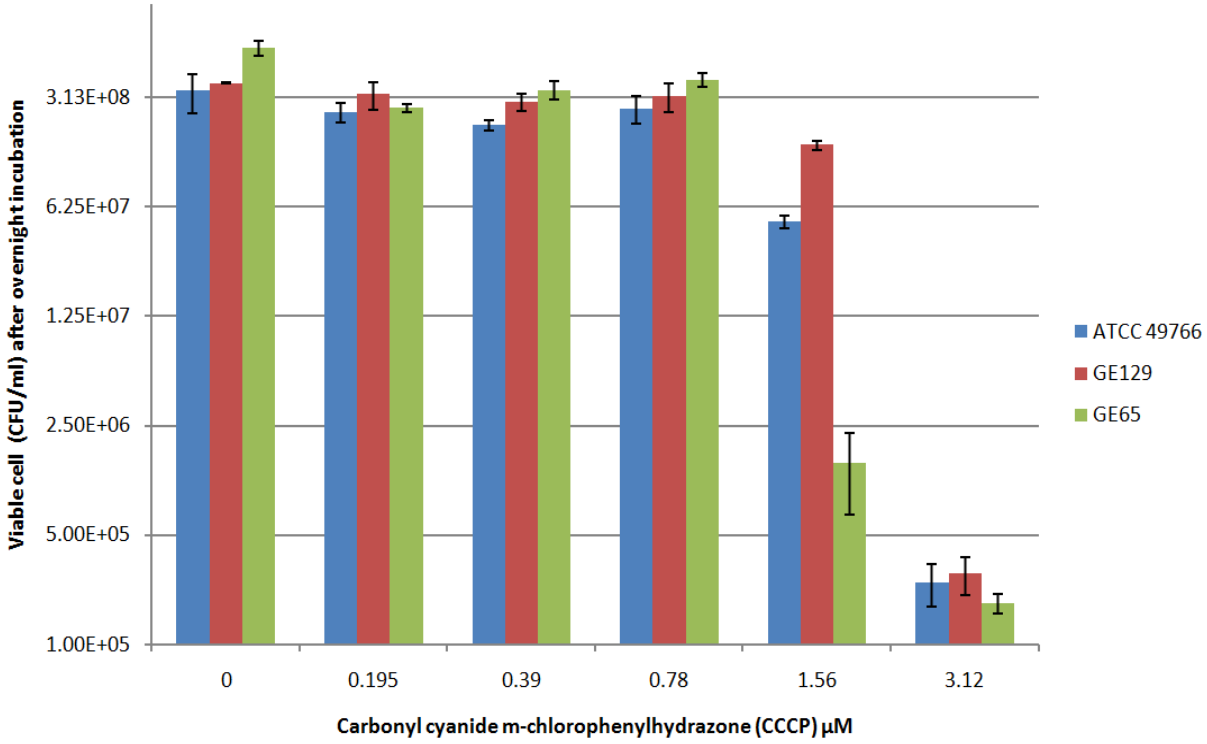
[1]Bacteriology Laboratory, Division of Laboratory Medicine, Department of Genetics and Laboratory Medicine, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

[2]Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

Supplementary Electronic Materials

	Name	Sequence	Size of PCR product (bp)	References
PBP4	*dacB*_F1	TGCGACAAACAGTTCAATGAG	1’605	[1]
	*dacB*_R1	TCGGGGCTTATTATTGTTCG		
	*dacB*_F1-d	TNCGNCAAACAGTTCAATGAG		This study
PBP4	*dacB*_F2	TGGCGATTTAGTATTAGATACCTCTG	674	This study
	*dacB*_R2	GAGATAGACCCGAACCATCG		
PBP5	*dacA*_F1	GACTGAAGTAAATCGCTCAGG	1’487	This study
	*dacA*_R1	CCCTTCTCGATTAATTCCCG		
	*dacA*_F1-d	GACTGAAGTNAATNGNNCAGG		
PBP5	*dacA*_F2	GGTTACCATTGGTGAAAGTGC	678	This study
	*dacA*_R2	TGATCCATTAAAGCACCAAGTTT		
The repressor gene of AcrAB-TolC efflux pump	*acrR*_F	TTGTGGGTTTACGGCTTACC	831	This study
	*acrR*_R	CCGATGACACCGACAAAAAT		
PBP3	*ftsI*_F1	GACGATTTGGATAACCCATA	2’265	[1]
	*ftsI*_R1	CTGGATAATTCTGTCTCAGA		
PBP3	*ftsI*_F2	GCGGATAAAGAACGAATTGC	1’295	[2]
	*ftsI*_R2	TCTCCTGCTTTTGGATCATTG		

Supplementary Table-S1: List of the primers designed and used in this study

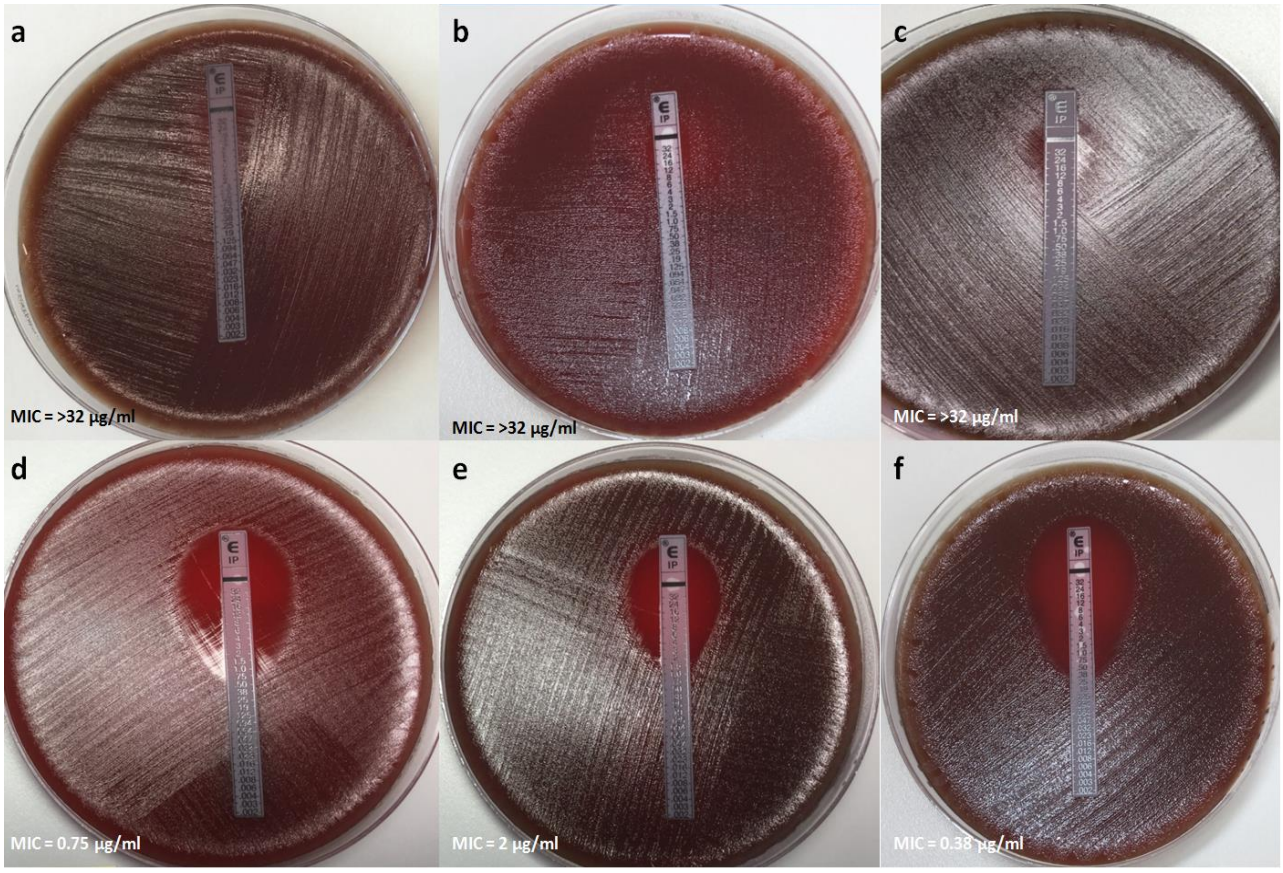


Supplementary Fig. S1: Effect of different concentrations of CCCP on *Haemophilus influenzae* growth. Values represent the mean ± SD from two independent experiments.

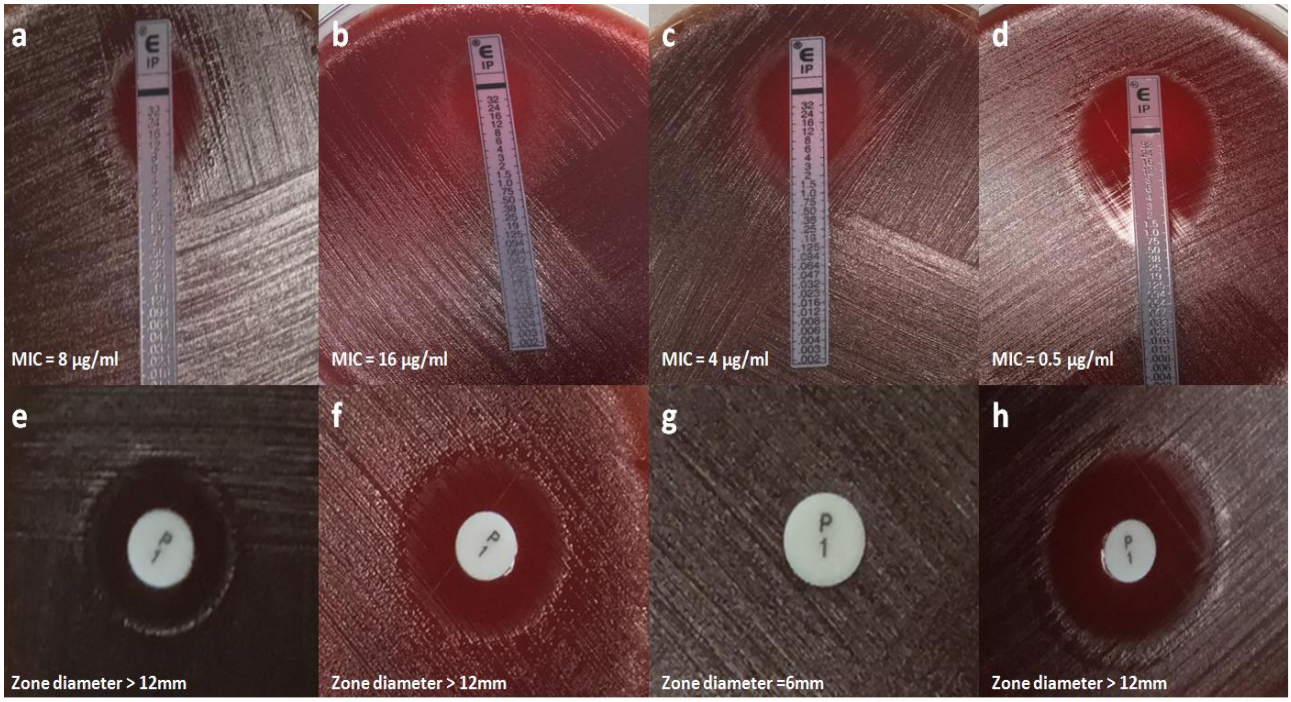
The inoculum suspension was prepared by selecting several colonies from overnight growth (16–24h of incubation) on chocolate agar plates with a cotton swab and suspending the colonies in sBHI to a McFarland 0.5 standard density.

Strain ID	Imipenem MIC (µg/ml)	Source	Accession number	Genome coverage (X)	Genome size (bp)	No. of contigs	Average contig sizes (bp)	No. of ORFs	No of RNAs	No of tRNAs	% GC
GE3	>32	Blood culture	LVZC00000000	399	1'904'241	55	34'773	1874	19	51	40,15
GE6	24	Blood culture	LVZD00000000	544	1'839'572	33	55'834	1748	19	52	39,45
GE42	>32	Sputum	LVZE00000000	562	1'789'234	24	74'515	1669	25	52	40,96
GE49	>32	Bronchoalveolar lavage	LVZF00000000	523	1'890'006	30	62'996	1813	21	53	39,93
GE68	8	Lung tissues	LVZG00000000	665	1'826'497	24	76'060	1723	13	51	38,27
GE71	12	Bronchoalveolar lavage	LVZH00000000	548	1'877'948	34	55'213	1799	23	54	40,01
GE117	>32	Bronchial aspirate	LVZI00000000	393	1'827'414	27	67'649	1729	18	54	39,45
GE146	>32	Bronchial aspirate	LVZJ00000000	555	1'895'444	29	65'754	1850	12	51	39,18

Supplementary Table-S2: Summary of genome sequencing for 8 nontypeable imipenem heteroresistant *H. influenzae* (IMI^{hR} NTHi) isolates exhibiting different *ftsI* gene mutation patterns.



Supplementary Fig. S2
a, **b,** and **c** = imipenem heteroresistant *H. influenzae* isolates by the Etest method
d, **e,** and **f** = imipenem-susceptible *H. influenzae* isolates by the Etest method
d = ATCC 49766; **e** = ATCC 49247; **f** = clinical isolate (GE20)
a, **b, c,** and **f** isolates were collected at Geneva University Hospitals between 2009 and 2014

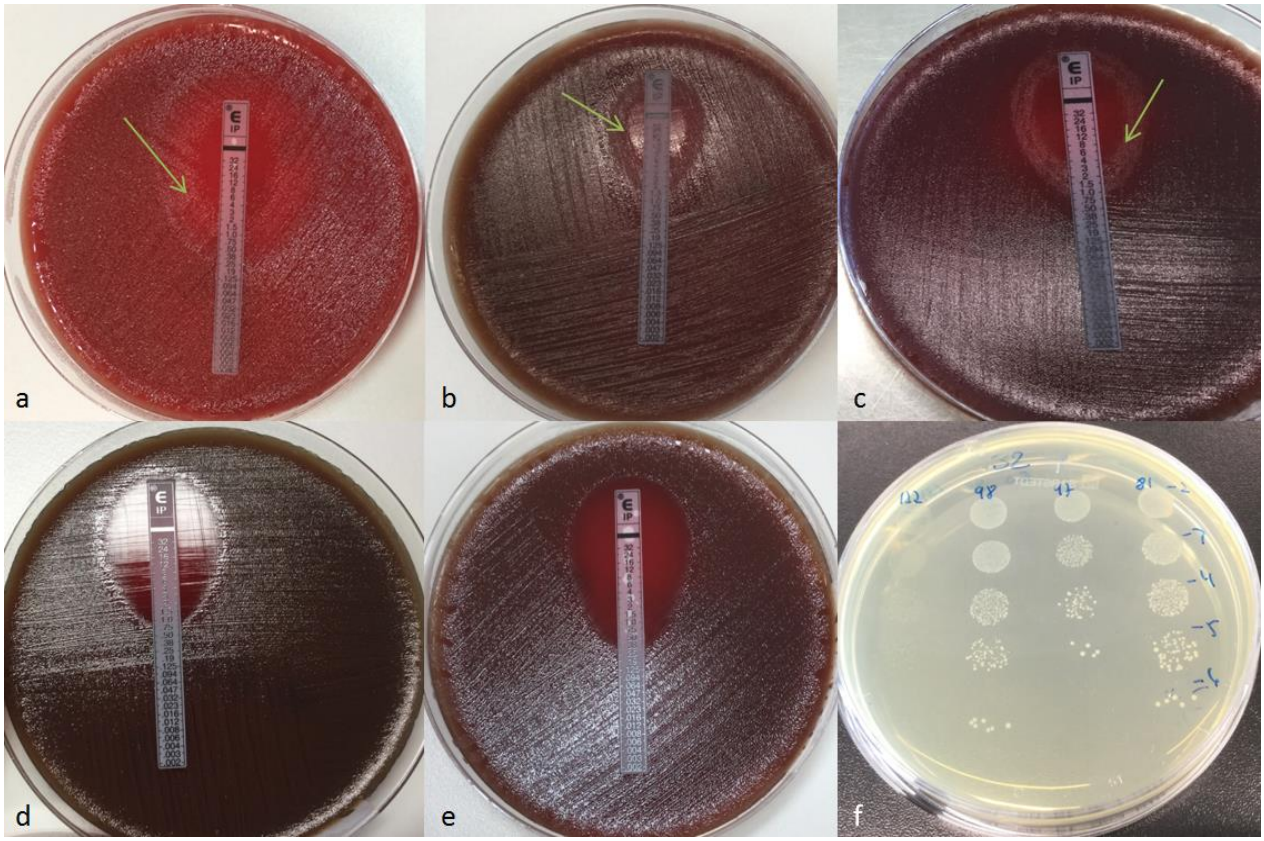


GE71 (a, e) **GE26** (b, f) **GE129** (c, g) **ATCC49766** (d, h)

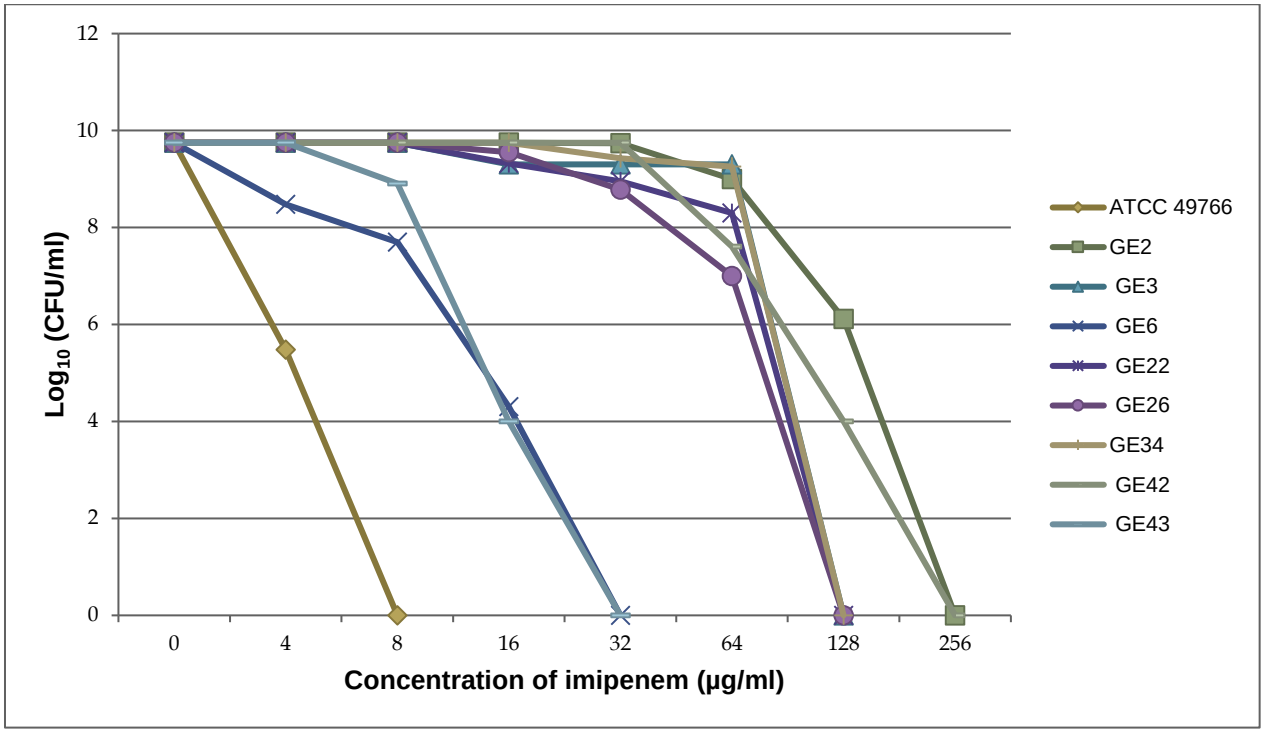
Supplementary Fig. S3

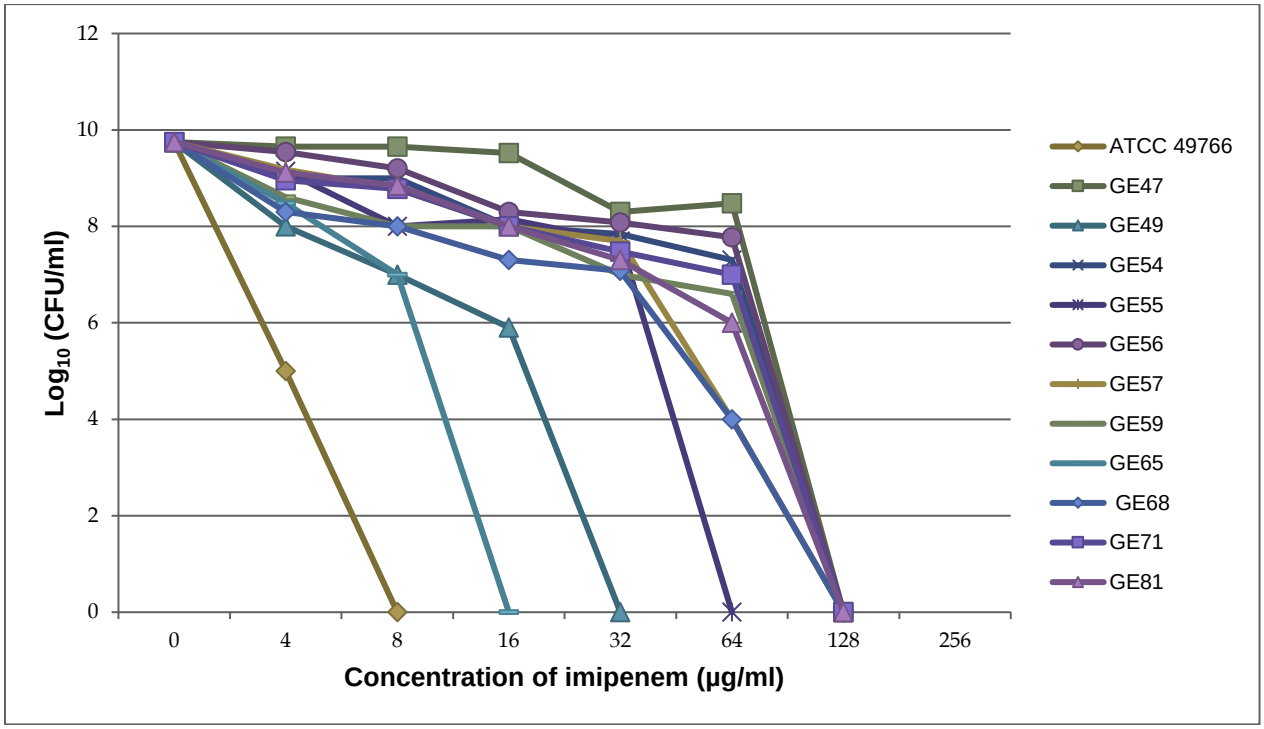
a, **b, c,** and **d** = Etest method ; **e, f**, **g**, and **h** = Disk diffusion (1unit benzylpenicillin)

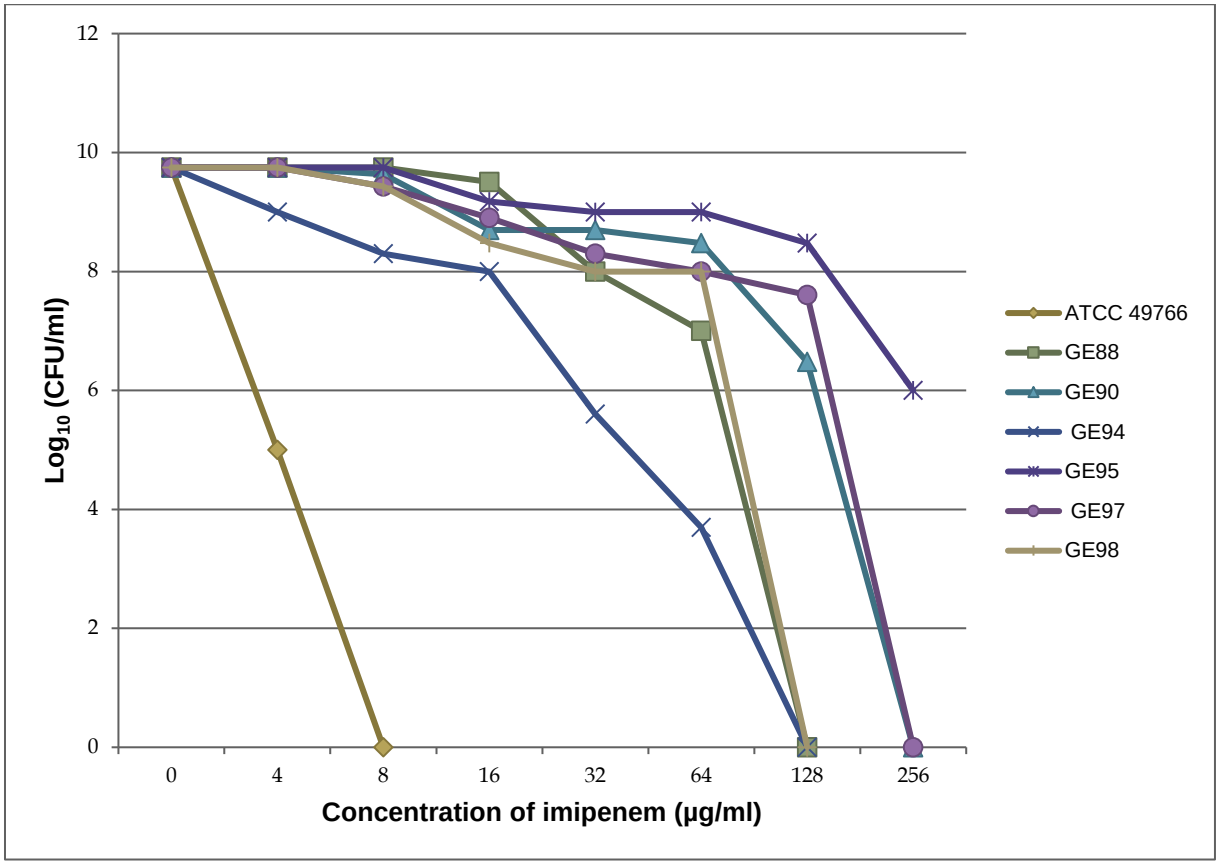
GE71, **GE26** and **GE129** imipenem heteroresistant *H. influenzae* isolates were collected at Geneva University Hospitals between 2009 and 2014

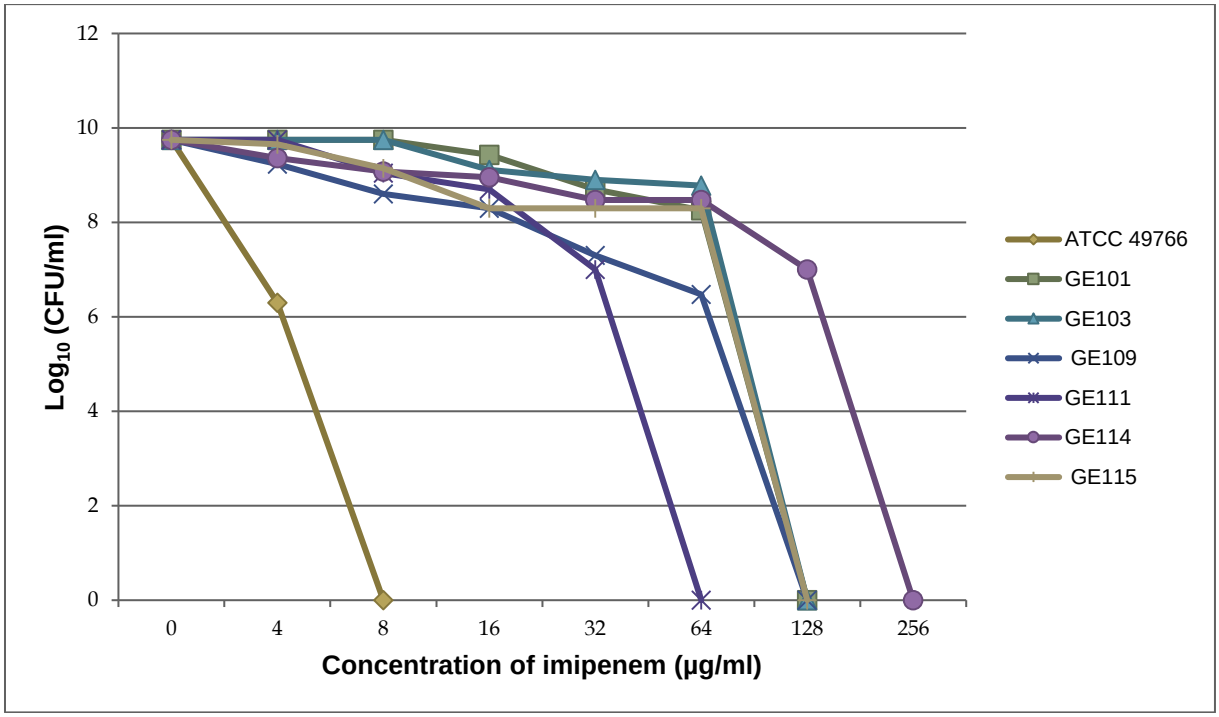


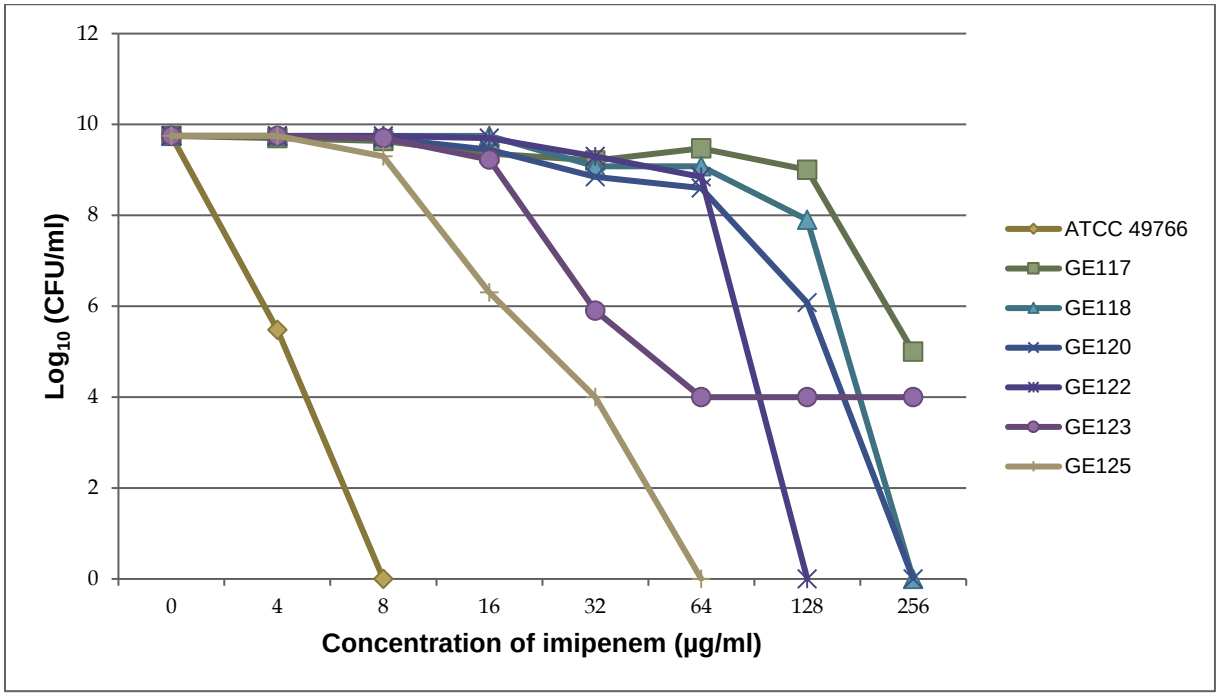
Supplementary Fig. S4
MICs determination using an imipenem Etest for five nontypable *H. influenzae* (NTHi) isolates
a, **b,** and **c** = imipenem heteroresistant *H. influenzae* isolates (the arrows indicate the imipenem resistant sub-populations)
d, and **e** = imipenem susceptible *H. influenzae* isolates (control group)
a, **b, c, d, and e** = isolates collected at Geneva University Hospitals between 2009 and 2014
f = microdilution plate of four imipenem heteroresistant NTHi. The 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions were each plated on HTM with 32µg/ml of imipenem

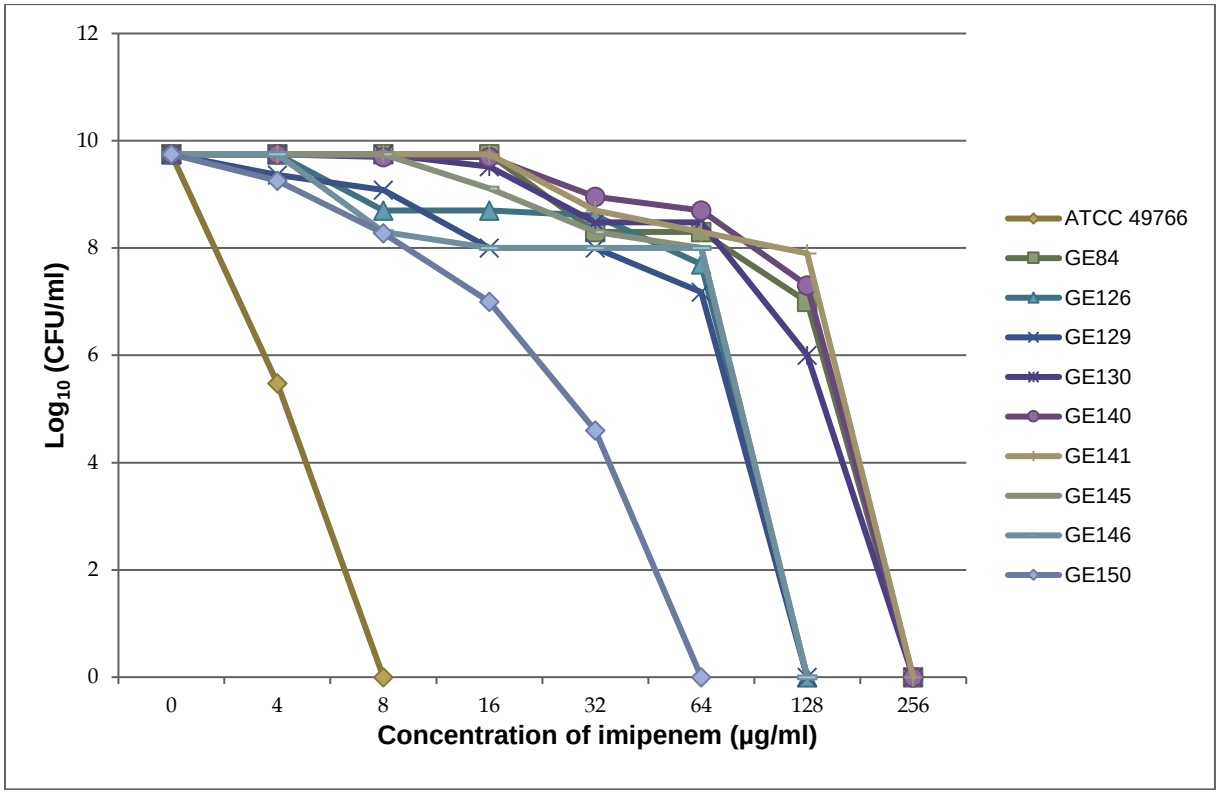

12
10
8
6
4
2
0
Log10 (CFU/ml)
0
4
8
16
32
64
128
256
Concentration of imipenem (μg/ml)
ATCC 49766
GE2
GE3
GE6
GE22
GE26
GE34
GE42
GE43


12
10
8
6
4
2
0
Log10 (CFU/ml)
0
4
8
16
32
64
128
256
Concentration of imipenem (μg/ml)
ATCC 49766
GE47
GE49
GE54
GE55
GE56
GE57
GE59
GE65
GE68
GE71
GE81


12
10
8
6
4
2
0
Log10 (CFU/ml)
0
4
8
16
32
64
128
256
Concentration of imipenem (μg/ml)
ATCC 49766
GE88
GE90
GE94
GE95
GE97
GE98

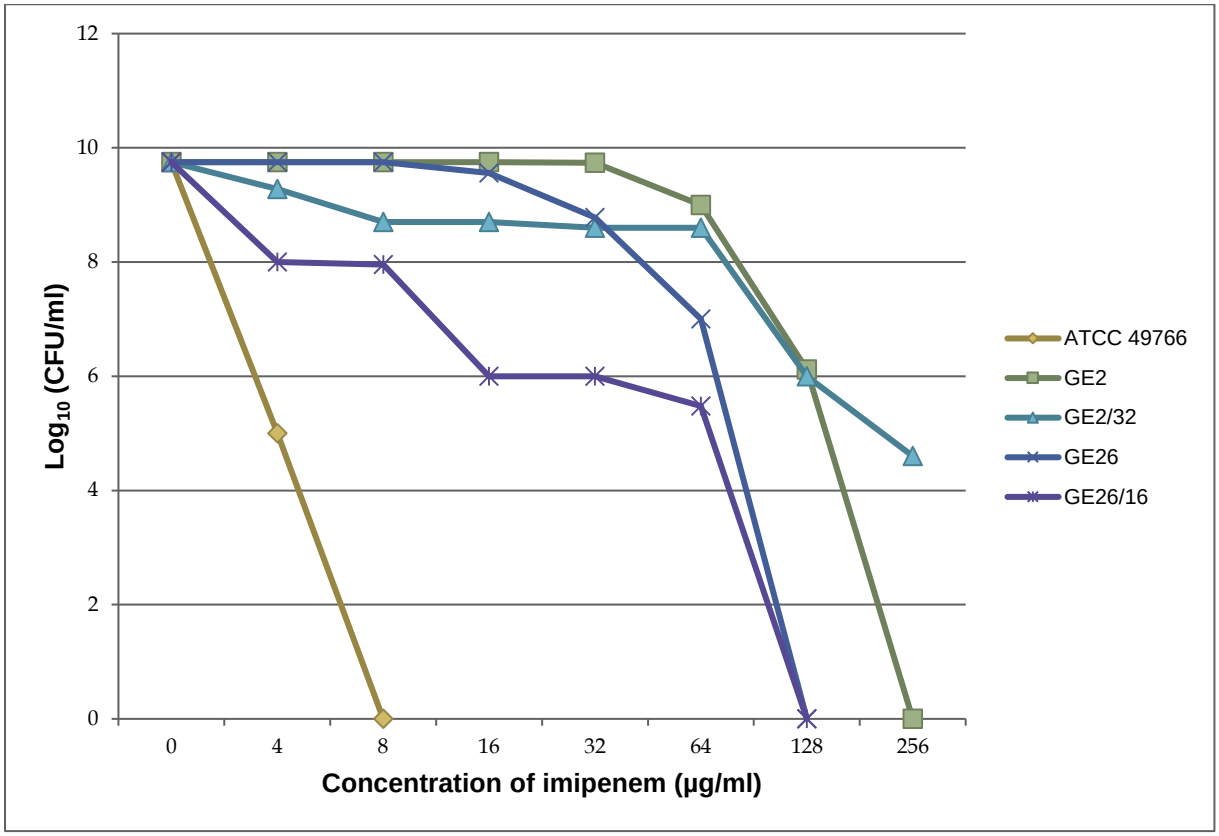

12
10
8
6
4
2
0
Log10 (CFU/ml)
0
4
8
16
32
64
128
256
Concentration of imipenem (μg/ml)
ATCC 49766
GE101
GE103
GE109
GE111
GE114
GE115

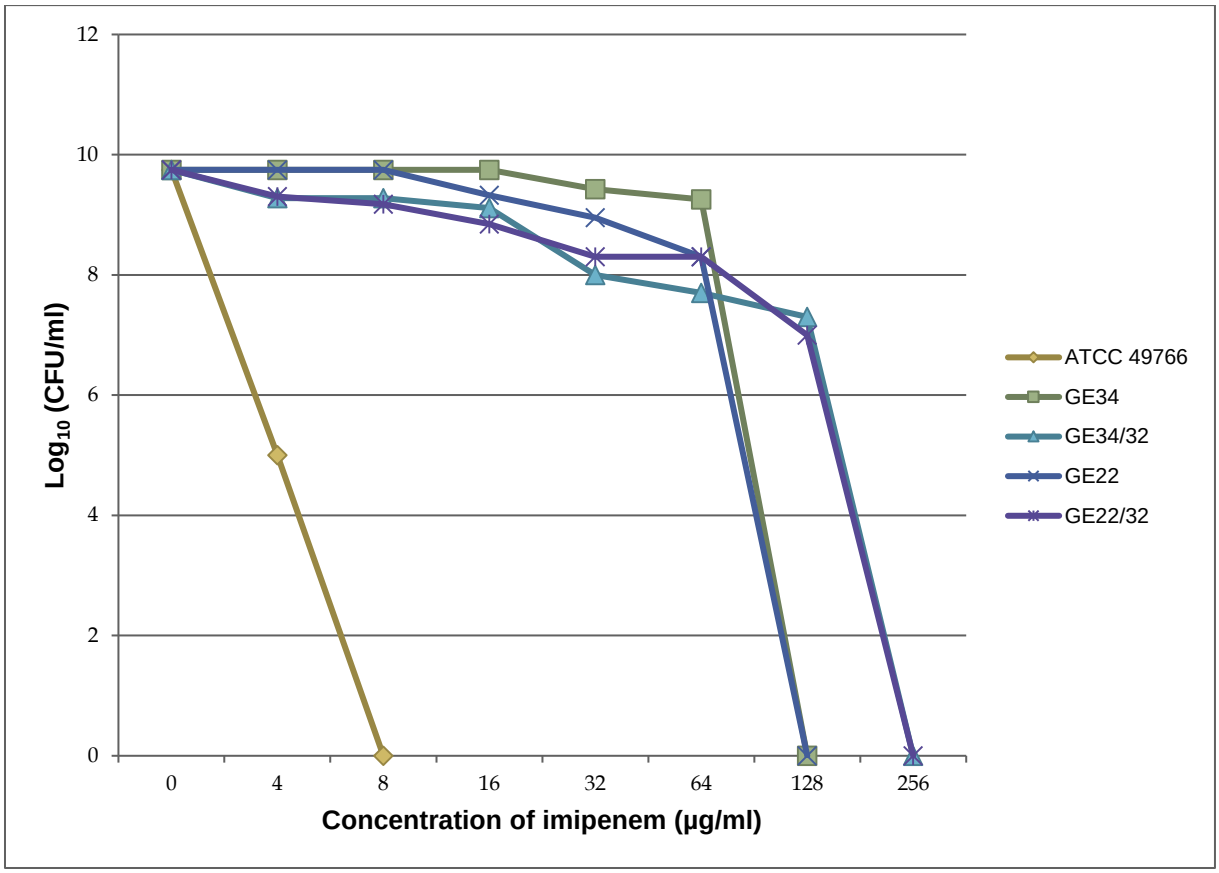


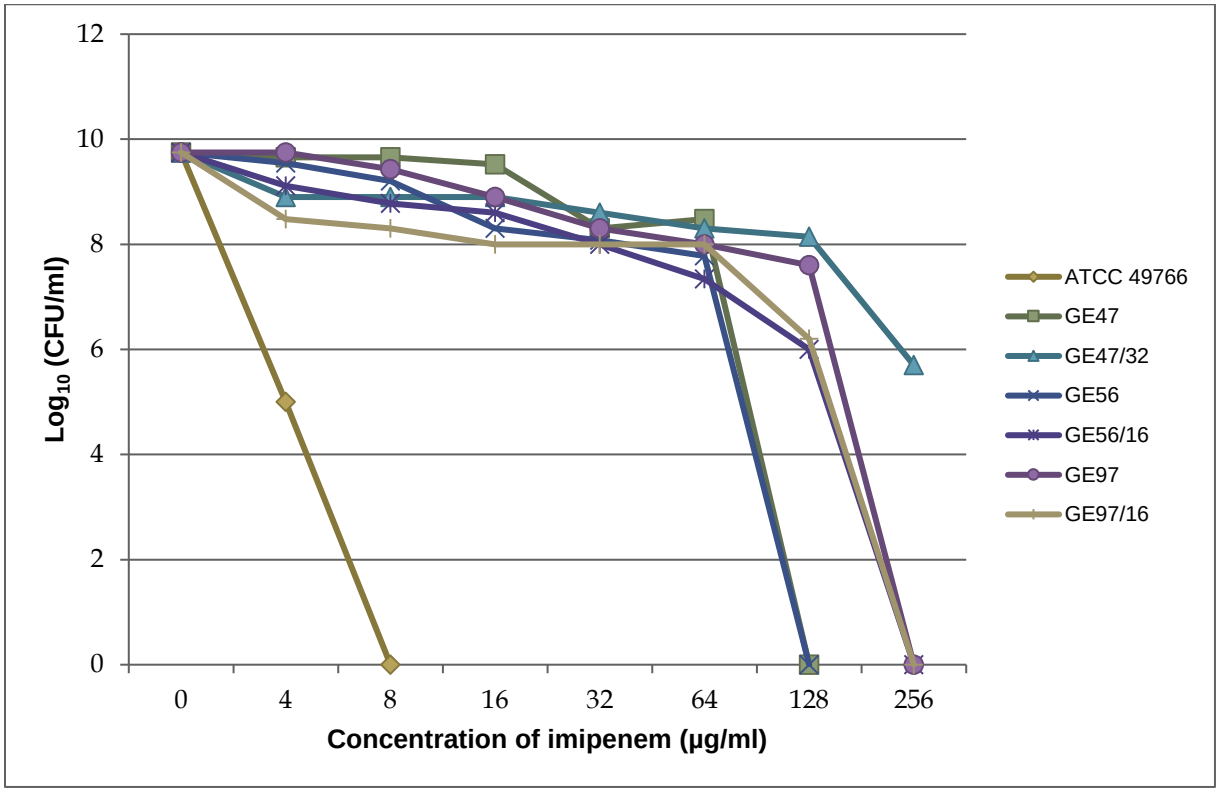


Supplementary Fig. S5

Population analysis profiles of 46 IMI^{hR} NTHi strains, and ATCC49766 with imipenem as determined by microdilution plate counts.


12
10
8
6
4
2
0
Log10 (CFU/ml)
0
4
8
16
32
64
128
256
Concentration of imipenem (μg/ml)
ATCC 49766
GE2
GE2/32
GE26
GE26/16

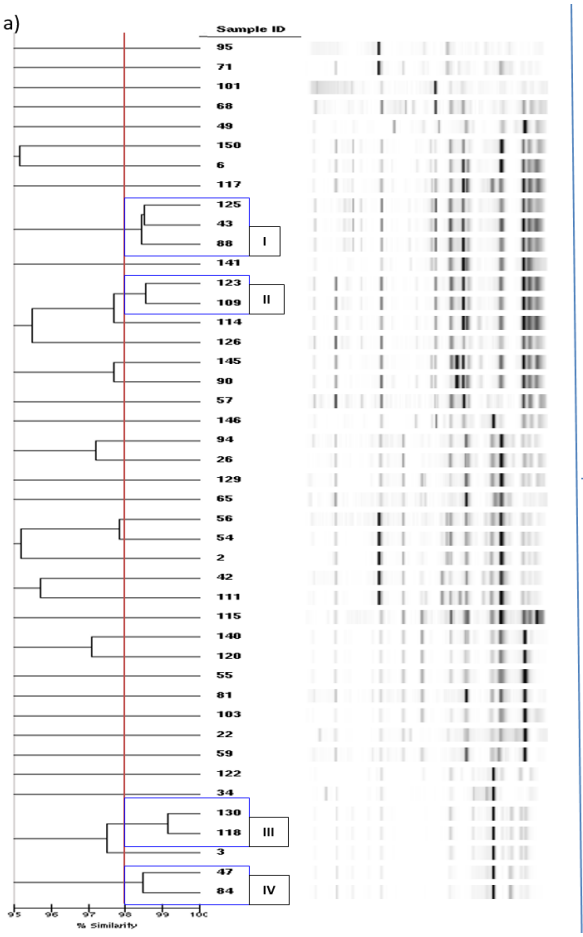

12
10
8
6
4
2
0
Log10 (CFU/ml)
0
4
8
16
32
64
128
256
Concentration of imipenem (μg/ml)
ATCC 49766
GE34
GE34/32
GE22
GE22/32

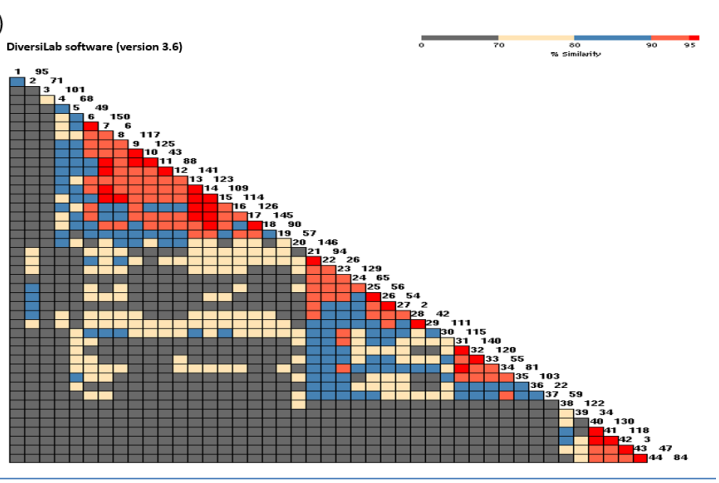


Supplementary Fig. S6

Population analysis profiles of 7 IMI^{hR} NTHi strains as determined by microdilution plate counts

One colony of each of 7 IMI^{hR} NTHI strains was picked from HTM agar containing 16µg or 32µg of imipenem/ml and passaged in imipenem free HTM agar. After overnight growth, these cultures were named strainID/16 or 32. These cultures were used as a starting cell suspension for further population analysis. Strain ATCC49766 was used as a negative control.





c)

	Strain ID	Age (years)	Sample type	Year of strain isolation	Amino acid substitutions in the *ftsI, dacA, dacB, and acrR* genes
I	43	55	Cerebrospinal fluid	December 2011	Identical
	125	74	Sputum	July 2013	
	88	72	Sputum	October 2013	Different
II	109	82	Tracheal aspirate	November 2013	Identical
	123	59	Tracheal aspirate	September 2013	
III	118	43	Sputum	January 2014	Identical
	130	67	Bronchoalveolar lavage	March 14	
IV	47	75	Sputum	January 2012	Different
	84	57	Sputum	January 2013	

Supplementary Fig. S7: Rep-PCR analysis of imipenem heteroresistant *H. influenzae* isolates.

The DNA was extracted and amplified using the DiversiLab *Haemophilus* kit (bioMérieux, La Balme-les-Grottes, France) for DNA fingerprinting according to the manufacturer's instructions. The rep-PCR products were detected, and the amplicons were separated using microfluidics lab-on-a-chip technology and analyzed using the DiversiLab system. Further analysis was performed with the web-based DiversiLab software (version 3.6) using the band-based modified Kullback–Leibler distance for the calculation of the percent similarities. A cluster of closely related isolates was defined as isolates sharing ≥95% similarity, and for isolates to be defined as identical, a similarity of ≥98% was used.
a) rep-PCR-based dendrogram, and virtual gel image; **b)** similarity matrix; and **c)** molecular and clinical characteristics for strains with a similarity of ≥98%.

Group	Strain (n)	Ampicillin MIC range (µg/ml)	Imipenem MIC range (µg/ml)	TEM-1	Amino acid substitution for :												
					Ala-19	Glu-25	Val-31	Thr-53	Pro-57	Met-69	Asn-205	Ala-214	Leu-312	Val-343	Asp-356	Asp-359	Phe-377
	Rd KW20	0.064 - 0.094	0.25 - 0.38														
I	1	256	>32	+	.	.	.	.	.	.	Asp	.	.	Ala	.	.	.
	18	0.19 - 4	4 - >32		.	.	.	.	.	.	Asp	.	.	Ala	.	.	.
II	1	12	>32		.	.	Ile	.	Leu	.	Asp	.	.	.	.	.	.
	3	1 - 3	>32		.	.	Ile	.	Leu	.	Asp	.	.	.	.	.	.
III	3	1 - 1.5	12 - >32		.	.	Ile	.	.	.	Asp	.	Phe	Ala	.	.	.
IV	1	1	>32		Ser	.	.	.	.	.	.	.	.	.	.	.	.
V	3	1 - 1.5	8 - >32		.	.	.	.	.	.	Asp	.	Phe	Ala	.	.	.
VI	1	1.5	>32		.	.	.	.	.	.	Asp	.	Phe	Ala	.	.	Leu
	1	256	>32	+	.	.	.	.	.	.	Asp	.	Phe	Ala	.	.	Leu
VII	1	0.75 - 0	>31	+	Ser	.	.	.	.	Ile	Asp	Pro	Phe	Ala	.	.	Leu
	2	0.75 - 1	>32		Ser	.	.	.	.	Ile	Asp	Pro	Phe	Ala	.	.	Leu
Control group (imipenem susceptible isolates)																	
	ATCC 49247	3	2		.	Lys	.	.	.	.	Asp	.	Phe	Ala	.	Asn	.
	ATCC 49766	0.125 - 0.19	0.75 - 1		.	.	.	.	.	.	Asp	.	.	Ala	.	.	.
	1	0.38	2		.	.	.	.	.	.	Asp	.	.	Ala	.	.	.
	1	8	1.5	+	.	.	.	.	.	.	Asp	.	.	Ala	.	.	.
	1	0.19	0.38		.	.	.	Ala	.	.	Asp	.	.	Ala	Gly	.	.
	3	0.5 - 1.5	1.5 - 2		.	.	Ile	.	.	.	Asp	.	Phe	Ala	.	.	.

Supplementary Table-S3: Amino acid substitutions identified in the transpeptidase domain of the *dacA* gene encoding PBP5 from 43 isolates of *H. influenzae.*

"." indicates no amino acid substitution

Nineteen IMI^{hR} and 3 IMI^{S} isolates including control strain ATCC 49766 had the most common mutation pattern, which was characterized by the following two amino acid substitutions: Asn250Asp and Val343Ala. The IMI^{hR} isolate (GE49) mutation pattern was characterized by only one amino acid substitutions (Ala19 to Ser). Three amino acid substitutions were found only among the IMI^{hR} isolates: Ala19Ser (4 isolates), Ala214Pro (3 isolates), and Phe377Leu (5 isolates).

Group	Strain (n)	Ampicillin MIC range (µg/ml)	Imipenem MIC range	TEM-1	Amino acid substitution for :																	
	Rd KW20	0.064 - 0.094	0.25 - 0.38		Ser-14	Ala-15	Ala-21	Arg-22	Asn-26	Gln-27	Ser-29	Leu-31	Leu-33	Lys-51	Thr-77	Ile-121	His-131	Gln-134	Ser-143	Ala-159	Lys-170	Ile-185
I	10	0.75 - 3	8 - >32		.	.	.	.	.	.	.	His	.	.	.	.	.	.	.	.	.	.
	3	12 - 256	>32	+	.	.	.	.	.	.	.	His	.	.	.	.	.	.	.	.	.	.
II	9	0.5 - 1.5	>32		.	.	.	.	.	.	.	His	.	.	.	Val	.	.	.	.	.	.
	2	32 - 256	8 - >32	+	.	.	.	.	.	.	.	His	.	.	.	Val	.	.	.	.	.	.
III	1	24	>32	+	.	.	.	.	.	.	.	His	.	Arg	.	Val	.	.	.	.	.	.
IV	4	0.19 - 3	6 - >32		.	.	.	.	.	.	.	His	.	.	.	Val	Asp	Lys	.	.	.	.
V	1	0.75	>32		Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	Pro	.	Asn	.
VI	1	1	>32		Leu	.	Val	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	.	.	.	.
VII	12	0.38 - 4	4 - >32		Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	.	.	.	.
	1	256	>32	+	Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	.	.	.	.
1 (GE65)		8	6	+	Deletion of 1 base (T) behind nucleotide 482 / Genetic consequence : Early stop codon																	
1 (GE129)		256	>32	+	Deletion of 1 base (A) behind nucleotide 65 / Genetic consequence : Early stop codon																	
Control group (imipenem susceptible isolates)																						
ATCC49247		3	2		.	.	.	.	.	.	.	His	.	.	.	Val	.	Lys	.	.	.	.
ATCC49766		0.125 - 0.19	0.75 - 1		Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	.	.	.	.
	3	0.5 - 1.5	1.5 - 2		.	.	.	.	.	.	.	His	.	.	.	.	.	.	.	.	.	.
	1	0.25	2		.	Glu	.	.	.	.	.	His	.	.	.	.	.	.	.	.	.	.
GE119	1	1.5	1		.	.	.	.	.	.	.	His	.	.	.	Val	.	.	.	.	.	.
	1	0.19	0.38		.	.	.	.	.	.	Pro	His	.	.	.	Val	.	Lys	.	.	.	Met
	1	24	0.38	+	Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	Pro	Val	Asn	.
	1	0.094	0.38		.	.	.	Lys	.	.	.	His	.	.	.	Val	.	.	.	.	.	.
	1	1	2		Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	Pro	Val	Asn	.
	2	0.38 - 0.75	0.38		Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	Pro	.	Asn	.
	1	0.38	2		Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	.	.	.	.
	1	8	1.5	+	Leu	.	.	Lys	Asp	Arg	.	His	Ile	.	Ser	Val	Asp	Lys	.	.	.	.

Supplementary Table-S4: Amino acid substitutions identified in *acrR* regulatory gene of the AcrAB-TolC efflux from 61 strains of *H. influenzae* strains according to their imipenem MICs.

"." indicates no amino acid substitution

	Amino acid substitution for :																																
H. influenzae Rd KW20	Val-10	Phe-13	Ala-21	Asn-30	Leu-37	Ile-39	Asn-45	Ser-46	Val-48	Asp-49	Asn-50	Gln-53	Ala-57	Gln-61	Gly-62	Phe-73	Phe-87	Thr-89	Lys-90	Ala-91	Ser-92	Glu-93	Asn-94	Gly-95	Asn-98	Gly-100	Asp-101	Thr-103	Ser-104	Lys-114			
GE3	.	.	.	.	.	.	.	.	Ile	Lys	Asp	.	.	.	.	.	Leu	Ser	Ala	Gln	.	Gly	Thr	Glu	.	.	His	Ile	Thr	.			
GE6	.	.	.	.	.	Val	.	.	Arg	Lys	Asp	.	Glu	Ala	.	.	Leu	Ser	Asp	Tyr	.	-	Ser	Arg	.	.	Asn	Ile	Thr	.			
GE42	Ile	.	.	Lys	Val	.	Thr	.	Gly	.	Asp	His	.	.	.	.	.	.	Asp	.	.	Lys	.	.	.	.	.	.	Thr	.			
GE49	.	.	Val	Lys	.	.	Ser	Ala	Glu	.	Asp	.	Thr	.	.	Leu	.	Ser	Asp	Tyr	Thr	Lys	Glu	Lys	His	Ala	.	Ser	Thr	Asn			
GE68	Ile	Leu	.	Lys	.	.	.	.	Ile	Lys	Asp	.	.	.	.	.	.	Ser	Ala	Gln	.	Gly	Thr	Lys	Asp	.	His	Ile	Thr	.			
GE71	.	.	.	.	.	.	.	.	Ile	Lys	Asp	.	.	.	.	.	Leu	Ser	Ala	Gln	.	Gly	Thr	Glu	.	.	His	Ile	Thr	.			
GE117	.	.	.	.	.	.	.	.	Ile	Lys	Asp	.	.	.	Ser	.	Leu	Ser	Pro	Gln	.	Gly	Thr	Glu	.	.	His	Ile	Thr	.			
GE146	.	.	.	.	.	.	.	.	Ile	Lys	Asp	.	.	.	Ser	.	Leu	Ser	Ala	Gln	.	Gly	Thr	Glu	.	.	His	Ile	Thr	.			

	Ala-115	Phe-116	Arg-123	Ala-128	Gly-130	Ser-133	Val-141	Asn-143	Asn-144	Ser-145	Asp-146	Thr-150	Ser-151	Thr-154	Val-155	Phe-159	Lys-160	Gly-161	Ile-162	Lys-176	Ala-180	Lys-181	Asn-182	Thr-183	Asn-184	Lys-185	Gln-186	Pro-187	Asn-188	Asp-189
GE3	.	Leu	.	.	.	.	.	.	.	.	Lys	Ile	Asp	.	.	.	.	.	.	Glu	.	.	.	.	.	.	.	.	.	.
GE6	Gly	.	.	Ser	.	.	.	Glu	.	Lys	Glu	Lys	Asp	Ser	.	.	.	.	.	.	Val	Ala	Tyr	Ser	Ser	Gly	Gly	Ser	.	.
GE42	.	.	Lys	.	.	Thr	.	Asp	Ser	Lys	Lys	.	Asn	.	Ile	Tyr	.	Val	Thr	Glu	.	Asn	Tyr	Asp	Asp	.	.	.	.	.
GE49	Thr	.	.	.	Glu	Thr	Leu	.	.	Lys	Lys	.	Asn	.	.	.	Asn	Ala	.	Glu	Asp	Val	Leu	Ser	Val	Pro	Gly	Val	Gly	.
GE68	.	.	.	.	.	.	.	.	.	.	Lys	.	Asn	.	.	.	.	.	.	Asp	Ser	.	Tyr	.	Ala	Ser	Asn	Asn	.	.
GE71	.	Leu	.	.	.	.	.	.	.	.	Lys	.	Asp	.	.	.	.	.	.	Glu	His	.	Tyr	.	Thr	Ala	Ala	Ala	Gly	Asn
GE117	.	Leu	.	.	.	.	.	.	.	.	Lys	.	Asn	.	.	.	.	.	.	Glu	His	.	Tyr	.	Ala	Gly	Gly	Val	Gly	Gly
GE146	.	.	.	Ser	.	.	.	.	.	.	Lys	.	Asn	.	.	.	.	.	.	Glu	Tyr	.	Tyr	Gly	Gly	Ala	Ala	Gly	Gly	Gly

	190	191	192	193	194	Lys-195	Ala-196	Arg-200	Ile-201	Gly-202	Glu-203	Ile-204	Asn-205	Ile-208	Asp-218	Val-220	Ala-221	Lys-222	Lys-231	Tyr-232	Asn-233	Glu-234	Ala-235	Asp-236	237	238	239	240	241	242
GE3	.	.	.	.	.	Val	.	Tyr	Pro	Gln	Lys	.	Ser	Val	Asn	Ile	.	Gly	Arg	.	.	.	.	.	.	.	.	.	.	.
GE6	.	.	.	.	.	.	Pro	Ser	Pro	Gln	.	.	Ser	Val	Asn	Ile	.	Gly	Arg	Glu	Asp	Ile	Thr	Ile	Gly	Pro	Gly	.	.	.
GE42	.	.	.	.	.	.	.	Ile	Pro	Gln	Lys	Val	Ser	Val	Asn	Ile	.	Arg	.	Glu	.	Thr	Ile	Glu	Lys	Leu	Asp	.	.	.
GE49	.	.	.	.	Thr	.	Thr	Thr	Val	.	.	.	.	.	Asn	Ile	Val	Gly	Arg	Asp	Ser	Asn	Lys	Gln	Arg	Ser	Ser	Arg	Gly	Ala
GE68	.	.	.	.	.	Val	.	Thr	Ser	Gln	Ser	.	Ser	Val	Asn	Ile	Ile	Gly	Arg	Glu	Asp	Ile	Ile	Asn	Glu	Pro	Asp	.	.	.
GE71	Pro	Arg	Ala	Gly	Thr	Val	.	Tyr	Pro	Gln	Lys	.	Ser	Val	Asn	Ile	.	Gly	Arg	Glu	Asp	Ile	Val	.	Leu	Asp	.	.	.	.
GE117	.	Val	Pro	Arg	Thr	Val	.	Tyr	Pro	Gln	Lys	.	Ser	Val	Asn	Ile	.	Gly	.	Glu	Asp	Ile	Thr	Ile	Val	Ala	Gly	.	.	.
GE146	Ala	Gly	Ala	Gly	Ala	Val	.	Tyr	Pro	Gln	Lys	.	Ser	Val	Asn	Ile	.	Gly	Arg	Glu	Ser	Ile	His	Glu	Thr	Asp	.	.	.	.

	243	244	245	246	247	248	Glu-249	His-250	The-251	Gln-253	Leu-254	Val-257	Ala-259	Thr-260	Arg-264	Asn-283	Thr-286	Arg-291	Val-308	Thr-317	Glu-323	Thr-325	Arg-326	Gln-328	Val-334	Leu-342	Ala-351	Lys-354	Thr-356	Asn-357
GE3	.	.	.	.	.	.	Gly	Lys	Lys	.	Val	Ala	Ser	.	.	.	Asp	Ser	.	Asp	Lys	.	.	.	.	Val	.	Arg	.	.
GE6	.	.	.	Asp	Asn	Ser	Gly	Lys	Lys	.	Val	Ala	Ser	.	His	.	Asp	.	.	Asn	Lys	.	.	.	.	Val	.	Arg	.	Glu
GE42	.	.	.	.	.	Leu	Gly	Lys	Lys	.	Val	Ala	Ser	.	.	.	Asp	.	.	.	.	.	His	.	.	Val	.	.	Asn	Glu
GE49	Thr	Thr	Tyr	Thr	Tyr	Gln	Gly	Lys	Lys	Glu	Val	Ala	.	Ser	.	Lys	Ala	Ser	Leu	Asn	.	Glu	.	.	Ile	Val	Ser	Arg	Ala	Ala
GE68	.	.	.	Asp	Leu	Val	Gly	Thr	Lys	.	Val	Ala	Ser	.	.	.	Ala	Ser	.	Asp	Lys	Ala	.	His	.	Val	.	Arg	.	Glu
GE71	.	.	.	.	.	Leu	Gly	Lys	Lys	.	Val	Ala	Ser	.	.	.	Asp	Ser	.	Asp	Lys	.	.	.	.	.	.	Arg	.	Ser
GE117	.	.	.	Asp	Asn	Ser	Gly	Lys	Lys	.	Val	Ala	Ser	.	His	.	Asp	.	.	Asn	Lys	Glu	.	.	Ile	Val	.	Arg	Asn	.
GE146	.	.	.	.	.	Leu	Gly	Lys	Lys	.	Val	Ala	Ser	.	.	.	Ala	Ser	.	Asn	Lys	Ala	.	His	.	Val	.	Arg	Asn	Asp

	Gly-358	359	360	Gly-361	362	363	Val-364	Lys-365	Glu-367
GE3	.	.	.	.	.	.	.	.	.
GE6	.	.	.	.	.	.	Lys	.	.
GE42	Lys	.	.	.	Lys	Ala	Glu	.	Gly
GE49	Asn	Asp	Gly	Lys	Thr	Lys	Glu	.	.
GE68	Ser	.	.	Lys	Lys	Gly	.	.	.
GE71	.	.	.	.	Thr	Asn	.	Thr	.
GE117	Lys	.	.	Ser	Lys	Ala	Glu	.	.
GE146	Gln	.	.	.	Lys	Thr	Glu	.	.

Supplementary Table-S5: *ompP2* gene sequences for 8 imipenem heteroresistant *H. influenzae* isolates collected at Geneva University Hospitals between 2009 and 2014.

"." indicates no amino acid substitution
Red box indicates amino acid insertion

Murein hydrolase activator NlpD precursor

H. influenzae Rd KW20	Amino acid substitution for : Lys-2	Thr-62	Glu-71	Thr-81	Gly-119	Ala-175	Asp-201	Ile-202	Thr-222	Thr-259	Ser-260	Ser-291	Thr-302	Thr-311	Thr-402
GE3	.		Pro	.	.	.	.	.	.	.	Leu	.	.	.	.
GE6	.	Thr	Pro	.	.	.	Ser	Thr	.	.	.	.	.	.	.
GE42	.	Ile	Pro	.	.	Glu	Ser	Thr	Met	.	.	.	.	.	Ala
GE49	.	Met	Pro	.	.	.	.	.	.	.	.	.	.	.	.
GE68	.	Thr	Pro	.	.	.	Ser	Thr	.	.	.	.	.	.	.
GE71	.	Ile	Pro	.	.	.	Ser	Thr	Met	.	.	.	.	.	Ala
GE117	Asn	Met	Pro	Ala	.	.	Gly	Thr	Met	Ile	.	Ala	Ala	.	.
GE146	.	.	Pro	.	Arg	.	.	.	.	.	.	.	.	Ala	.

Murein hydrolase activator EnvC precursor

H. influenzae Rd KW20	Amino acid substitution for : Arg-53	Glu-54	Ala-56	Glu-76	Ala-112	Thr-115	Ile-118	Gln-141	Asn-166	Lys-178	Ser-182	Thr-233	Ala-273	Leu-292	Leu-307
GE3	Leu	Gln	Thr	Ala	.	Ala	.	Lys	.	.	.	Ala	.	.	.
GE6	Leu	Gln	.	.	Lys	Ala	Met	.	His	Glu	Ala	Ala	Thr	.	Ser
GE42	Leu	Gln	Thr	Ala	.	Ala	.	Lys	.	.	.	Ala	.	.	.
GE49	Leu	Gln	Thr	Ala	.	Ala	.	Lys	.	.	.	Ala	.	.	.
GE68	Leu	Gln	Thr	Ala	.	Ala	.	Lys	.	.	.	Ala	.	.	.
GE71	Leu	Gln	Thr	Ala	.	Ala	.	Lys	.	.	.	Ala	.	.	.
GE117	.	.	.	.	.	Ala	Met	.	.	Glu	Ala	Ala	.	Ile	.
GE146	Leu	Gln	Thr	Ala	.	Ala	.	Lys	.	.	.	Ala	.	.	.

Protein MraZ

H. influenzae Rd KW20	Amino acid substitution for : Leu-27	Gln-33	Ser-43	Ala-91	Ser-98	Gly-99	Pro-100	Thr-146
GE3	.	.	.	.	.	.	.	Ala
GE6	.	Lys	Pro	.	.	.	.	.
GE42	.	.	.	.	.	.	.	Ala
GE49	.	.	.	.	.	.	.	.
GE68	.	Lys	Pro	.	.	.	.	.
GE71	.	.	.	.	.	.	.	Ala
GE117	.	Lys	Pro	.	.	.	.	.
GE146	Ile	Lys	.	Ser	Gly	Ser	Leu	Ala

Cell division protein FtsL

H. influenzae Rd KW20	Amino acid substitution for : Leu-29
GE3	.
GE6	.
GE42	.
GE49	.
GE68	.
GE71	Val
GE117	.
GE146	.

UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2, 6-diaminopimelate ligase (*murE*)

H. influenzae Rd KW20	Amino acid substitution for : Pro-11	Glu-12	Asn-15	Leu-53	His-54	Ala-77	Ala-91	Val-100	Asn-107	Asn-108	Ala-142	Ile-156	Ala-175	His-178	Thr-184	His-200	Val-202	Ala-209	Tyr-259	Ser-439	Thr-449
GE3	.	.	.	.	.	Glu	Ser	.	Lys	.	.	.	Thr	.	.	.	.	.	.	Pro	Ala
GE6	.	.	.	.	.	Glu	Ser	.	Lys	.	.	.	Thr	.	.	.	.	.	.	Pro	Ala
GE42	.	.	.	.	.	Glu	.	.	Lys	.	.	.	Thr	.	.	.	.	.	.	Pro	Ala
GE49	.	.	.	.	.	Glu	.	.	Lys	.	.	.	Thr	.	.	.	.	.	.	Pro	Ala
GE68	Leu	.	.	Ile	.	.	.	Ile	Lys	Lys	.	Val	Thr	.	.	.	.	.	.	Pro	Ala
GE71	.	.	Asp	Ile	Tyr	.	.	.	Lys	.	Ser	.	Asn	Gln	Ala	Tyr	Ala	.	His	Pro	Ala
GE117	.	.	.	.	.	Glu	Ser	.	Lys	.	.	.	Thr	.	.	.	.	.	.	Pro	Ala
GE146	.	Lys	Asp	.	.	Asp	.	.	.	.	.	.	Thr	.	.	.	.	Val	.	Pro	Ala

UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D- alanine ligase (*murF*)

H. influenzae Rd KW20	Amino acid substitution for : Glu-26	Lys-27	Ser-79	Ala-213	Ala-267	His-272	Thr-273	Ser-274	Ile-282	Glu-289	Thr-291	Val-300	Thr-322	Phe-339	Gly-370	Arg-375	Gly-381	Ser-427
GE3	.	Glu	Leu	Val	Val	Gly	Asn	Glu	Val	Asp	Ser	.	Ala	.	Ser	Leu	Ala	Leu
GE6	.	Glu	Leu	.	Val	Gly	Asn	Glu	Val	Asp	Ser	.	Ala	.	Ser	Leu	Ala	Leu
GE42	.	Glu	Leu	.	Val	Gly	Asn	Glu	Val	Asp	Ser	Ile	Ala	Leu	Ser	Leu	Ala	Leu
GE49	Ala	Glu	Leu	.	Val	.	.	.	Val	Asp	Ser	.	Ala	.	Ser	Leu	Ala	Leu
GE68	.	Glu	Leu	.	Val	Gly	Asn	Glu	Val	Asp	Ser	.	Ala	.	Ser	Leu	Ala	Leu
GE71	.	Glu	Leu	.	Val	Gly	Asn	Glu	Val	Asp	Ser	Ile	Ala	Leu	Ser	Leu	Ala	Leu
GE117	.	Glu	Leu	.	Val	Gly	Asn	Glu	Val	Asp	Ser	.	Ala	.	Ser	Leu	Ala	Leu
GE146	.	Glu	Leu	.	Val	Gly	Asn	Glu	Val	Asp	Ser	Ile	Ala	Leu	Ser	Leu	Ala	Leu

Phospho-N-acetylmuramoyl-pentapeptide- transferase (*mraY*)

	Amino acid substitution for :		
H. influenzae **Rd KW20**	**Asn-27**	**Pro-99**	**Ala-112**
GE3	Ile	Ser	.
GE6	Ile	Ser	.
GE42	Ile	Ser	.
GE49	Ile	.	Thr
GE68	Ile	Ser	.
GE71	Ile	Ser	.
GE117	Ile	Ser	.
GE146	Ile	Ser	.

UDP-N-acetylmuramoylalanine--D-glutamate ligase (*murD*)

	Amino acid substitution for :																		
H. influenzae **Rd KW20**	**Asn-40**	**Val-184**	**His-214**	**Lys-217**	**Arg-226**	**Asn-232**	**Lys-237**	**His-238**	**Thr-239**	**Glu-269**	**Ala-277**	**Ile-298**	**Thr-307**	**Val-326**	**Asp-371**	**Ala-398**	**Leu-399**	**Phe-406**	**Asp-411**
GE3	.	.	Gln	.	.	.	.	.	.	.	Val	.	.	.	.	.	.	.	Glu
GE6	.	.	Gln	.	.	.	.	.	.	.	Val	.	.	.	.	.	.	.	Glu
GE42	Lys	.	.	Glu	Lys	Gly	Arg	Gln	.	.	Val	Val	.	.	.	Val	Gln	.	Glu
GE49	.	Ile	.	.	.	.	.	.	.	.	.	.	.	Ala	Asn	.	.	.	Glu
GE68	Lys	.	.	Glu	Lys	Gly	Arg	Gln	.	.	Val	Val	.	.	.	Val	Gln	.	Glu
GE71	Lys	Ile	.	.				Gln	Ile	Lys	.	.	Ala	Ala	.	.	Gln	Leu	Glu
GE117	Lys	.	.	Glu	Lys	Gly	Arg	Gln	.	.	Val	Val	.	.	.	Val	Gln	.	Glu
GE146	Lys	.	.	Glu	Lys	Gly	Arg	Gln	.	.	Val	Val	.	.	.	Val	Gln	.	Glu

Lipid II flippase FtsW

	Amino acid substitution for :	
H. influenzae **Rd KW20**	**Glu-247**	**Met-365**
GE3	Asp	.
GE6	Asp	.
GE42	Asp	.
GE49	Asp	Ile
GE68	Asp	.
GE71	Asp	Ile
GE117	Asp	.
GE146	Asp	.

UDP-N-acetylglucosamine--N-acetylmuramyl- (pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (*murG*)

	Amino acid substitution for :											
H. influenzae **Rd KW20**	**Ile-81**	**His-159**	**Met-174**	**Asn-176**	**Phe-223**	**Ala-260**	**Ala-286**	**Gly-311**	**Tyr-333**	**Leu-337**	**Met-354**	**Asn-370**
GE3	.	Tyr	.	Asp	Leu	.	.	.	Ser	Phe	.	.
GE6	.	Tyr	.	.	Leu	.	.	.	.	.	Ile	Ser
GE42	.	.	.	.	Leu	.	Val	Asp	.	.	.	.
GE49	Val	.	Val	.	Leu	Thr	.	.	Ser	Phe	.	.
GE68	.	.	.	.	Leu	.	Val	Asp	.	.	.	.
GE71	.	.	.	.	Leu	.	Val	Asp	.	.	.	.
GE117	.	.	.	.	Leu	.	Val	Asp	.	.	.	.
GE146	.	.	.	.	Leu	.	Val	Asp	.	.	.	.

UDP-N-acetylmuramate--L-alanine ligase (*murC*)

	Amino acid substitution for :							
H. influenzae **Rd KW20**	**Glu-79**	**Val-98**	**Thr-99**	**Ser-100**	**Val-321**	**Ala-328**	**Gly-372**	**Gln-412**
GE3	.	.	.	.	Ile	Asp	.	.
GE6	.	Ile	Ala	Ala	.	.	Asp	Leu
GE42	Lys	.	.	.	.	.	Asp	Leu
GE49	.	.	.	.	Ile	Asp	.	.
GE68	.	.	.	.	Ile	Asp	.	.
GE71	Lys	.	.	.	.	.	Asp	Leu
GE117	Lys	.	.	.	.	.	Asp	Leu
GE146	Lys	.	.	.	.	.	Asp	Leu

D-alanine--D-alanine ligase B (*ddlB*)

	Amino acid substitution for :								
H. influenzae **Rd KW20**	**Leu-23**	**His-41**	**Met-78**	**Ser-133**	**Pro-202**	**Ala-247**	**Ser-249**	**Thr-250**	**Val-263**
GE3	Phe	.	.	Ala	Ser	Glu	Ala	Ile	.
GE6	Phe	.	.	.	.	Glu	Ala	Ile	Leu
GE42	Phe	.	.	.	.	Glu	Ala	Ile	Leu
GE49	.	Tyr	Ile	.	.	.	.	.	.
GE68	Phe	.	.	Ala	Ser	Glu	Ala	Ile	.
GE71	Phe	.	.	.	.	Glu	Ala	Ile	Leu
GE117	Phe	.	.	.	.	Glu	Ala	Ile	Leu
GE146	Phe	.	.	.	.	Glu	Ala	Ile	Leu

Cell division protein FtsQ

	Amino acid substitution for :			
H. influenzae **Rd KW20**	**Ala-76**	**Val-210**	**Asp-224**	**Val-267**
GE3	Asp	.	.	Ile
GE6	.	Ile	.	.
GE42	.	Ile	.	.
GE49	.	.	Tyr	.
GE68	Asp	.	.	Ile
GE71	.	Ile	.	.
GE117	.	Ile	.	.
GE146	.	Ile	.	.

Cell division protein FtsA

	Amino acid substitution for :		
H. influenzae **Rd KW20**	**Val-22**	**Val-182**	**Tyr-411**
GE3	Ala	Ile	His
GE6	Ala	.	His
GE42	Ala	.	His
GE49	Ala	Ile	.
GE68	Ala	Ile	.
GE71	Ala	.	His
GE117	Ala	.	His
GE146	Ala	.	His

Cell division protein FtsZ

	Amino acid substitution for :	
H. influenzae **Rd KW20**	**Thr-168**	**Thr-410**
GE3	Ala	.
GE6	Ala	Ala
GE42	Ala	Ala
GE49	Ala	.
GE68	Ala	.
GE71	Ala	.
GE117	Ala	Ala
GE146	Ala	Ala

Supplementary Table-S6: Amino acid substitutions identified in *envC*, *nlpD* and the *dcw* cluster of 8 imipenem heteroresistant *H. influenzae* isolates collected at Geneva University Hospitals between 2009 and 2014

"." indicates no amino acid substitution

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Research article

Transcriptional Modulation of Penicillin-Binding Protein 1b, Outer Membrane Protein P2 and Efflux Pump (AcrAB-TolC) during Heat Stress Is Correlated to Enhanced Bactericidal Action of Imipenem on Non-typeable *Haemophilus influenzae*

Abdessalam Cherkaoui, Seydina M. Diene, Adrien Fischer, Stefano Leo, Patrice François and Jacques Schrenzel

Authors' contribution statements

Abdessalam CHERKAOUI designed the study and all the experiments. He carried out the following elements:

- Selection of the clinical bacterial strains included in this study
- Antimicrobial susceptibility testing (disk diffusion and E-test assays)
- Quantification of viable NTHi cells with a range of imipenem concentrations
- Determination of growth and viability of NTHi cells at either 37 or 42°C
- Killing activity of imipenem on NTHi cells at either 37 or 42°C
- Binding affinity to PBPs by using the competitive fluorescent penicillin Bocillin-FL assay
- Interpretation of the transcriptome analysis data
- Interpretation of the qRT-PCR data
- He analysed all the data and wrote the manuscript

S.M. Diene and **S. Leo** carried out the transcriptome analysis

A. Fischer provided help and support for the statistical analyses

P. François helped in the transcriptome analysis, and revised the manuscript.

J. Schrenzel supervised the research and revised the manuscript

This study originated with the unexpected observation that highly imipenem resistant *H. influenzae* strains (MIC > 32 mg/L) , although viable when exposed at 37°C to high concentrations of imipenem, revealed more susceptible to lower concentrations of this antibiotic when the cells were grown at 42°C. Nowadays, several reports are published regarding the regulation of gene expression in response to heat stress in various bacterial species. However, there is currently no specific work dealing with the ability of *H. influenzae* cells to increase their antimicrobial susceptibility after heat stress. In this study, we observed that on the basis of their viability and growth levels at either 37 or 42°C without imipenem, the quantitation of viable cells pre-exposed to 0.25 mg/mL of imipenem revealed more than a twofold decrease in *H. influenzae* viable cells at 42°C as compared to 37°C. This interesting observation raises many interrogations on the modulation of PBPs functions, cell wall structure and imipenem susceptibility by heat stress. Thus, we investigated binding affinity to PBPs by using Bocillin-FL and bacterial cells growth at either 37 or 42°C, monitored transcriptome changes by RNA-seq after pre-incubation of bacterial cells at either 37 or 42°C, and confirmed by real-time quantitative reverse transcription-PCR (qRT-PCR) the transcriptional changes of different key genes (*ponA, ponB, pbp2, ftsI, acrR, acrB, ompP2*). Transcriptome analysis showed that the expression levels of *ponB* (encoding PBP1b) and *acrR* (regulator of AcrAB-TolC efflux pump) were significantly increased at 42°C. In contrast, the transcript levels of *ompP2* (encoding the outer membrane protein P2) and *acrB* gene (encoding AcrB) were significantly lower under heat stress condition. No significant differences were observed in expression level of *ftsI* (encoding PBP3) and cluster of genes required in cell division and cell wall (dcw) biosynthesis. This study shows the correlation in the heat stress response between the transcriptional modulation of *ponB, ompP2, acrR*, and *acrB* and the enhancement of antimicrobial effects of imipenem on *H. influenzae*.

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ORIGINAL RESEARCH
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Transcriptional Modulation of Penicillin-Binding Protein 1b, Outer Membrane Protein P2 and Efflux Pump (AcrAB-TolC) during Heat Stress Is Correlated to Enhanced Bactericidal Action of Imipenem on Non-typeable *Haemophilus influenzae*

Abdessalam Cherkaoui[1], Seydina M. Diene[2], Adrien Fischer[1], Stefano Leo[2], Patrice François[2] and Jacques Schrenzel[1,2]*

[1] Bacteriology Laboratory, Division of Laboratory Medicine, Department of Genetics and Laboratory Medicine, Geneva University Hospitals, Geneva, Switzerland, [2] Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, Geneva, Switzerland

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Edited by:
Miguel Cacho Teixeira,
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Reviewed by:
Raymond Allan,
University of Southampton, United Kingdom
Jan Rupp,
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Thomas Dandekar,
University of Würzburg, Germany

***Correspondence:**
Abdessalam Cherkaoui
abdessalam.cherkaoui@hcuge.ch

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Objective: The purpose of the present study was to investigate the penicillin binding proteins (PBPs), drug influx and efflux modulations during heat stress and their effects on the bactericidal action of imipenem on non-typeable *Haemophilus influenzae* (NTHi).

Methods: The two NTHi clinical isolates (GE47 and GE88, imipenem MICs by E-test > 32 μg/mL) examined in this study were collected at Geneva University Hospitals. The imipenem killing activity was assessed after incubation of the NTHi strains at either 37 or 42°C for 3 h with increasing concentrations of imipenem. The detection of PBPs was carried out by Bocillin-FL. Global transcriptional changes were monitored by RNA-seq after pre-incubation of bacterial cells at either 37 or 42°C, and the expression levels of relevant target genes were confirmed by qRT-PCR.

Results: Quantitation of NTHi viable cells after incubation with 0.25 μg/mL of imipenem for 3 h revealed more than a twofold decrease in GE47 and GE88 viable cells at 42°C as compared to 37°C. Transcriptome analysis showed that under heat stress conditions, there were 141 differentially expressed genes with a $|\log2(\text{fold change})| > 1$, including 67 up-regulated and 74 down-regulated genes. The expression levels of *ponB* (encoding PBP1b) and *acrR* (regulator of AcrAB-TolC efflux pump) were significantly increased at 42°C. In contrast, the transcript levels of *ompP2* (encoding the outer membrane protein P2) and *acrB gene* (encoding AcrB) were significantly lower under heat stress condition.

Conclusion: This study shows that the transcriptional modulation of *ponB, ompP2, acrR,* and *acrB* in the heat stress response is correlated to enhanced antimicrobial effects of imipenem on non-typeable *H. influenzae.*

Keywords: heat stress, NTHi, RNA-seq, AcrAB-TolC, OmpP2, PBP1b, Bocillin-FL

INTRODUCTION

Non-typeable *Haemophilus influenzae* (NTHi) commonly resides in the human nasopharynx, from which it can disseminate to other organs and cause two types of infections, differing in their epidemiology and severity. The non-invasive infections usually affect the upper respiratory tract, whereas the invasive infections include bacteremia, and meningitis (Tristram et al., 2007; Murphy et al., 2009; Langereis and de Jonge, 2015). The asymptomatic colonization of the human nasopharynx is considered as the first step in the pathogenesis. NTHi is able to persist in the respiratory tract and also to be responsible of recurrent infections (Leach et al., 1994). The treatment of invasive NTHi infection can be seriously affected by antibiotic resistance. For a long time, ampicillin (alone or in combination with chloramphenicol) was considered as the first-line antibiotic in the treatment of invasive *H. influenzae* (Cole et al., 1979). However, since the emergence of strains resistant to ampicillin, amoxicillin/clavulanic acid and second-generation cephalosporins (e.g., cefuroxime); extended-spectrum cephalosporins (e.g., ceftriaxone) have been extensively used. Therefore, finding alternatives to extended-spectrum cephalosporins for the treatment of invasive infections caused by resistant strains was considered important. This holds especially true in the context of the global spread of extended-spectrum-β-lactamases (ESBLs) in *Enterobacteriaceae*, as well as of the recent reports of ESBL (bla_{TEM-15}) in the closely related *Haemophilus parainfluenzae* (Tristram et al., 2008). Nowadays carbapenems are largely used as an alternative to extended-spectrum cephalosporins for initial empirical treatment of severe infections until definitive microbiological analysis results are available. A steady and worrying increase in the incidence of invasive infections caused by NTHi associated to the global spread of resistant strains (Whittaker et al., 2017) highlights the importance to identify potential new antibiotic targets, or to enhance the activity of existing antibiotics. Furthermore, our previous data imply that the resistance to imipenem in NTHi should be seriously considered. Two β-lactams resistance mechanisms have been largely reported in *H. influenzae*. One requires TEM-1 or ROB-1 β-lactamases. The other one requires decreased β-lactams affinity for penicillin binding protein (PBP) 3. Nowadays, it is noticeable that the functions of PBPs are multiple and may differ according to growth conditions and physiological status. In addition, as it has been shown previously, β-lactams resistance in *H. influenzae* is often multi-factorial (Tristram et al., 2007; Cherkaoui et al., 2016).

In their hosts, NTHi must continuously cope with a broad spectrum of stress factors such as elevated temperatures, some nutrient limitation, host defense mechanisms, and antibiotics effects. Depending on their proprieties, stress triggers different highly regulated adaptive responses that do not only preserve bacteria from the environmental changes, but can besides lead to bacterial transformations that affect their susceptibility to antimicrobials (Poole, 2012). In response to heat stress conditions, bacteria induce many proteins called heat-shock proteins (HSPs). These proteins encompass chaperones and proteases that are important for overcoming the imbalance of protein homeostasis caused by heat stress. The importance of HSPs within the proteome can vary substantially from species to species. For instance, in *Escherichia coli* the level of HSPs decreases rapidly once the stress disappears. In contrast, in *Streptococcus pneumoniae*, the HSPs induced by heat stress remain detectable 1 h after the re-establishment of normal conditions (Tran et al., 2011). Regarding NTHi, there are only scarce studies on the function of stress-induced proteins, and the regulation of gene expression in response to heat stress. To further investigate relationships between β-lactams and PBPs, studies should consider temperature-sensitive.

To gain insights into the effect of heat stress on imipenem resistance in *H. influenzae,* we measured the interaction between controlled heat stress and imipenem resistance in NTHi by four mutually supportive approaches: (i) we simultaneously measured growth and cell viability at either 37 or 42°C in NTHi cells exposed to increasing concentrations of imipenem; (ii) we investigated PBPs by using Bocillin-FL and bacterial cells growth at either 37 or 42°C; (iii) we monitored transcriptome changes by RNA-seq after pre-incubation of bacterial cells at either 37 or 42°C; and (iv) we confirmed by real-time quantitative reverse transcription-PCR (qRT-PCR) the transcriptional changes of different key genes (*ponA, ponB, pbp2, ftsI, acrR, acrB, ompP2*) that can be implicated in the resistance of *H. influenzae* to β-lactam antibiotics.

MATERIALS AND METHODS

Bacterial Strains

The two highly imipenem resistant non-typeable *H. influenzae* strains (GE47 and GE88) examined in this study were taken from a collection of 46 NTHi strains previously studied (Cherkaoui et al., 2016). In more detail, GE47 and GE88 were grown in the presence of high concentrations of imipenem (**Figure 1**). Using E-test assay (bioMérieux), the imipenem MIC was greater than 32 μg/mL for the both strains (Supplementary Figure S1) and neither of them produces a β-lactamase, assessed by the chromogenic cephalosporin assay using a nitrocefin disk (Becton Dickinson) and the hydrolysis of penicillin G in the API *Neisseriae–Haemophilus* system (bioMérieux). Furthermore, these strains present amino acid substitutions in PBP3, PBP4, and AcrR (Supplementary Table S1). Susceptibility profiles of GE47 and GE88 strains to 14 antibiotics are reported in Supplementary

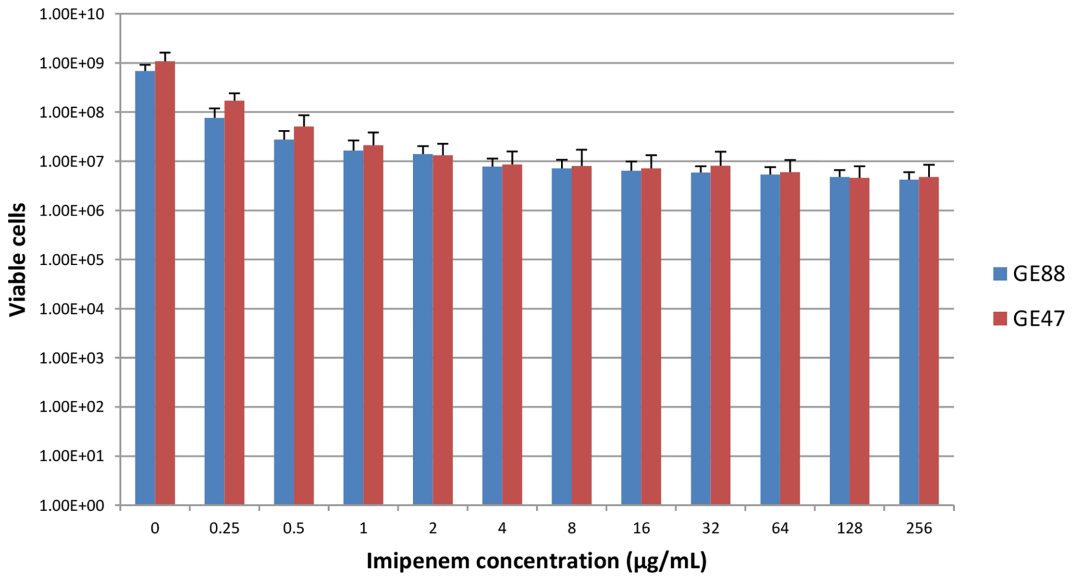

FIGURE 1 | Viable NTHi cells after growth at 37°C in the presence of increasing concentrations of imipenem. Data are presented as means ± SD of 8 independent biological replicates.

Table S2. Finally, the two strains were isolated from the sputum of two 75 year-old patients.

The reference strain *H. influenzae* Rd KW20 was also used for the preparation of membrane proteins-enriched fraction used for the detection of PBPs.

Growth and Viability of NTHi Cells at either 37 or 42°C

The inoculum suspension (50 mL) was prepared by picking several colonies from an overnight culture at 37°C on chocolate agar plates and suspending the colonies in Brain Heart Infusion (BHI) broth supplemented with NAD 2 µg/mL and hemin 10 µg/mL (sBHI) to a density of 0.5 McFarland. This inoculum suspension was transferred into two sets of 10 vials, each containing 2 mL of that inoculum suspension. One set was then incubated at 37°C and the other at 42°C. The optical densities (OD_{600}) of 1 mL aliquots were plotted against time; and the amount of viable cells was determined at selected time points by plating culture dilutions onto chocolate agar plates, followed by overnight incubation at 37°C, and viable cell counting. The longest incubation time at 42°C that did not significantly affect cell growth was 3 h (Supplementary Figure S2). Therefore this incubation time was used in all following experiments. All culture incubations were carried out in the presence of 5% CO_2.

Killing Activity of Imipenem on NTHi Cells at either 37 or 42°C

The killing activity of imipenem on NTHi cells was assessed after 3 h incubation at either 37 or 42°C with increasing concentrations of imipenem ranging from 0 to 256 µg/mL, following the procedure depicted in Supplementary Figure S3. The incubation of NTHi cells during 3 h at either 37 or 42°C with 0 µg/mL of imipenem was considered as a control condition in each experiment. The assay was performed in eight independent biological replicates. The number of viable cells was determined by plating culture dilutions on chocolate agar plates followed by overnight incubation at 37°C, and viable cell counting. The amount of GE47 and GE88 viable cells at either 37 or 42°C after incubation for 3 h with increasing concentrations of imipenem (range: 0.25–256 µg/mL), were normalized based on their amount of viable cells in a control condition. The data are expressed as a percentage of viable cells relative to the control condition for the different concentrations of imipenem. A paired Student's *t*-test was performed with GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, United States) and comparisons were considered statistically significant when associated *p*-values were < 0.05.

Detection of Penicillin Binding Proteins

The detection of PBPs in NTHi was carried out by the non-radioactive synthetic fluorescent penicillin Bocillin-FL (Zhao et al., 1999). GE47, GE88 and the reference strain *H. influenzae* Rd KW20 were used for the preparation of membranes for the detection of PBPs by following the procedures described previously, with a few modifications (Morikawa et al., 2004; Asli et al., 2016). The overnight cultures (4 mL each) were inoculated into 400 mL of fresh *Haemophilus* Test Medium broth (HTM broth). Cell cultures were grown for 3 h at either 37 or 42°C, and harvested by centrifugation at 6,000 × *g* for 15 min. The cells were washed once with 67 mM potassium phosphate (pH 7.0), and re-suspended in the same buffer. Cells were treated with 3 µg/mL of protease inhibitor cocktail (Sigma-Aldrich), DNase (6 µg/mL), RNase (6 µg/mL), and lysozyme (400 µg/mL). After 30 min of treatment, cells were disrupted with Bioruptor® Pico sonication device (Europe Diagenode SA/Seraing, Belgium).

The resulting cell lysates were centrifuged at 15,000 × *g* for 30 min. The supernatant fractions were collected and centrifuged at 136,000 × *g* for 30 min. The pellets were collected, washed once, and re-suspended in the same phosphate buffer. The resulting suspensions were considered as membrane preparations and were used for the fluorescent Bocillin-FL binding assays. Determination of protein concentration was performed using the Bio-Rad protein assay.

Cells were labeled at either 37 or 42°C for 30 min with a Bocillin-FL. The reaction mixtures (55 μL each), which contained 35 μL of each membrane preparation (∼200 μg of protein), 20 μL of 50 μM (final concentration) Bocillin-FL, were incubated at either 37 or 42°C for 30 min. Then, 4 μL of 10% sodium sarcosine, including 180 μg of penicillin G per mL, were added to the reaction mixture and the mixture was centrifuged at 10,000 × *g* for 30 min. Twenty microliters of each of the resulting supernatants were denatured with 20 μL of sodium dodecyl sulfate denaturing solution at 100°C for 3 min. Then, for the sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (10% polyacrylamide), 10 μL of each reaction mixture were used. After electrophoresis the gels were rinsed with water for 1 h. To visualize the labeled PBPs, the gels were directly scanned with the Gel Doc™ XR+ System (Bio-Rad Laboratories). The fluorescence intensity of each band was quantified by using Image LAB™ software (version 5.1; Bio-Rad).

Transcriptome Analysis

The transcriptome analysis was assessed only for GE47 strain. This strain was chosen because the cell growth was higher than that of GE88 during the three first hours of incubation at 42°C. Global transcriptional changes were monitored by RNA-seq after pre-incubation of bacterial cells for 3 h at either 37 or 42°C. Experiments were performed in two independent biological replicates. Total RNA of GE47 was purified from bacterial pellets re-suspended in 1 mL of TRIzol (Invitrogen) with 0.1-mm glass/zirconia beads and disrupted in a Bead Beater apparatus. The RNA in the aqueous phase was precipitated with ethanol, rinsed several times and solubilised in 50 μL of diethylpyrocarbonate-treated water. The concentration and purity of total RNA were determined by measuring the absorbance at 230, 260, and 280 nm using a Nanodrop-8000 spectrophotometer (Thermo Fisher). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies). RNA libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB), and sequenced on an Illumina Hiseq 2500 platform. The raw sequence data were filtered by removing reads containing adapter, reads containing poly-N, and low-quality reads. The filtered reads were aligned against the genome of *H. influenzae* Rd KW20 (NC_000907.1).

RNA-Seq data were further analyzed for statistics in the software R v3.2.3 with the package edgeR v3.10.5 (Robinson et al., 2010) as described previously (Anders et al., 2013). Briefly, we filtered out 164 out of 1846 genes which had counts per million lower than 1 in at least two samples (as two was the minimum number of samples used for each condition). The reads mapping to the remnant 1682 genes were normalized by weighted trimmed mean of M-values method and modeled according to a negative binomial distribution. To detect differentially expressed genes, we eventually performed pairwise comparisons between the two temperatures with the exact test. *p*-Values were corrected for false discovery rate (FDR). RNA-seq data have been submitted to ArrayExpress[1] with the accession number: E-MTAB-6237.

Real-Time Quantitative Reverse Transcription-PCR (qRT-PCR)

To confirm the RNA-Seq results, the expression levels of eight target genes were determined by qRT-PCR using the primers listed in Supplementary Table S3. First-strand cDNA was synthesized from total RNA extracted after 3 h incubation at either 37 or 42°C using SuperScript II (Invitrogen) on batch of 250 ng of total RNA. The mRNA levels of target genes (*ponA, ponB, pbp2, ftsI, acrR, acrB,* and *ompP2*) extracted from the GE47 strain grown at either 37 or 42°C were normalized based on their ribosomal RNA small subunit methyltransferase H (*rsmH*, originally designated as *mraW*) transcript level, which were assayed in each round of qRT-PCR. Data were presented as means ± SD of 4 independent biological replicates. Pairwise comparisons and paired Student's *t*-test were done with GraphPad Prism 6 software as described above.

RESULTS

Killing Activity of Imipenem on NTHi Cells at either 37 or 42°C

As depicted in **Figure 1**, the two NTHi strains analyzed in this study were highly imipenem resistant. Using E-test and macrodilution methods, the imipenem MIC was larger than 32 μg/mL for both strains. Moreover, the amount of viable NTHi cells after incubation at 37°C in the presence of increasing concentrations of imipenem revealed different according to imipenem concentration, suggesting the presence of heteropopulations. The population analysis profiles of GE47 and GE88 (**Figure 2A**) showed that with increasing concentrations of imipenem (range: 0–8 μg/mL), the subpopulations of cells did not differ considerably from each other in their imipenem susceptibility. To determine whether colonies of NTHi cells growing in the presence of high concentrations of imipenem were originating from populations of cells with a different resistant phenotypes, one colony of each of the two NTHi strains was picked from HTM agar containing 16 or 32 μg of imipenem/mL and passaged in imipenem free HTM agar. After overnight growth, these cultures named GE47/32 and GE88/16 were used as a starting cell suspension for further population analyses. Cultures of GE47/32 and GE88/16 were enriched in sub-populations resistant to 128 and 256 μg/mL of imipenem, respectively (**Figure 2B**).

The effect of heat stress on imipenem susceptibility was assessed on two highly imipenem resistant NTHi isolates (GE47,

[1]https://www.ebi.ac.uk/arrayexpress/

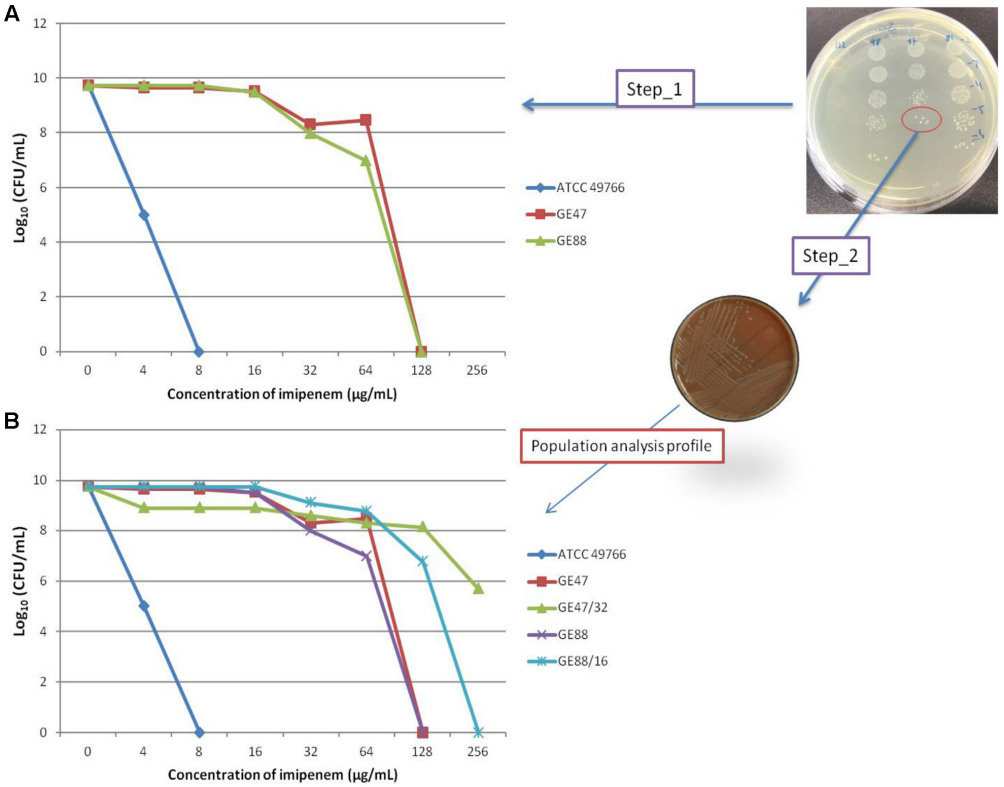

FIGURE 2 | (A) Population analysis profiles of GE47 and GE88 strains as determined by microdilution plate counts. *Haemophilus influenzae* ATCC 49766 strain was used as a negative control for all experiments. **(B)** One colony of each of two NTHi strains was picked from HTM agar containing 16 or 32 µg of imipenem/mL and passaged in imipenem free HTM agar. After overnight growth, these cultures were named GE47/32 and GE88/16.

and GE88). As shown in Supplementary Figure S2, the amount of viable cells continued to increase significantly during the three first hours of incubation at 42°C, but the two isolates were differently affected by the heat stress. The GE47 cell growth was larger than that of GE88 during the three first hours of incubation at 42°C (after 3 h of incubation at 42°C, the number of viable cells was 1.21×10^9 and 6.65×10^8 for GE47 and GE88, respectively).

Based on their viability and growth levels at either 37 or 42°C without imipenem (control condition), quantitation of viable cells incubated with 0.25 µg/mL of imipenem revealed more than a twofold decrease in GE47 and GE88 viable cells at 42°C as compared to 37°C (**Figures 3A,B**). Unlike GE47, a significant difference was also observed for GE88 with imipenem concentrations ranging from 0.5 to 2.0 µg/mL (**Figure 3B**). However, no significant difference was observed on viable cells between 37 and 42°C when the bacterial cells were incubated with higher imipenem concentrations (>0.25 µg/mL for GE47 and >2.0 µg/mL for GE88) (Supplementary Figure S4), indicating that the increased imipenem susceptibility detected under heat stress conditions does not seem to be linked to imipenem heteroresistance. Importantly, for both strains, at 0.25 µg/mL of imipenem, the number of viable cells dropped substantially at 42°C as compared to 37°C, suggesting that heat stress potentiates bactericidal drug effects when the cells are incubated with low concentrations of imipenem.

Binding of Bocillin-FL to Penicillin Binding Proteins

The Bocillin-FL binding to PBPs was investigated and performed at two different growth and labeling temperatures (37 or 42°C) for the two NTHi isolates. As depicted by Bocillin fluorescence measurements in SDS-PAGE gels (**Figure 4A**), the labeling at either 37 or 42°C when NTHi cells were grown at 42°C, showed for both strains (GE47 and GE88) a marked increase in fluorescence intensity corresponding to PBP3. However, under the same growth temperature, no significant difference in fluorescence intensity was observed after labeling at 37 or 42°C. Coomassie blue staining of SDS-PAGE gels showed that after growth at 42°C, the amount of proteins corresponding to PBP3 molecular weight was at least fourfold higher (**Figure 4B**). The NTHi strains studied here have altered PBP3 (Asp350Asn, Ala502val, Ala502Thr, Asn526Lys, combined with other mutations), which do not bind Bocillin-FL as well as the reference strain *H. influenzae* Rd KW20. Importantly, when labeled at 42°C, the Rd KW20 PBPs did not show significant difference in fluorescence intensities as compared to 37°C (**Figure 5**). Therefore, the increase in fluorescence intensity corresponding to PBP3 was not due

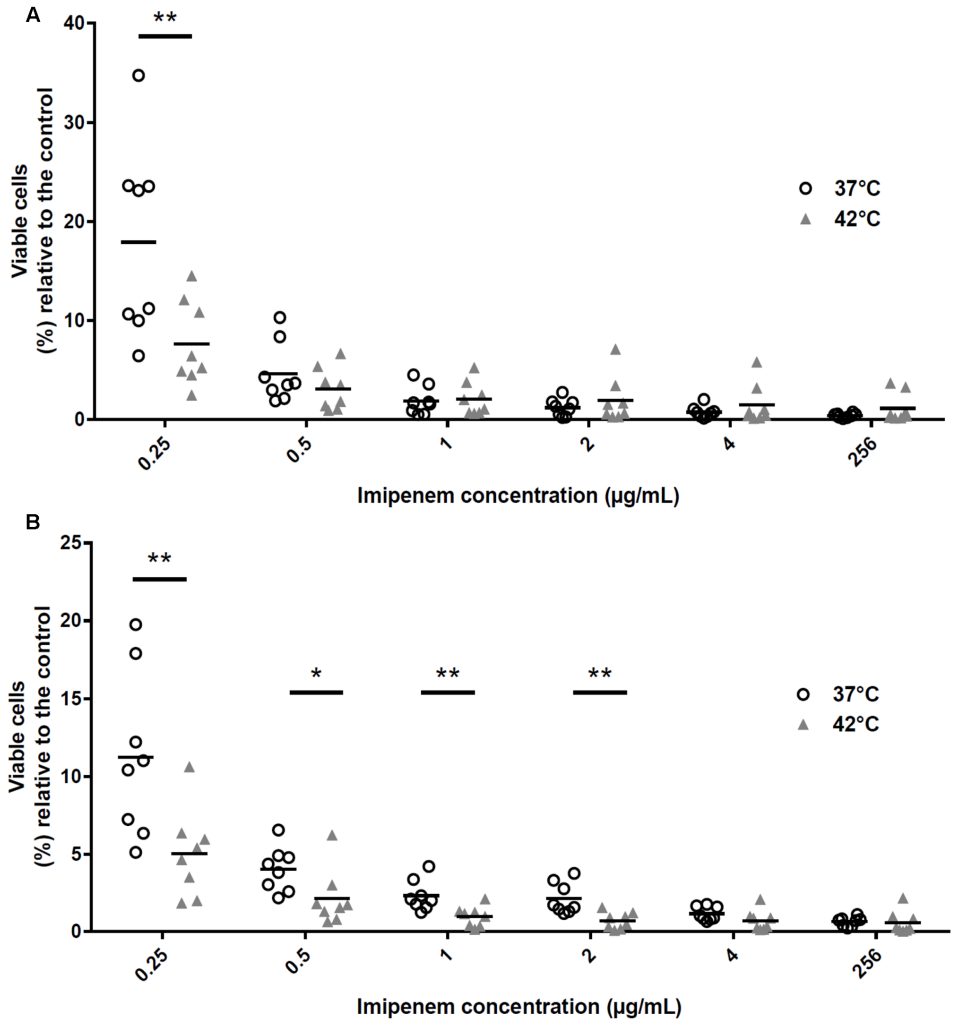

FIGURE 3 | Percentage of viable NTHi cells relative to the control condition after exposition to increasing concentration of imipenem during 3 h at either 37 or 42°C. The amounts of GE47 and GE88 viable cells after incubation with increasing concentration of imipenem ranged from 0.25 to 256 µg/mL at either 37 or 42°C were normalized based on their amount of viable cells in a control condition (i.e., growth at either 37 or 42°C with 0 µg/mL of imipenem). No significant difference was observed on viable cells between 37 and 42°C when the bacterial cells were incubated with higher imipenem concentrations (>0.25 µg/mL for GE47 and >2.0 µg/mL for GE88) (Supplementary Figure S4). **(A)** GE47 strain (imipenem MIC by E-test = > 32 µg/mL); **(B)** GE88 strain (imipenem MIC by E-test = > 32 µg/mL). Experiments were performed in eight independent biological replicates. *$p < 0.05$, **$p < 0.01$ (paired Student's *t*-test).

to a change of the Bocillin-FL properties when incubated at 42°C.

Taking into account the specificity of Bocillin-FL binding affinity and the potential of the heat stress to induce the production of a large amount of proteins with molecular weights close to that of PBP3 (e.g., HSP70/DnaK and HSP60/GroEL, as confirmed by liquid chromatography tandem-mass spectrometry analysis, data not shown), it is likely that the observed fluorescence increase cannot be completely related to PBP3.

Transcriptome Analysis of NTHi Strains Incubated at either 37 or 42°C

To investigate whether the use of two replicates was suitable for differential expression analysis, we generated a multidimensional scaling (MDS) plot to show the relationship existing between groups of pairs. In other words, a MDS plot was used as a variation of the usual principle coordinate plot where groups of samples are maximally separated based on their reciprocal differences. In more detail, we first filtered out genes that had an expression measured in counts per million higher than 1 in at least two out of all samples analyzed (we chose two since it is the minimum number of replicates present per condition). This procedure allowed us to exclude genes that had no counts in almost the totality of the samples. We therefore kept 1682 genes out of 1846. We then considered: (A) the top 500 genes with the largest standard deviation between samples; (B) the top 500 differentially expressed genes having the largest fold-changes between samples. We computed a pairwise-distance between samples for (A) and for (B) (Euclidean distance) and

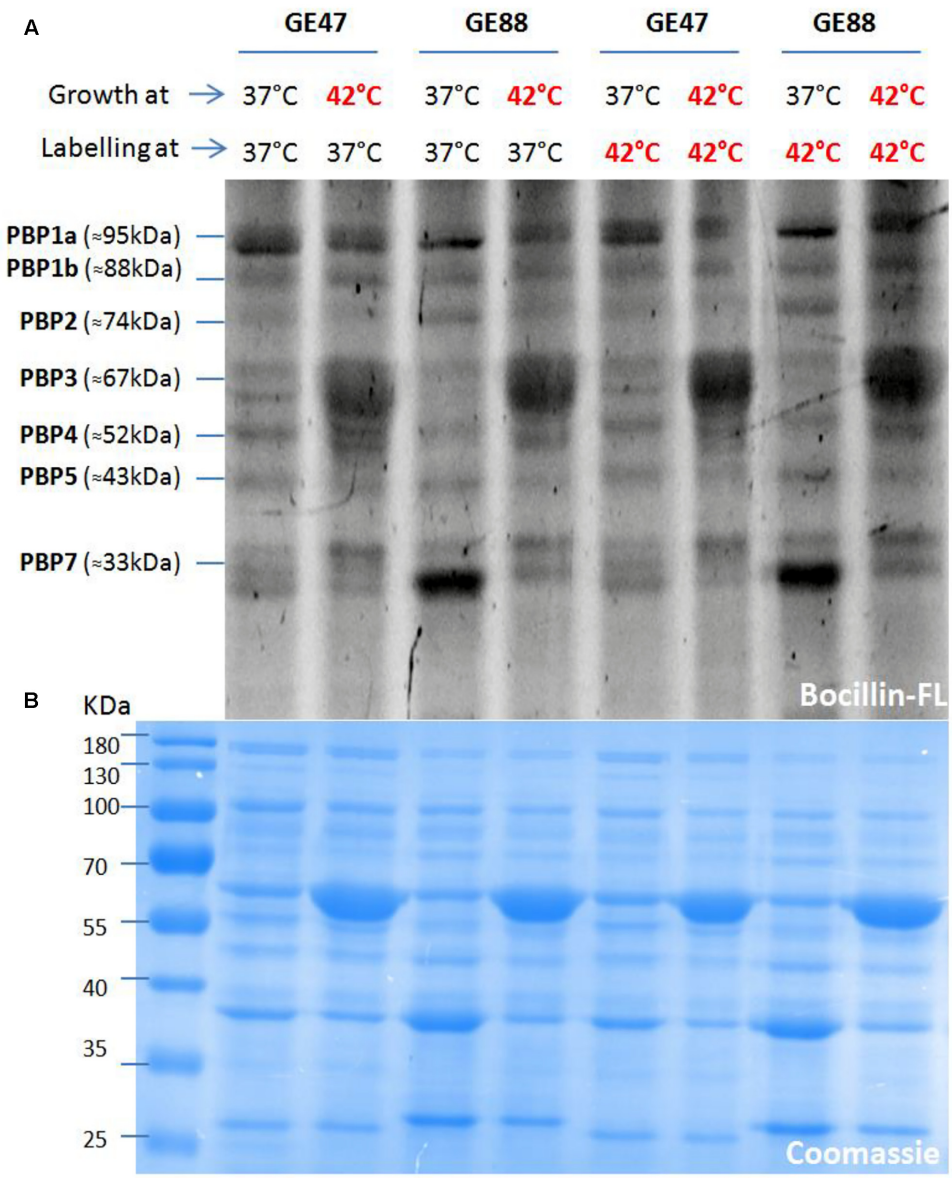

FIGURE 4 | Binding of Bocillin-FL to penicillin binding proteins (PBPs) at two different growth and labeling temperatures (37 or 42°C). **(B)** Shows a stained image (Coomassie) of the top gel **(A)**, which indicates that for both strains (GE47 and GE88) the same amounts of membrane proteins were loaded in all the lanes.

generated for both (A) and (B) a MDS plot (Supplementary Figure S5). In both cases, replicates clustered according to their own conditions and were separated from those of other temperatures. We conclude that the use of two replicates per condition was adequate here to perform differential expression analyses since intra-replicate variability was lower than inter-replicate variability. We would like to emphasize that the selection of differentially expressed genes has been performed by computing FDR-corrected *p*-values with the intent to limit as much as possible false positive results.

There were 141 differentially expressed genes with a $|\log2(\text{fold change})| > 1$ and a FDR < 0.05 under heat stress conditions, including 67 up-regulated and 74 down-regulated genes (Supplementary Table S4). A volcano plot was generated to visualize the distribution of these differentially expressed genes (**Figure 6**). The transcriptional changes of genes encoding for PBPs, outer membrane protein P2, efflux pumps (AcrAB-TolC) and the repressor AcrR, according to RNA-seq analysis were confirmed by qRT-PCR. The results were in good agreement with those from the RNA-seq analysis (**Figure 7** and **Table 1**). The expression levels at 42°C of *ponB* (encoding PBP1b) and *acrR* were significantly increased, but the expression of *ompP2* and *acrB* were significantly decreased.

Coomassie blue staining of SDS-PAGE gels showed that after growth at 42°C, the amount of proteins corresponding to PBP3 molecular weight was at least fourfold higher. However, it was not

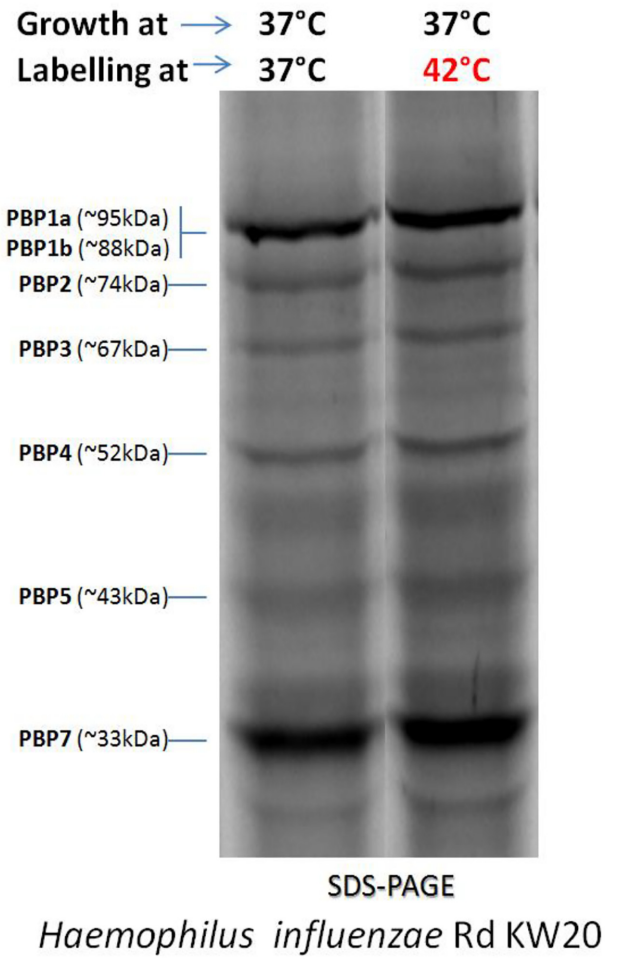

FIGURE 5 | Bocillin-FL binding to PBPs of the reference *H. influenzae* strain Rd KW20 at two different labeling temperatures (37 or 42°C).

correlated with the important increase in the expression level of *ftsI* gene in same condition (42°C). We postulate that the increase in the amount of the PBP3 may contribute to more stability of mRNA than increase in the expression of the *ftsI* gene.

Starting with our observation that the *ompP2* was down-regulated at 42°C but, strikingly, the exposition to heat stress enhanced NTHi susceptibility to low concentrations of imipenem, we investigated the expression levels of genes encoding different HSPs (SurA, DnaK, GroL, ClpB, and GroS) (Supplementary Figure S6A). All these proteins displayed increased transcript levels at 42 versus 37°C. In addition, no significant differences were observed in expression level of cluster of genes required in cell division and cell wall (*dcw*) biosynthesis (Supplementary Figure S6B), except for *fstQ*, *fstA*, and *ddlB*.

DISCUSSION

This study originated with the unexpected observation that highly imipenem resistant NTHi strains, although viable when exposed at 37°C to high concentration of imipenem (**Figure 1**), revealed more susceptible to lower concentrations of this antibiotic (0.25–2 μg/mL) when the cells were grown at 42°C. This raised the question of how does heat stress contribute to the active enhancement of imipenem susceptibility. Heat stress is one of the most significant environmental factors impacting bacterial physiology, which trigger adaptive and protective responses, notably by altering gene expression patterns. These major changes can affect cell physiology in different ways that can ultimately influence antimicrobial susceptibility profiles (Laport et al., 2001; Singh et al., 2007; Poole, 2012; Tomoyasu et al., 2012). Nowadays, a lot of information is available regarding the regulation of gene expression in response to heat stress in various bacterial species. However, there is currently no specific work dealing with the ability of NTHi cells to increase their antimicrobial susceptibility after heat stress. In this investigation, we observed that on the basis of their viability and growth levels at either 37 or 42°C without imipenem, the quantitation of viable cells pre-exposed to 0.25 μg/mL of imipenem revealed more than a twofold decrease in GE47 and GE88 viable cells at 42°C as compared to 37°C. This interesting observation raises many interrogations on the modulation of PBPs functions, cell wall structure and imipenem susceptibility by heat stress. As observed previously with *E. coli*, PBP1b interacts directly with PBP3 (Bertsche et al., 2006), which is required for the septal peptidoglycan synthesis (Nguyen-Disteche et al., 1998). B-lactams affect PBP1b or both, PBP1a and PBP1b, to rapidly lyse cells in *E. coli* (Yousif et al., 1985). Importantly, the inactivation of PBP2 or PBP3 by other target-specific β-lactam drugs causes different effects on bacteria cells. In more details, the inactivation of PBP2 results in the formation of spherical cells while the inhibition of septation and the occurrence of filamentous cells are induced by the inactivation of PBP3 (Tuomanen et al., 1986). Like in *E. coli*, the inactivation of PBP3 by target-specific β-lactams in *H. influenzae* leads to the formation of filamentous cells. Malouin and Bryan (1988) previously reported that *H. influenzae* cells grown at 42°C show filamentous cell formation. This growth aspect can explain a steady increase in the absorbance after 3 h at 42°C but a reduction in the overall viability of bacterial cells (Supplementary Figure S2). We previously observed that imipenem shows high affinity for PBP3 (IC_{50}, 0.004 μg/mL) and PBP1b (IC_{50}, 0.75 μg/mL) (Cherkaoui et al., 2016).

In a previous study it has been shown that *H. influenzae* displays a characteristic temperature-sensitive PBP3 with a reduced penicillin-binding activity at 42°C (Malouin et al., 1988). Taking into account the fact that in *E. coli*, the inhibition of PBP1b alone or combined with that of PBP1a by β-lactams causes the rapid lysis of bacteria cells, it is more than likely that an additional inhibitory effect linked to the temperature-sensitive PBP3 should be considered for the increased imipenem sensitivity of GE47 and GE88 strains grown at 42°C. Furthermore in *E. coli*, a mutation impacting the *ftsA* gene expression, which is a part of cluster of genes required in cell division and cell wall (*dcw*) biosynthesis, induces alterations in PBP3 phenotype at 42°C (Tormo et al., 1986). In GE47 strain, the *ftsA* was shown down-regulated at 42°C.

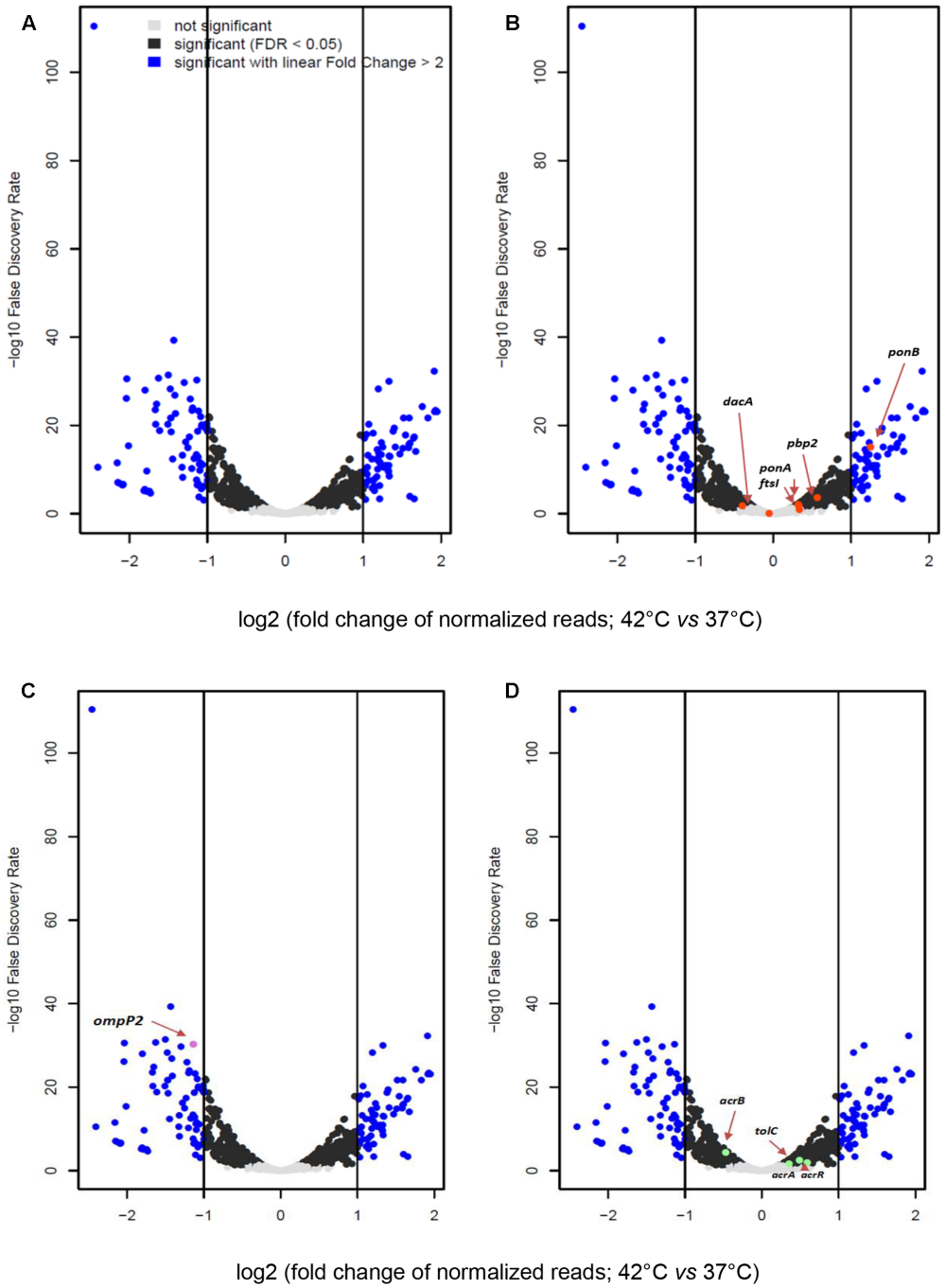

FIGURE 6 | (A) Volcano plot reporting the –log10 false discovery rate (FDR) versus the log2 transformed fold change in read counts between 37 and 42°C. Each dot represents a given gene. Fold change is computed by considering the expression level at 42°C compared to 37°C, thereby all genes at the right of 0 of x-values are meant to be up-regulated at 42°C and those at the left of 0 of x-values are down-regulated at 42°C. Gray = genes that do not change in significantly expression; black = genes with expression values associated with FDR-corrected *p*-values; blue = genes expression values associated with *p*-value < 0.05 and linear fold change between 37 and 42°C higher than 2. **(B–D)** Same as **(A)** except that in **(B)** genes coding for PBPs are marked in red; in **(C)** outer membrane protein P2 gene is in violet, **(D)** efflux pumps (AcrAB-TolC) and the repressor AcrR-coding genes are depicted in green. *ponA* gene encoding PBP1a; *ponB* gene encoding PBP1b; *pbp2* gene encoding PBP2*; ftsI* gene encoding PBP3*; dacA* gene encoding PBP5.

The observation that at 42°C, *H. influenzae* PBP1b and PBP3 showed different transcript levels suggests that the optimal activities of these PBPs were modulated by the physiological status of the bacteria cells. Modifications in PBP patterns like the reduction of PBP affinity to β-lactams, the decrease in the PBP transcript levels, or the presence of new PBPs, were reported

in several other bacterial species. For example, *Enterococcus faecium* mutants that are PBP3 temperature-sensitive, do also overproduce PBP5. This allows these mutants to grow for 150 min at 42°C before cell lysis (Canepari et al., 1987; Fontana et al., 1994). Thus, the data reported in this study suggest that PBP1b, although not essential for imipenem resistance at 37°C, becomes important under heat stress conditions, yet without being able to strictly isolate the role of each molecular component, due to the compensatory effects of other PBPs.

Mechanisms affecting porins and efflux pumps enable bacteria cells to overcome the action of antibiotics, as they can restrict the interactions between the drug and its intracellular targets. Outer membrane protein P2 (OMP P2), representing the most abundant protein in the NTHi outer membrane, has porin activity and is considered as the target of bactericidal antibodies. The quality control of outer membrane proteins biogenesis and the structure of the bacterial membranes or cell wall appears negatively affected under heat stress conditions, thereby affecting the import or export fluxes of antibiotics through such bacterial membranes (McMahon et al., 2007; Ge et al., 2014). This suggests that the down-regulation of *ompP2* under heat stress conditions could be functionally involved in the increased imipenem killing observed with 0.25 μg/mL.

AcrAB-TolC belongs to the resistance-nodulation-division (RND) family of transporters. AcrAB is encoded by the *acr* operon that includes *acrA*, and *acrB* genes. The transcription of *acr* operon is negatively regulated by its repressor *acrR* (Dean et al., 2005). The combined increase of *acrR* and decrease of *acrB* expression levels, reveals that under heat stress, imipenem overcomes efflux-mediated multidrug resistance. These observations strongly imply that down-regulation of AcrAB-TolC is one of the major mechanisms accounting for enhanced NTHi susceptibility to imipenem under heat stress conditions.

Different chaperones have been reported in *H. influenzae,* including HSP70/DnaK and HSP60/GroEL and most of them are heat inducible (Hartmann and Lingwood, 1997). Major HSPs are implicated in the correct folding and assembly of proteins. In addition they are required in various cell processes (DNA replication, RNA transcription, bacterial growth). According to the duration and severity of the heat stress condition, the accumulation of misfolded proteins can lead to the death of the bacteria. Nonetheless, if the heat stress is not lethal, the bacterial cells become more tolerant against more severe stress conditions (Ellis and van der Vies, 1991). As depicted in Supplementary Figure S6, SurA, DnaK, GroL, ClpB, and GroS displayed increased transcript levels at 42°C, suggesting their roles in the growth of NTHi cells at such temperatures, again by the evidence of correlations but not formally from a mechanistic standpoint.

In NTHi, LytM factors are reported to have a direct effect on cell division by influencing the structure of the peptidoglycan (Ercoli et al., 2015). In the present study, no differences were observed in the transcription levels of LytM factors under heat stress.

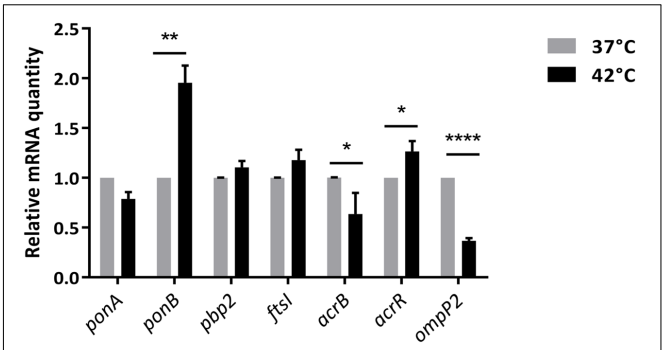

FIGURE 7 | qRT-PCR of differentially expressed genes. The mRNA levels of target genes were normalized based on their ribosomal RNA small subunit methyltransferase H (RsmH) transcript level, which were assayed in each round of qRT-PCR. Data were presented as means ± SD of 4 independent biological replicates. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ (paired Student's *t*-test).

TABLE 1 | Summary statistics of RNA-seq profiles between 37 and 42°C of genes encoding in *H. influenzae* for different PBPs, OmpP2, AcrAB-TolC efflux pump and this negative regulator.

Gene	Annotation	Log2 (fold change)	LogCPM (logarithm of counts per million reads)	*p*-Value	False discovery rate (FDR)
ponA	Penicillin-binding protein 1a	0.320	10.333	0.004	0.010
ponB	Penicillin-binding protein 1b	1.250	9.000	0.000	0.000
pbp2	Penicillin-binding protein 2	0.563	9.422	0.000	0.000
ftsI	Penicillin-binding protein 3	0.329	10.162	0.007	0.016
dacB	Penicillin-binding protein 4	−0.050	7.812	0.714	0.785
dacA	Penicillin-binding protein 5	−0.396	8.766	0.007	0.017
pbp7	Penicillin-binding protein 7	0.337	6.681	0.068	0.118
acrA	Multidrug efflux system protein AcrA	0.354	9.604	0.012	0.027
acrB	Multidrug efflux system protein AcrB	−0.470	10.268	0.000	0.000
tolC	Membrane protein TolC	0.489	9.114	0.001	0.003
acrR	Regulator of the AcrAB-TolC efflux pump gene expression	0.589	7.438	0.004	0.011
ompP2	Outer membrane protein P2 (OmpP2)	−1.136	13.128	0.000	0.000

CONCLUSION

This study has identified the specific heat stress-regulated genes and processes involved in imipenem resistance. The growth of NTHi cells at 42°C rendered the organism unable to withstand a subsequent imipenem challenge as compared to NTHi cells grown at 37°C. The increased expression level of *ponB* (encoding PBP1b) shown in this study may reflect an increased physiological activity of this PBP toward imipenem. In addition, PBP1b could take over the function of the temperature-inactivated PBP3. Yet, this postulate still needs to be proven in future studies, since these elements are derived from correlations.

In this study we provided also evidence that the relationship between imipenem susceptibility and heat stress response depends largely on the down-regulation of OmpP2 and AcrAB-TolC. This suggests that bacterial regulatory networks may play a role in the control of the of the imipenem resistance phenotype, and in the enhancement of NTHi susceptibility to imipenem under heat stress conditions. Dissecting such pathways in further genetic studies might help identifying potential antibiotic targets.

AUTHOR CONTRIBUTIONS

AC: performed the experiments and drafted the manuscript. SD: carried out the transcriptome analysis and participated in drafting the manuscript. AF: carried out the statistical analysis of killing activity of imipenem. SL: carried out the transcriptome analysis. PF and JS: supervised the research and participated in drafting the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02676/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

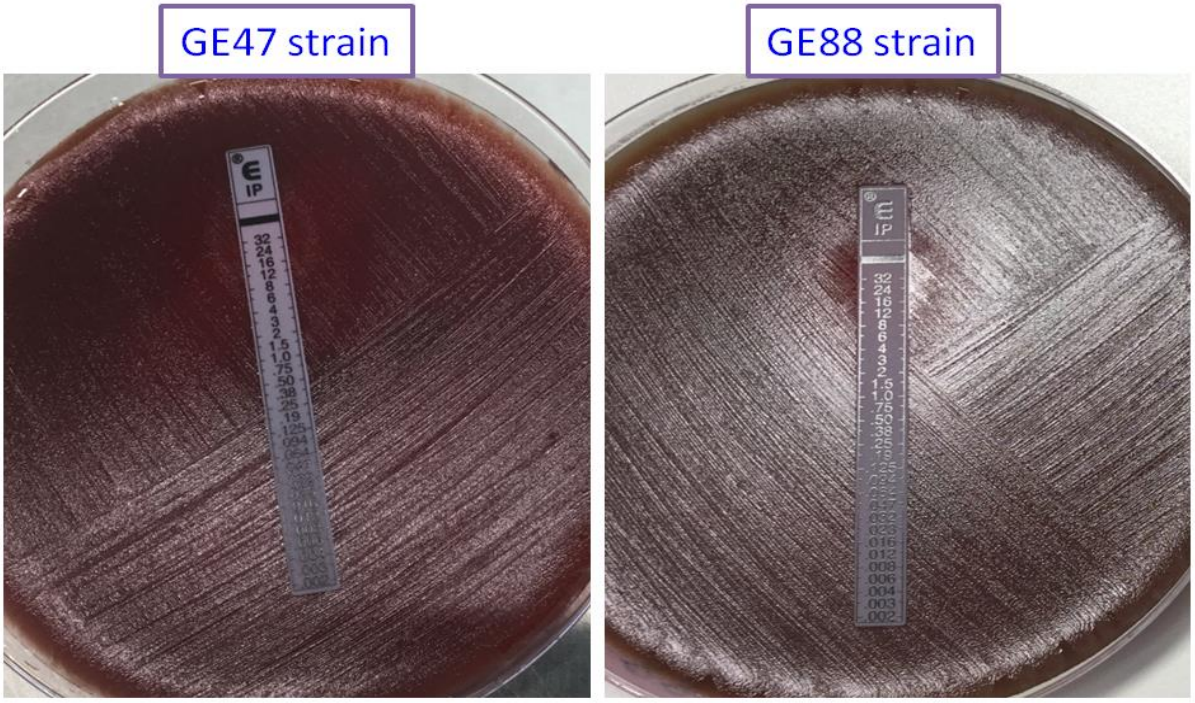
Transcriptional modulation of penicillin-binding protein 1b, outer membrane protein P2 and efflux pump (AcrAB-TolC) during heat stress enhances the bactericidal action of imipenem on nontypeable *Haemophilus influenzae*

A. Cherkaoui[1], S. M. Diene[2], A. Fischer[2], S. Leo[2], P. François[2], J. Schrenzel[1,2]

[1]Bacteriology Laboratory, Department of Genetics and Laboratory Medicine, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

[2]Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

Supplementary Materials



Supplementary Fig.S1: Imipenem MICs determination using E-test method

The imipenem MIC for both strains was greater than 32µg/mL

Group	Strain (n)	Ampicillin MIC range (µg/ml)	Imipenem MIC range (µg/ml)	Amino acid substitution for :																												
					Near *STVK motif					Close to SSN motif							Surrounding KTG motif																
	Rd KW20	0.064 - 0.094	0.25 - 0.38	Glu-141	Ser-273	Glu-274	Ser-311	Asp-350	Ser-357	Met-377	Ser-385	Leu-389	Ala-437	Ile-449	Gly-490	Ala-502	Val-511	Arg-517	Asn-526	Ala-530	Thr-532	Val-547	Asp-569	Ala-586	Ala-587	Asp-589	Thr-591	Ile-601	Glu-603				
IIb	GE47	2	>32	.	.		.	Asn	.	.	.	.	.	.	.	Val	.	.	Lys	.	.		.	Pro	Val	Lys	Ala	Val	Asp				
IIc	GE88	0.19	>32	Lys	.	Asp	.	Asn	.	.	.	.	.	.	.	Thr	.	.	Lys	.	.	Ile	Ser	.	.	.	.	.	Asn				

Amino acid substitutions identified in the transpeptidase domain of the ***ftsI* gene** (encoding for PBP3)
*shows the position of the catalytic serine residue

Strain ID	Amino acid substitution for :					
Rd KW20	Asp-84	Asn-101	Asn-107	Ala-172	Glu-262	Gln-270
GE88	Gly	Ser	His	.	Ala	.
GE47	.	.	.	Ser	Ala	Lys

Amino acid substitutions identified in the transpeptidase domain of the ***dacB* gene** (encoding for PBP4)

Strain ID	Amino acid substitution for :			
Rd KW20	Leu-31	Ile-121	His-131	Gln-134
GE47	His	.	.	.
GE88	His	Val	Asp	Lys

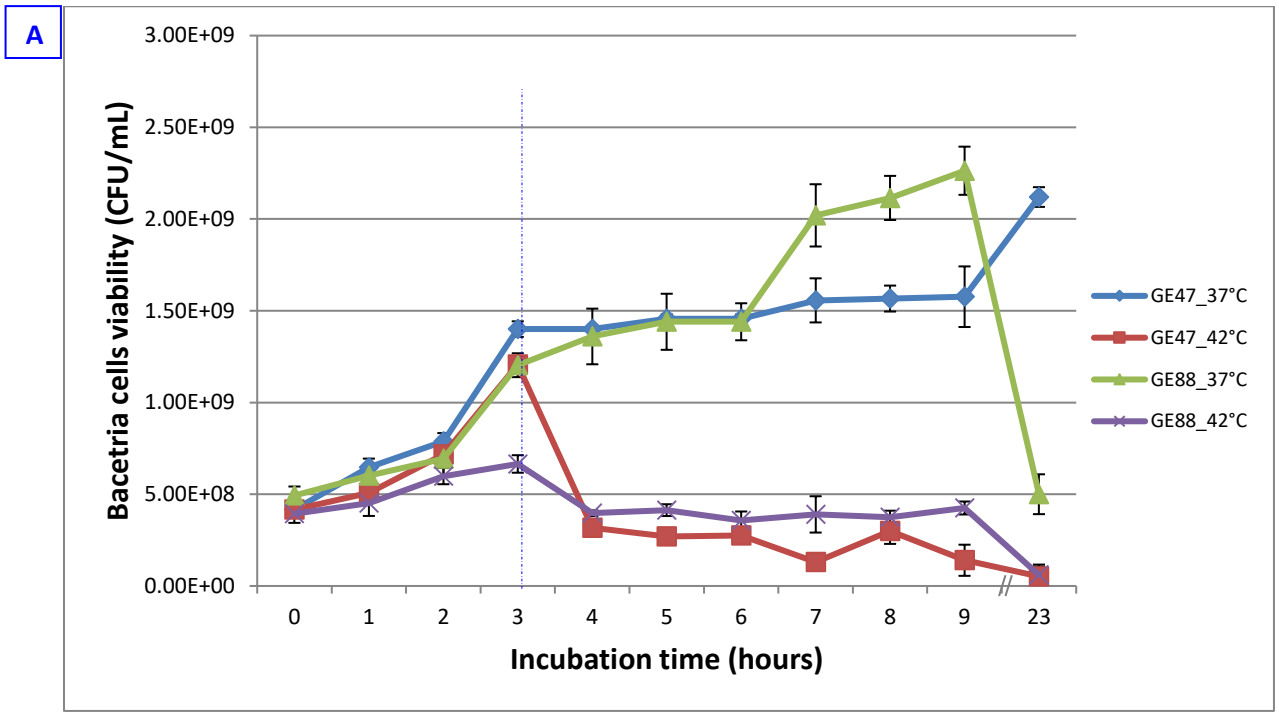
Amino acid substitutions identified in the transpeptidase domain of the ***acrR* regulatory gene of the AcrAB-TolC efflux**

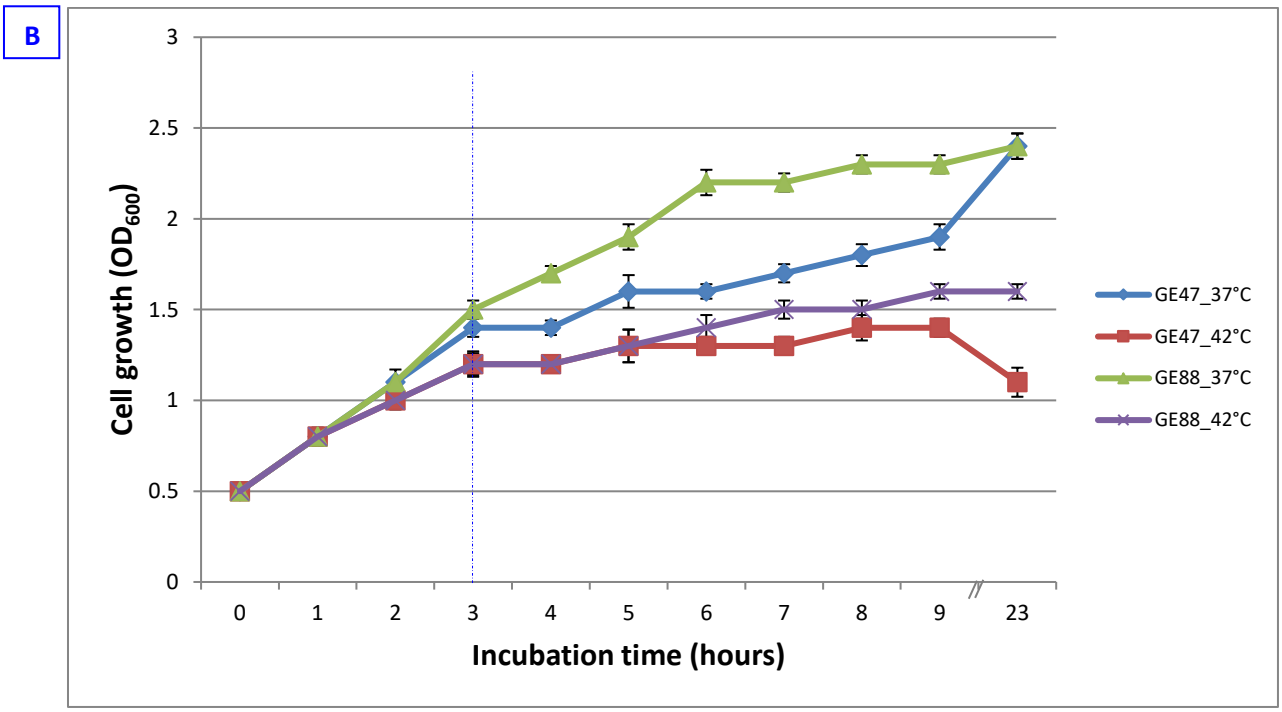
Supplementary Table S1: Amino acid substitutions in the *ftsI*, *dacB*, and *acrR* genes for two imipenem resistant nontypeable *H. influenzae* strains (GE47 and GE88)

Antibiotic	Strain ID	MIC µg/ml	EUCAST clinical breakpoints	CLSI clinical breakpoints
Imipenem	GE 47	>32	R	R
	GE88	>32	R	R
Ampicillin	GE 47	2	R	I
	GE88	0.19	S	S
Amoxicillin / clavulanic acid	GE 47	1.5	S	S
	GE88	0.125	S	S
Piperacillin/ tazobactam	GE 47	0.023		S
	GE88	0.016		S
* Cefuroxime	GE 47	3	R	S
	GE88	0.75	S	S
Cefotaxime	GE 47	0.047	S	S
	GE88	0.047	S	S
Ceftriaxone	GE 47	0.023	S	S
	GE88	0.032	S	S
Cefixime	GE 47	0.032	S	S
	GE88	0.047	S	S
Ceftobiprole	GE 47	0.064		
	GE88	0.064		
Ertapenem	GE 47	0.094	S	S
	GE88	0.25	S	S
** Meropenem	GE 47	0.19	S	S
	GE88	0.75	S	R
Levofloxacin	GE 47	0.023	S	S
	GE88	0.023	S	S
Co-trimoxazol	GE 47	4	R	R
	GE88	>32	R	R
Clarithromycine	GE 47	12	I	I
	GE88	24	I	I
Benzylpenicillin (screen) 1 unit	GE 47	11 mm	R	
	GE88	6 mm	R	
Ampicillin (2 µg)	GE 47	14 mm	R	
	GE88	6 mm	R	
Imipenem (10 µg)	GE 47	6 mm	R	R
	GE88	10 mm	R	R

Supplementary Table S2: Susceptibilities of GE47 and GE88 to 14 antibiotics

* EUCAST clinical breakpoints for intravenous drug administration
** EUCAST clinical breakpoints for infections other than meningitis

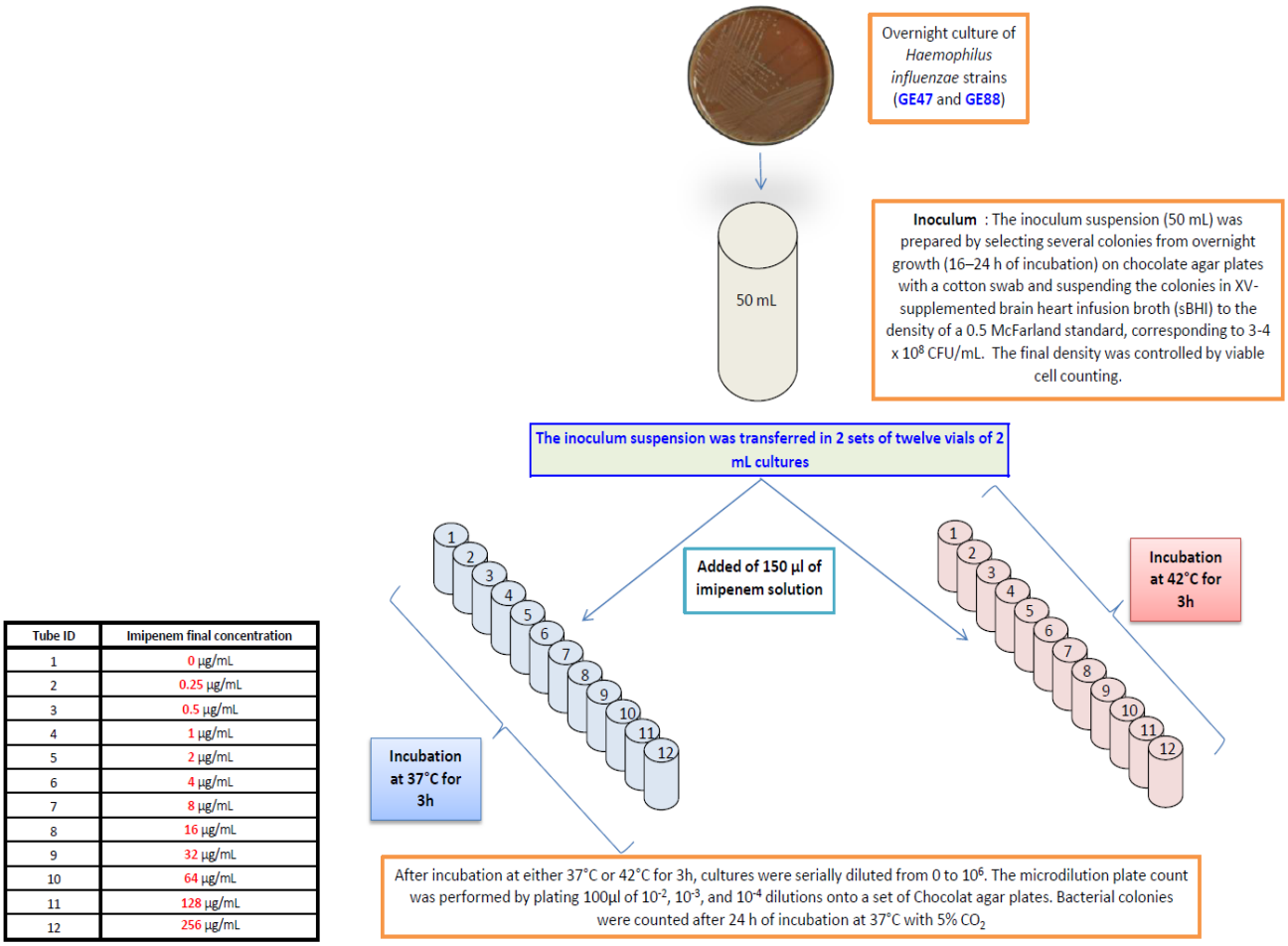




Supplementary Fig.S2: (**A**) Bacterial cell viability and (**B**) growth curves of GE47 and GE88 strains at either 37°C or 42°C in XV-supplemented brain heart infusion broth (sBHI).

The inoculum suspension was prepared by picking several colonies from an overnight growth on chocolate agar plates and suspending the colonies in sBHI to a McFarland 0.5 standards density. Inoculated sBHI media were incubated at either 37 or 42°C. Values represent the mean ± SD from 2 independent biological replicates.

The longer incubation time at 42°C that did not significantly affect cell growth was 3h; this incubation time was then used in all experiments.



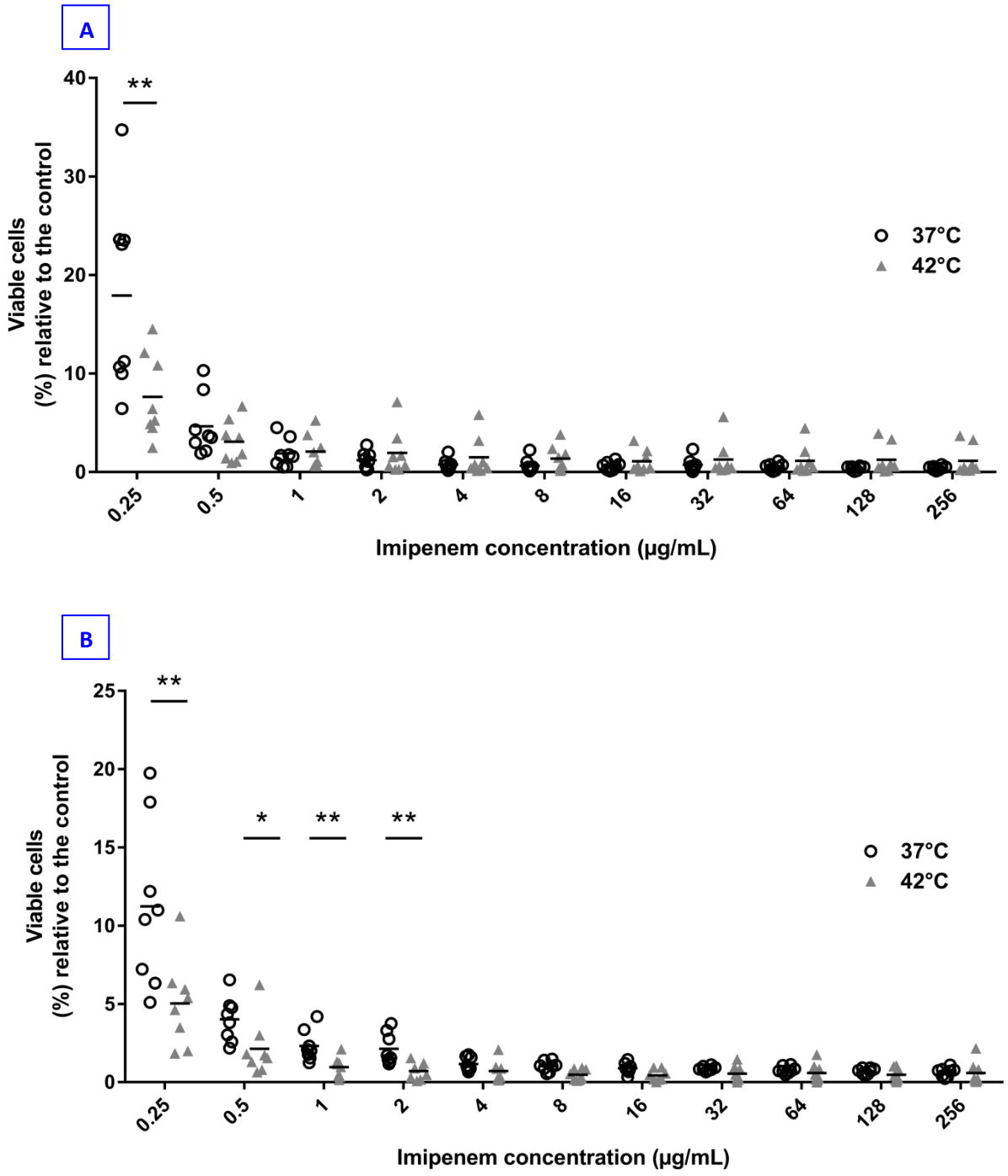
Tube ID	Imipenem final concentration
1	0 µg/mL
2	0.25 µg/mL
3	0.5 µg/mL
4	1 µg/mL
5	2 µg/mL
6	4 µg/mL
7	8 µg/mL
8	16 µg/mL
9	32 µg/mL
10	64 µg/mL
11	128 µg/mL
12	256 µg/mL

Supplementary Fig.S3: Assay procedure for viability measurement of NTHi cells after incubation with increasing concentrations of imipenem at either 37 or 42°C.

Target	Gene		Sequence	References
PBP1a	***ponA***	*Forward*	TCGGCGAGCAAATTTGGATT	
		Reverse	AAGCCACCGACCACTGCTTC	This study
		Probe	GCGTGCTAATGGGGAATGGCA	
PBP1b	***ponB***	*Forward*	CGGTACAGGGCGGAAGTACG	
		Reverse	CAAGGCTTCGTTGGCTTTGC	This study
		Probe	TTTATCGCGCGAACGCACCA	
PBP2	***pbp2***	*Forward*	GGTATGCCAACGGGGATTGA	
		Reverse	CCGAAATCGTATCGCCTTGC	This study
		Probe	CGGCTGCCAATATACCAACTCGAGA	
PBP3	***ftsI***	*Forward*	CCGCCAGTTATTGGGAAACG	
		Reverse	TTTACGCCGACACGGTAGCC	This study
		Probe	GCAATTAAAAATAAACGCGCAATGGTG	
AcrB	***acrB***	*Forward*	AGTTTCTTATCTGGTGCGACAGTTAC	
		Reverse	ATCTCGTTTTACCTGCGAAATGAC	This study
		Probe	TGTGGATGTGGATGGACGCGCTTA	
AcrR	***acrR***	*Forward*	GCGACAGATCGTTTAATGGCAAG	
		Reverse	GGTAAATCGTTCCTGCGGCTA	This study
		Probe	TGCTCAAACTTGCGAAAGAAGCAAA	
Omp2	***omp2***	*Forward*	CGTTGGTGCATTCGCAGCTT	
		Reverse	TCTGCGATAATGCTTAAACGACCA	This study
		Probe	CAGCAGCAAACGCAGCTGTTGT	
RsmH*	***rsmH***	*Forward*	GGGCAAAATTGACGGTATTTTG	
		Reverse	AAAACCACGTTCTGCTTCATCA	This study
		Probe	TTGATCTTGGTGTGTCTTCCCCTCAGC	

Supplementary Table S3: List of the primers designed and used in this study

*Ribosomal RNA small subunit methyltransferase H (originally designated as MraW).

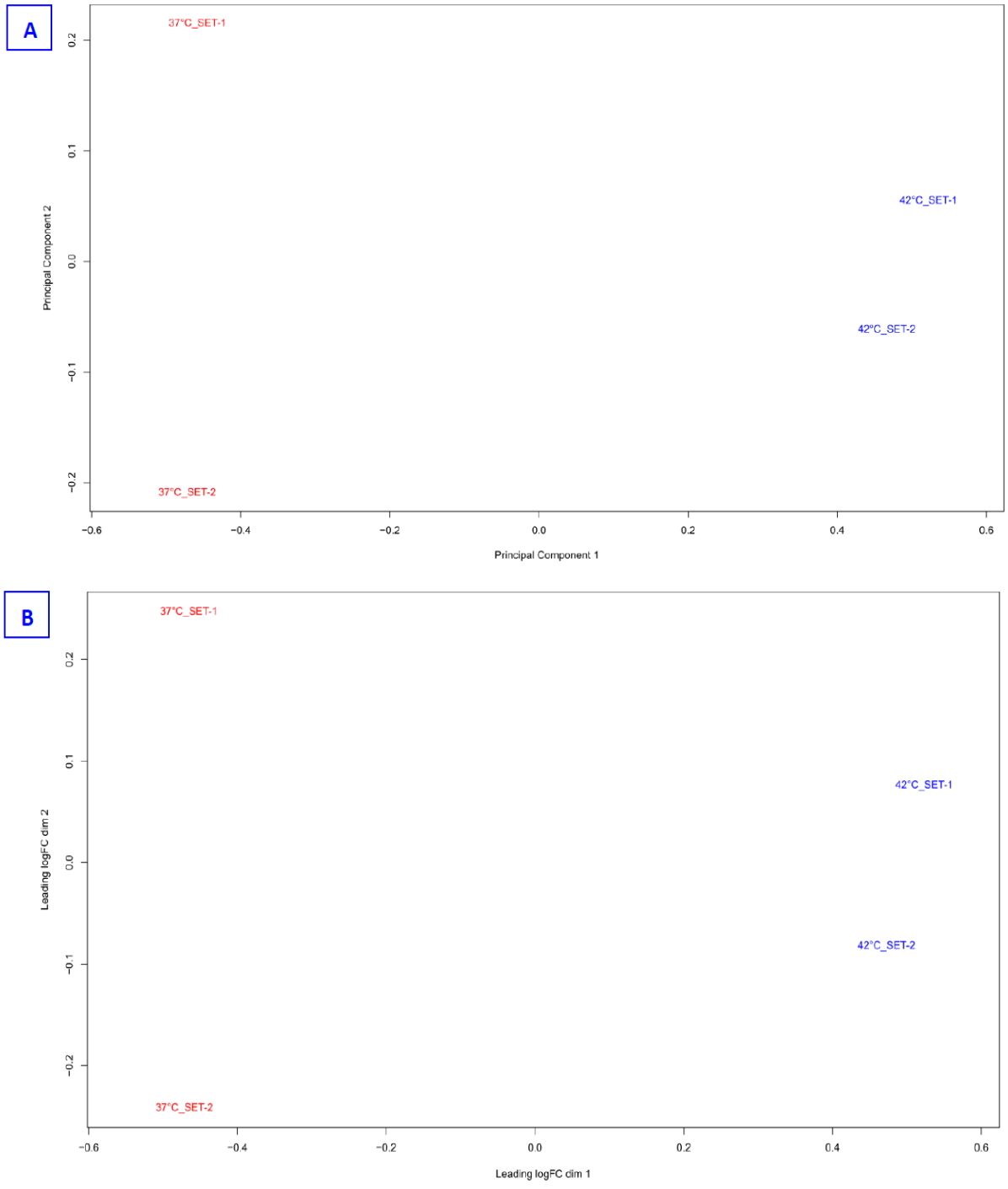


Supplementary Fig.S4: Percentage of viable NTHi cells relative to the control condition after exposition to increasing concentration of imipenem during 3h at either 37 or 42°C.

The amounts of GE47 and GE88 viable cells after incubation with increasing concentration of imipenem ranged from 0.25 to 256 µg/mL at either 37 or 42°C were normalized based on their amount of viable cells in a control condition (i.e. growth at either 37 or 42°C with 0µg/mL of imipenem).

(A): GE47 strain (imipenem MIC by E-test = >32 µg/mL)
(B): GE88 strain (imipenem MIC by E-test = >32 µg/mL)

Experiments were performed in 8 independent biological replicates.
* = p <0.05, ** =p <0.01 (paired Student's t test).

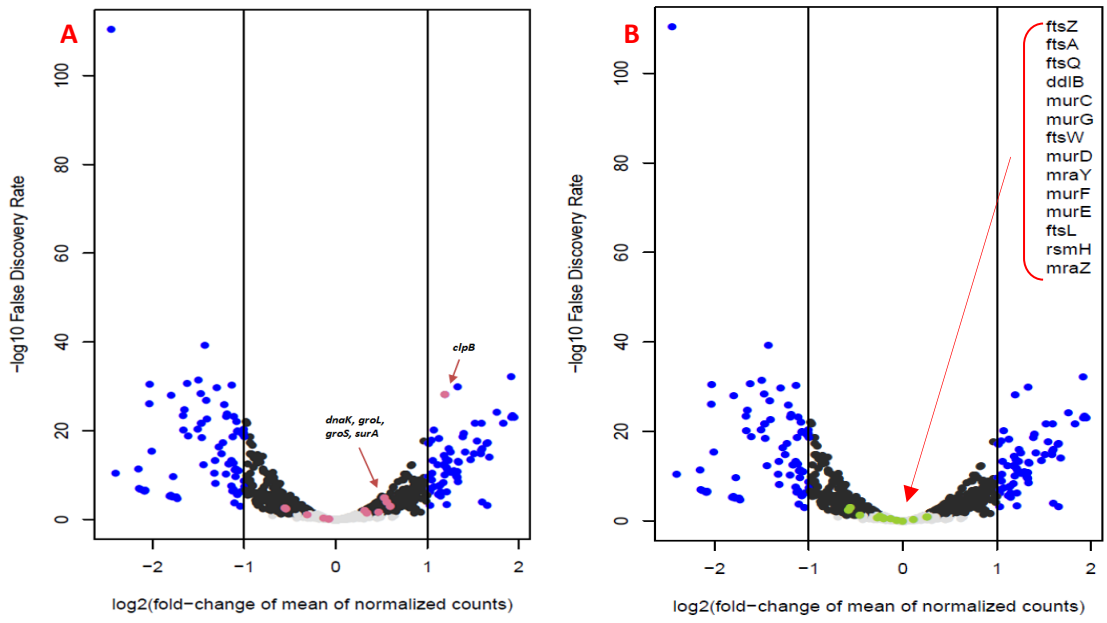


Supplementary Fig.S6

A: MDS plot with top 500 genes with the highest standard deviation in expression between samples.

B: MDS plot with top 500 genes with the highest fold change in expression between samples. The distance between each pair of samples is the root-mean-square deviation (Euclidean distance) for the top genes. Distances on the plot can be

interpreted as leading log2-fold-change, meaning the typical (root-mean-square) log2-fold-change between the samples for the genes that distinguish those samples.



Supplementary Fig.S7: Volcano plot of differentially expressed genes

(A) chaperon proteins, (B) division cell wall (dcw) gene cluster

Gray = genes that do not change in significantly expression; black = genes with expression values associated with FDR-corrected p-values < 0.05; blue = genes expression values associated with with FDR-corrected p-value < 0.05 and linear fold change between 37°C and 42°C higher than 2.

Reference genome: *H. influenzae* Rd KW20 (NC_000907.1)	Gene	Function	GE47 strain - Growth at 37°C		GE47 strain - Growth at 42°C		Statistical analysis			
			SET-1	SET-2	SET-1	SET-2	log2 (Fold change)	logCPM	*P* Value	FDR
			Total reads=	Total reads=	Total reads=	Total reads=				
			12471697	12706187	13245098	13125243				
			Mapped reads (79.9%)=	Mapped reads (80.2%)=	Mapped reads (81.9%)=	Mapped reads (82.1%)=				
			9958912	15852996	16172459	15994253				
HI0002	-	Long-chain-fatty-acid--CoA ligase FadD15	5494	8181	20023	18520	1.13	9.98	1.9E-20	6.4E-19
HI0039	mreD	Rod shape-determining protein MreD	810	829	2514	2396	1.19	6.99	7.4E-12	9.9E-11
HI0040	-	hypothetical protein	4161	4470	11868	11032	1.01	9.26	1.4E-11	1.8E-10
HI0041	xthA	Exodeoxyribonuclease III	5722	6692	17412	16048	1.04	9.80	3.3E-20	1.1E-18
HI0042	rluA_1	Ribosomal large subunit pseudouridine synthase A	2872	3887	11769	11112	1.38	9.15	6.9E-21	2.5E-19
HI0043	-	hypothetical protein	4324	5562	15100	15091	1.23	9.59	3.4E-18	9.5E-17
HI10044.1 (HI0044.1)	-	tRNA-Ser	56	63	211	181	1.33	3.35	3.0E-10	3.3E-09
HI0070	recN	DNA repair protein RecN	5347	6631	20465	18782	1.33	9.94	4.8E-33	1.0E-30
HI0075	nrdD	Anaerobic ribonucleoside-triphosphate reductase	820	1048	2561	2467	1.05	7.07	1.0E-10	1.2E-09
HI0086.1	-	tRNA-Cys	1348	1664	6129	5126	1.52	8.09	4.2E-24	2.2E-22
HI0086.2	-	tRNA-Gly	92	148	483	457	1.60	4.52	4.4E-18	1.2E-16
HI0086.3	-	tRNA-Leu	1969	1781	8190	6745	1.59	8.48	8.5E-18	2.3E-16
HI0086.4	-	tRNA-Lys	294	258	965	729	1.21	5.46	6.0E-08	4.8E-07
HI0088	thrB	Homoserine kinase	5236	6948	3648	3775	-1.09	8.70	2.3E-13	3.7E-12
HI0089	thrA	Bifunctional aspartokinase/homoserine dehydrogenase 1	14718	23134	9572	11138	-1.23	10.28	1.3E-19	3.9E-18
HI0092	gntP	High-affinity gluconate transporter	1853	2704	1201	1360	-1.20	7.24	4.0E-12	5.6E-11
HI0113.3 (HI_r02)	-	23S ribosomal RNA	10209	3970	2113	2498	-2.09	8.76	4.7E-08	3.8E-07
HI1003.5 (HI_r03)	-	16S ribosomal RNA	2302	995	703	597	-1.80	6.70	5.8E-07	3.8E-06
HI0113.6	-	tRNA-Pro	73	63	200	188	1.12	3.40	2.5E-07	1.8E-06
HI0113.7	-	tRNA-His	172	174	848	768	1.83	5.24	4.2E-24	2.2E-22
HI0122	metC	Cystathionine beta-lyase MetC	4098	7555	2509	3449	-1.32	8.54	5.9E-10	6.2E-09
HI0123.1	-	tRNA-Gly	111	141	505	429	1.51	4.53	6.8E-17	1.6E-15

HI0123.2	-	tRNA-Leu	1992	1740	8030	6800	1.58	8.47	5.7E-17	1.4E-15
HI0136.1	-	tRNA-Asp	4	15	34	41	1.60	1.03	2.5E-05	1.2E-04
HI0139	**ompP2**	Outer membrane protein P2 OmpP2	116878	146711	78732	77408	-1.14	13.13	2.4E-33	5.7E-31
HI0157	fabH	3-oxoacyl-[acyl-carrier-protein] synthase 3	6643	7470	19306	18056	1.01	9.97	2.7E-19	7.9E-18
HI0189	gdhA	NADP-specific glutamate dehydrogenase	3216	5263	1538	1894	-1.66	8.00	1.3E-22	6.0E-21
HI0220.4 (HI_r05)	-	23S ribosomal RNA	10275	4070	2126	2388	-2.13	8.77	1.4E-08	1.2E-07
HI0220.6	-	tRNA-Glu	6	8	32	26	1.65	0.68	1.3E-04	5.3E-04
HI0220.5 (HI_r06)	-	16S ribosomal RNA	2402	984	684	643	-1.81	6.74	1.0E-06	6.3E-06
HI0223	-	rarD protein, putative	516	527	1395	1423	1.04	6.24	8.8E-09	8.1E-08
HI0255	dapA	4-hydroxy-tetrahydrodipicolinate synthase	13912	17654	9090	8554	-1.22	10.04	9.8E-29	1.1E-26
HI0256	-	hypothetical protein	6926	7004	4570	4016	-1.10	8.92	1.5E-11	1.9E-10
HI0274.1	-	tRNA-Val	102	105	298	314	1.17	4.02	4.7E-10	5.0E-09
HI0274.2	-	tRNA-Val	98	101	283	292	1.14	3.94	1.9E-09	1.9E-08
HI0274.3	-	tRNA-Val	98	93	295	334	1.33	4.02	2.5E-11	3.1E-10
HI0274.4	-	tRNA-Val	93	93	283	327	1.32	3.98	1.7E-11	2.2E-10
HI0287	mtr	Tryptophan-specific transport protein	852	1072	2834	2948	1.21	7.22	1.1E-13	1.8E-12
HI0319	cmoA	tRNA (cmo5U34)-methyltransferase	2483	2854	7547	6937	1.05	8.59	2.5E-12	3.5E-11
HI0325	-	Na+/H+ antiporter family protein	9846	11033	6610	5587	-1.17	9.47	1.7E-14	3.1E-13
HI0331	-	hypothetical protein	373	550	1432	1424	1.26	6.19	1.9E-12	2.8E-11
HI0332	recO	DNA repair protein RecO	622	718	2496	2607	1.54	6.95	5.4E-20	1.7E-18
HI0333	rlmD	23S rRNA (uracil(1939)-C(5))-methyltransferase RlmD	673	798	2642	2469	1.41	6.98	2.9E-17	7.5E-16
HI0380.2	-	tRNA-Lys	289	265	922	707	1.15	5.42	9.7E-08	7.5E-07
HI0438	comB	competence protein B	178	164	110	104	-1.07	3.57	2.4E-07	1.7E-06
HI0443	recR	Recombination protein RecR	1812	2089	5488	6015	1.17	8.22	2.7E-14	4.7E-13
HI0444	topB	DNA topoisomerase 3	1389	1779	7528	7976	1.91	8.46	9.9E-36	5.5E-33
HI0465	serA	D-3-phosphoglycerate dehydrogenase	5739	7735	4065	3708	-1.17	8.82	2.0E-14	3.5E-13
HI0548	infA	Translation initiation factor IF-1	1077	1235	3466	3430	1.19	7.48	5.0E-14	8.3E-13
HI0561	-	OPT oligopeptide transporter protein	11630	17660	6995	7137	-1.42	9.86	1.2E-29	1.6E-27
HI0583	cpdB	2',3'-cyclic-nucleotide 2'-phosphodiesterase/3'-nucleotidase precursor	27355	31084	19020	19206	-1.00	11.01	5.4E-21	2.0E-19
HI0584	iaaH	Indole-3-acetyl-aspartic acid hydrolase	7380	8980	4507	4281	-1.28	9.08	1.9E-18	5.3E-17

HI0595	arcC1	Carbamate kinase 1	5386	7570	1843	2244	-2.04	8.55	1.2E-33	3.4E-31
HI0596	arcB_3	Ornithine carbamoyltransferase, catabolic	5236	7670	1742	2307	-2.04	8.54	5.7E-29	6.9E-27
HI0601.2 (HI_r07)	-	16S ribosomal RNA	2389	958	712	678	-1.73	6.74	3.9E-06	2.2E-05
HI0601.6	-	tRNA-Ile	120	140	490	579	1.66	4.69	1.4E-19	4.4E-18
HI0601.7	-	tRNA-Ala	80	114	477	482	1.93	4.48	4.6E-26	3.8E-24
HI0601.3 (HI_r08)	-	23S ribosomal RNA	10484	4141	2187	2365	-2.15	8.79	1.1E-08	9.7E-08
HI0609.1	-	tRNA-Pro	71	52	174	185	1.14	3.28	9.9E-07	6.2E-06
HI0621.2 (HI_r10)	-	16S ribosomal RNA	2440	997	726	650	-1.78	6.77	1.5E-06	9.1E-06
HI0621.3 (HI_r11)	-	23S ribosomal RNA	10339	4054	2238	2423	-2.09	8.78	2.8E-08	2.4E-07
HI0630	rseB	Sigma-E factor regulatory protein RseB precursor	3829	3375	12334	11009	1.29	9.21	6.9E-13	1.0E-11
HI0642.2	-	tRNA-Arg	1356	1632	4330	3799	1.06	7.76	2.8E-15	5.6E-14
HI0648	mdaB	Modulator of drug activity B	3707	5746	2151	1831	-1.61	8.18	3.8E-21	1.5E-19
HI0683	glpC	Anaerobic glycerol-3-phosphate dehydrogenase subunit C	1319	2078	5818	5262	1.34	8.12	6.2E-15	1.2E-13
HI0684	glpB	Anaerobic glycerol-3-phosphate dehydrogenase subunit B	1138	1748	4900	4902	1.40	7.93	1.1E-21	4.5E-20
HI0685	glpA	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	1572	2274	5829	5830	1.23	8.22	2.5E-15	5.0E-14
HI0689	glpQ	Glycerophosphoryl diester phosphodiesterase precursor	37728	41704	20958	21348	-1.30	11.36	1.1E-32	2.1E-30
HI0693	hel	Lipoprotein E precursor	25668	31158	16726	17520	-1.11	10.92	7.7E-26	5.9E-24
HI0723.1 (HI_r13)	-	16S ribosomal RNA	2305	989	725	625	-1.75	6.71	1.3E-06	7.8E-06
HI0723.2	-	tRNA-Ile	138	140	473	537	1.47	4.65	1.4E-15	2.9E-14
HI0723.5	-	tRNA-Ala	85	101	451	466	1.92	4.42	1.2E-25	9.0E-24
HI0723.3 (HI_r14)	-	23S ribosomal RNA	10341	3997	2203	2439	-2.09	8.77	4.0E-08	3.3E-07
HI0749	lexA	LexA repressor	4203	4766	14432	12528	1.20	9.44	1.1E-15	2.3E-14
HI0761.1	-	tRNA-Phe	17	19	56	53	1.21	1.62	1.1E-04	4.7E-04
HI0761.2	-	tRNA-Asn	74	82	256	252	1.32	3.72	1.1E-11	1.4E-10
HI0775	cynR_1	HTH-type transcriptional regulator CynR	1474	1842	853	875	-1.32	6.76	2.9E-15	5.7E-14
HI0779	rplW / rpl23	50S ribosomal protein L23	34471	37692	23149	21874	-1.07	11.29	2.1E-22	9.5E-21
HI0781	rpsS19	30S ribosomal protein S19	30786	36301	22110	21318	-1.01	11.20	2.8E-22	1.2E-20
HI0818	galM	Aldose 1-epimerase	2070	3248	11696	11430	1.76	9.07	6.8E-27	6.7E-25
HI0819	galK	Galactokinase	2121	3135	10534	9905	1.59	8.93	3.9E-24	2.1E-22
HI0822	mglB	D-galactose-binding periplasmic protein precursor	31743	40153	23879	22643	-1.01	11.29	9.2E-23	4.5E-21

HI0848	trmA	tRNA/tmRNA (uracil-C(5))-methyltransferase	1752	1800	5548	5308	1.22	8.12	2.0E-13	3.3E-12
HI0853	hbpA_1 / dppA	Heme-binding protein A precursor	17907	22631	13062	13226	-1.01	10.47	8.7E-22	3.6E-20
HI0859	clpB	Chaperone protein ClpB	28409	38267	98886	98948	1.19	12.32	3.5E-31	5.4E-29
HI0875	pepB_2	Peptidase B	720	1039	456	591	-1.12	5.89	1.7E-08	1.5E-07
HI0974.1	-	hypothetical protein	744	756	459	437	-1.14	5.68	2.5E-09	2.5E-08
HI0975	panF	Sodium/pantothenate symporter	15592	19325	10955	10470	-1.09	10.23	1.6E-24	1.0E-22
HI0998	rpmH / rpL34	50S ribosomal protein L34	623	599	1670	1714	1.07	6.49	3.9E-09	3.7E-08
HI0999	rnpA	Ribonuclease P protein component	4668	4370	16039	14083	1.33	9.56	3.8E-15	7.4E-14
HI1000	yidD	Putative membrane protein insertion efficiency factor	1752	1386	7160	6218	1.67	8.30	3.9E-16	8.6E-15
HI1001	yidC	Membrane protein insertase YidC	18664	22126	59683	52236	1.07	11.53	1.4E-22	6.4E-21
HI1053	ahpD	Alkyl hydroperoxide reductase AhpD	1252	1529	889	923	-1.00	6.61	3.5E-09	3.3E-08
HI1120	oppF	Oligopeptide transport ATP-binding protein OppF	10233	13660	5448	5723	-1.47	9.56	3.4E-24	2.0E-22
HI1121	oppD_1	Oligopeptide transport ATP-binding protein OppD	7604	10316	5271	5419	-1.12	9.25	1.0E-14	1.9E-13
HI1122	oppC	Oligopeptide transport system permease protein OppC	7667	9825	5011	5295	-1.14	9.21	2.2E-15	4.4E-14
HI1123	oppB	Oligopeptide transport system permease protein OppB	10472	11040	4561	4426	-1.65	9.38	1.4E-27	1.5E-25
HI1124	oppA_2	Periplasmic oligopeptide-binding protein precursor	37004	46365	9816	9977	-2.46	11.17	2E-114	4E-111
HI1125	talB	Transaldolase B	18601	22770	12703	10832	-1.20	10.44	6.0E-26	4.8E-24
HI1167	serC	Phosphoserine aminotransferase	5321	6839	3220	3423	-1.25	8.65	5.3E-17	1.3E-15
HI1176	-	hypothetical protein	82	104	317	291	1.33	3.97	8.5E-13	1.3E-11
HI1177	artM	Arginine ABC transporter permease protein ArtM	339	461	1181	937	1.03	5.83	6.7E-08	5.3E-07
HI1180	artP	Arginine transport ATP-binding protein ArtP	275	344	958	800	1.12	5.54	2.9E-09	2.9E-08
HI1197	sucD	Succinyl-CoA ligase [ADP-forming] subunit alpha	3245	7009	2255	3752	-1.11	8.41	4.1E-05	1.8E-04
HI1217	-	putative TonB-dependent receptor precursor	12722	16950	8766	8109	-1.19	9.96	1.4E-26	1.3E-24
HI1225	-	translation initiation factor Sui1	1371	2216	861	977	-1.33	6.85	2.0E-12	2.9E-11
HI1247.1	-	tRNA-Asn	56	64	183	163	1.14	3.23	9.0E-08	7.0E-07
HI1359	glgC	Glucose-1-phosphate adenylyltransferase	5248	9730	4290	4963	-1.05	8.99	7.0E-08	5.5E-07
HI1360	glgA	Glycogen synthase	4509	8276	3257	4463	-1.08	8.75	3.1E-07	2.1E-06
HI1362	pntA	NAD(P) transhydrogenase subunit alpha	33960	49356	23090	26242	-1.12	11.46	7.5E-21	2.6E-19
HI1363	pntB	NAD(P) transhydrogenase subunit beta	25237	34717	17639	19191	-1.08	11.00	3.4E-22	1.4E-20
HI1379	phoB	Phosphate regulon transcriptional regulatory protein PhoB	10414	10475	7247	6487	-1.00	9.53	1.6E-10	1.8E-09

HI1380	pstB	Phosphate import ATP-binding protein PstB	9812	11190	5030	4905	-1.47	9.39	3.9E-24	2.1E-22
HI1381	pstA	Phosphate transport system permease protein PstA	11511	16809	6184	6949	-1.48	9.80	2.9E-31	4.9E-29
HI1382	pstC	Phosphate transport system permease protein PstC	19003	26948	9759	11231	-1.50	10.49	1.0E-34	4.4E-32
HI1383	pstS	Phosphate-binding protein PstS precursor	31430	46702	14672	17950	-1.63	11.22	6.7E-34	2.3E-31
HI1389.1	trpC	yadA	3065	3869	8377	10120	1.04	8.95	6.0E-11	7.2E-10
HI1403	-	Phage tail fiber repeat protein	203	360	167	163	-1.13	4.23	3.8E-08	3.1E-07
HI1405	-	hypothetical protein	42	60	29	35	-1.05	1.84	2.3E-04	8.7E-04
HI1424.1	-	tRNA-Leu	27	30	77	75	1.03	2.12	1.7E-04	6.6E-04
HI1530	gltS	Sodium/glutamate symport carrier protein	30992	37971	16818	16506	-1.43	11.11	6.2E-43	5.2E-40
HI1545	sstT	Serine/threonine transporter SstT	5471	7082	3881	3937	-1.06	8.76	6.3E-13	9.7E-12
HI1546	-	DNA polymerase V subunit UmuD	549	551	1574	1358	1.02	6.30	3.7E-08	3.1E-07
HI1601	-	hypothetical protein	1128	2449	543	775	-1.78	6.71	1.9E-11	2.4E-10
HI1603	-	Phosphate transport regulator	1624	1685	4809	5044	1.18	8.00	1.5E-16	3.4E-15
HI1617	aspC	Aspartate aminotransferase	3125	4219	1756	1831	-1.41	7.88	2.8E-25	1.9E-23
HI1632	lysC	Lysine-sensitive aspartokinase 3	6246	6826	2407	2514	-1.80	8.63	7.9E-31	1.1E-28
HI1702	metE	5-methyltetrahydropteroyltriglutamate-- homocysteine methyltransferase	16947	20126	11863	12007	-1.02	10.34	1.4E-21	5.3E-20
HI1707	qseC	Sensor protein QseC	4609	6296	2542	2426	-1.51	8.42	1.1E-22	5.1E-21
HI1708	qseB	Transcriptional regulatory protein QseB	3916	5421	2012	1793	-1.67	8.15	3.8E-26	3.3E-24
HI1709	-	hypothetical protein	8401	11751	5183	4275	-1.46	9.32	9.2E-21	3.2E-19
HI1725	**pbp1B (ponB)**	Penicillin-binding protein 1B	2805	3694	9918	10162	1.25	9.00	3.0E-17	7.5E-16
HI1728	mntH	Divalent metal cation transporter MntH	2454	4339	1371	1816	-1.45	7.73	2.3E-14	4.1E-13
HI1729	-	LamB/YcsF family protein	672	1410	219	371	-2.16	5.85	2.2E-13	3.5E-12
HI1730	kipA	KipI antagonist	903	2168	242	485	-2.41	6.36	2.0E-12	2.9E-11
HI1731	kipI	Kinase A inhibitor	770	1466	310	396	-2.01	5.99	1.8E-17	4.7E-16
HI1733	rnb	Exoribonuclease 2	3123	3841	9658	9924	1.11	9.01	3.1E-14	5.2E-13
HI1739.6 (HI_r17)	-	23S ribosomal RNA	10053	3975	2195	2377	-2.08	8.74	2.6E-08	2.3E-07
HI1739.5	-	tRNA-Ala	84	96	442	465	1.95	4.40	1.3E-25	9.2E-24
HI1739.4	-	tRNA-Ile	134	138	517	598	1.64	4.75	3.2E-19	9.2E-18
HI1739.3 (HI_r18)	-	16S ribosomal RNA	2327	996	723	641	-1.75	6.73	1.3E-06	7.7E-06

Supplementary Table S4: Summary statistics of RNA-seq profiles between 37°C and 42°C of the 141 differentially expressed genes with a |log2(fold change)| >1, including 67 up-regulated and 74 down-regulated.

LogCPM = Logarithm of counts per million reads

FDR = False discovery rat

12. Fluoroquinolones resistance

The quinolones are among the most prescribed classes of antibiotics over the world. In 1962, the first generation of quinolones (nalidixic acid), which displayed a narrow spectrum of activity, was established for uncomplicated urinary tract infections (105). The evolution of quinolones to wide-spectrum antibacterial agents was made possible by changing the chemical structure of the nalidixic acid at different positions (106). The fluoroquinolones (second generation of the quinolones) exhibit substantially improved activity against both Gram-positive and Gram-negative bacteria. Moreover, the pharmacokinetic and pharmacodynamic properties of fluoroquinolones were remarkably enhanced (107). Ciprofloxacin displays for example activity in different types of bacterial infections, including community-acquired and nosocomial pneumonia, bone and joint infections, skin and soft tissue infections (105). In 1996, levofloxacin which is the synthetic *L*-isomer of ofloxacin, was approved in the United States for medical use. Levofloxacin hampers crucial processes in the bacterial cell by inhibiting type II topoisomerases. In addition to its effects on Gram-positive and Gram-negative bacteria, levofloxacin displays additional activity on atypical bacteria, such as *Mycoplasma pneumoniae, Legionella pneumophila* and *Chlamydophila pneumoniae* (formerly *Chlamydia pneumoniae*) (108). Quinolones operate by inhibiting the activity of two enzymes critical for bacteria viability (DNA gyrase and topoisomerase IV). The resistance against quinolones is commonly linked to chromosomal mutations in *gyrA*, *gyrB*, *parC* and *parE genes,* or mutations affecting drug accumulation by an increased efflux *or* a decreased uptake. Additionally, quinolone resistance genes harbored by plasmids have been reported in different bacterial species, e.g. the *qnr* gene and the *aac(6')-Ib-cr* gene (107). The World Health Organization (WHO) has listed quinolones as a critically important antibiotic class. However, as a consequence of the wild use of this group of antibiotics in human and veterinary medicine, the rate of quinolone-

resistant strains has increased steadily, and is now reported in all bacterial species that can be treated by this class of antibiotics. The Antimicrobial Resistance Epidemiological Survey on Cystitis study conducted between 2003 and 2006 in nine European countries, highlighted that 8.3% of *Escherichia coli* strains isolated in the context of uncomplicated lower urinary tract infections in healthy women were resistant to ciprofloxacin (109). Furthermore, high ciprofloxacin resistance rates were reported in *E. coli* isolates from intraabdominal infections in China (>60%) (110). According to surveillance studies carried out up to 2005, levofloxacin resistance has not been reported in *H. influenzae.* Unfortunately, many recent reports have described several *H. influenzae* clinical strains resistant to ciprofloxacin, levofloxacin and moxifloxacin. Some of these clinical strains were clonal (35, 111).

This part of the thesis project originated with the steady increase of *H. influenzae* clinical strains resistant to fluoroquinolones and imipenem. In addition to the recent report indicating the clonal emergence of high level ciprofloxacin-monoresistant *H. influenzae* in the region of southern Denmark implicating both children and elderly in different hospitals and wards. *H. influenzae* clinical strains with the same MLST profile and mutation in quinolone resistance-determining regions compared to Danish strains were reported recently in Spain. This study is aimed at understanding better this worrisome observation, by analysing the underlying mechanisms and epidemiology.

Research article

Molecular characterization of fluoroquinolones, macrolides, and imipenem resistance in *Haemophilus influenzae*: analysis of the mutations in QRDRs and assessment of the extent of the AcrAB-TolC-mediated resistance

Abdessalam Cherkaoui, Nadia Gaïa, Damien Baud, Stefano Leo, Adrien Fischer, Etienne Ruppe, Patrice François, Jacques Schrenzel

Authors' contribution statements

Abdessalam CHERKAOUI designed the study and all the experiments. He carried out the following elements:

- Selection of the clinical strains included in this study
- Antimicrobial susceptibility testing (disk diffusion and E-test assays)
- Effect of CCCP on MIC and minimum bactericidal concentration assays
- Interpretation of the whole-genome sequencing data
- He analysed all the data and wrote the manuscript.

N. Gaïa, D. Baud, and **S. Leo** carried out whole-genome sequencing and core genome multi-locus sequence typing (cgMLST)

A. Fischer provided help and support for statistical analyses

P. François and **E. Ruppe** helped in the sequencing analysis and revised the manuscript

J. Schrenzel supervised the research and revised the manuscript

Objectives: The purpose of this study was to determine the mechanisms of resistance to fluoroquinolones, macrolides, and imipenem in *H. influenzae*, to evaluate the amount of the AcrAB-TolC-mediated resistance, and to establish a core genome multilocus sequence typing (cgMLST) scheme for *H. influenzae*.

Methods: In vitro susceptibility testing was performed for six fluoroquinolone-resistant *H. influenzae* isolates (FQR), GE47, and GE88 using the E-test and disk diffusion methods. To assess the contribution of the AcrAB-TolC-mediated resistance on imipenem, erythromycin, and levofloxacin, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) levels were determined using broth microdilution assay in the absence or presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP). Mutations in the quinolone resistance-determining regions and in PBPs were determined by whole-genome sequencing. We defined a core genome multi-locus sequence typing (cgMLST) by using all 19 *H. influenzae* closed genomes available, the genomes of 4 *H. influenzae* strains monoresistant to ciprofloxacin isolated in Denmark and the genomes of 8 *H. influenzae* strains heteroresistant to imipenem isolated at Geneva University Hospitals.

Results: All fluoroquinolone-resistant *H. influenzae* isolates included in this study possessed the *ermB* gene. Four amino acid substitutions in GyrA (at Ser84 and Asp88), ParC (at Ser84), and ParE (at Asp420) were found closely associated to the fluoroquinolones MICs. We did not find any plasmid-mediated quinolone resistance. In addition, no amino acid substitution surrounding the three highly conserved amino acid motifs in PBP3 related to imipenem resistance was defined. CCCP decreased the MIC of imipenem by twofold for FQR-6 and fourfold for GE47 and GE88 strains. For erythromycin, the MICs were decreased by twofold. We determined that the six FQR isolates clustered in two groups. The number of different loci within FQR-1_FQR-3_FQR-5 cluster was 6, while FQR-2 and FQR-4 differed for 21 loci. FQR-1_FQR-3_FQR-5 and FQR-2_FQR-4 clusters were distant among each other and compared to all other tested genomes.

Conclusions: We provide evidence that drug efflux is one of the major mechanisms of imipenem and erythromycin resistance in *H. influenzae*. We confirm that specific amino acid substitutions in GyrA, ParC, and ParE are implicated in quinolone resistance. Additionally, the degree of resistance is related to the number of these amino acid substitutions

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ORIGINAL ARTICLE

Molecular characterization of fluoroquinolones, macrolides, and imipenem resistance in *Haemophilus influenzae*: analysis of the mutations in QRDRs and assessment of the extent of the AcrAB-TolC-mediated resistance

Abdessalam Cherkaoui[1] · Nadia Gaïa[2] · Damien Baud[2] · Stefano Leo[2] · Adrien Fischer[1] · Etienne Ruppe[3] · Patrice François[2] · Jacques Schrenzel[1,2]

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Abstract

The aims of the present study were to characterize the mechanisms of resistance to fluoroquinolones, macrolides, and imipenem in *Haemophilus influenzae*, to assess the extent of the AcrAB-TolC-mediated resistance, and to define a core genome multilocus sequence typing (cgMLST) scheme for *H. influenzae* by using whole-genome sequencing. Four amino acid substitutions in GyrA (at Ser84 and Asp88), ParC (at Ser84), and ParE (at Asp420) were found to be closely associated to the MICs. We did not find any amino acid substitution surrounding the three highly conserved amino acid motifs in PBP3 related to imipenem resistance. All the isolates possessed the ermB gene. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) decreased the MIC of imipenem by twofold for FQR-6 and fourfold for GE47 and GE88 strains. For erythromycin, the MICs were decreased by twofold. We found that the six FQR isolates were clustered in two groups. The number of different loci within FQR-1_FQR-3_FQR-5 cluster was 6, while FQR-2 and FQR-4 differed for 21 loci. FQR-1_FQR-3_FQR-5 and FQR-2_FQR-4 clusters were distant among each other and compared to 19 genomes downloaded from NCBI, to 8 strains heteroresistant to imipenem, and to 4 strains monoresistant to ciprofloxacin isolated in Denmark. We confirmed that specific amino acid substitutions in GyrA, ParC, and ParE are implicated in quinolone resistance. Additionally, the degree of resistance is related to the number of these amino acid substitutions. We provide robust evidence that drug efflux is one of the substantial mechanisms of imipenem and erythromycin resistance in *H. influenzae*.

Keywords Imipenem · Fluoroquinolone · Macrolide · *H. influenzae* · CCCP · Whole-genome sequencing · cgMLST · Antibiotic resistance

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✉ Abdessalam Cherkaoui
abdessalam.cherkaoui@hcuge.ch

[1] Bacteriology Laboratory, Service of Laboratory Medicine, Department of Genetics, Laboratory Medicine and Pathology, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

[2] Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

[3] AP-HP, Hôpital Bichat, Laboratoire de Bactériologie, INSERM, IAME, UMR 1137, Université Paris Diderot, IAME, UMR 1137, Sorbonne Paris Cité, 75018 Paris, France

Introduction

Haemophilus influenzae, a human-restricted pathogen, is implicated in several diseases. The colonization of the respiratory tract, facilitated by the production of several colonization and virulence factors, constitutes the initial step for the development of otitis media, sinusitis, bronchitis, acute exacerbations of chronic obstructive pulmonary disease, community-acquired pneumonia, and meningitis [1]. The prevalence and treatment of each of these infections differ considerably between countries. However, accurate diagnosis and treatment of respiratory infections caused by *H. influenzae* remain imperative. *H. influenzae* type b (Hib) was considered, before the introduction of the conjugate vaccine, as the principal cause of meningoencephalitis in young children [2]. Nowadays, strain

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replacement has been largely reported and nontypeable *H. influenzae* (NTHi) have become the preponderant pathogens among both invasive and non-invasive diseases [3]. Beta-lactams, macrolides, and fluoroquinolones are frequently used as antimicrobial therapy for respiratory tract infections and have exhibited good antibacterial activity against pathogens such as *H. influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* [4, 5]. The resistance to ampicillin, amoxicillin/clavulanic acid, and second-generation cephalosporins in *H. influenzae* has emerged and is now globally widespread. Thus, the use of these drugs as front-line in infections for which *H. influenzae* is either suspected or proven becomes nowadays problematic. Three β-lactams resistance mechanisms have been previously reported in *H. influenzae*. One mechanism is the acquisition of the bla (TEM**)** and bla (ROB**)** genes. Another mechanism requires decreased β-lactams affinity for penicillin binding protein 3 (PBP3). The third mechanism is the overexpression of the chromosomal multidrug efflux pump AcrAB-TolC [6–8].

Since the first report in 1993, fluoroquinolone-resistant *H. influenzae* isolates have been described all over the world [9, 10], and treatment failure with levofloxacin has already been reported [11, 12]. The chromosomal point mutations which have been shown to occur in a stepwise manner in the quinolone resistance-determining regions (QRDRs) of the genes encoding the DNA gyrase (*gyrA* and *gyrB* genes encoding A_2B_2 complex), and the topoisomerase IV (*parC* and *parE* genes encoding C_2E_2 complex), constitute the principal mechanism of fluoroquinolone resistance in *H. influenzae* [13–15]. A first mutation in *gyrA* affects quinolones susceptibility, but minimum inhibitory concentrations (MICs) still remain in the susceptible range according to the current Clinical and Laboratory Standards Institute (CLSI) and European Society of Clinical Microbiology and Infectious Diseases (EUCAST) breakpoint interpretations. In contrast, double mutations in both DNA gyrase and topoisomerase IV cause a resistant phenotype [13].

Regardless of the fact that the majority of *H. influenzae* isolates show low-level intrinsic resistance to macrolide, the use of macrolides in treating respiratory infections where NTHi may be implicated is largely reported [16]. The resistance to macrolides in *H. influenzae* was associated to three different mechanisms: first, the acquisition of the resistance genes *mefA* (encoding a drug efflux pump) and *ermB* (encoding 23S rRNA methylase); second, amino acid substitutions in ribosomal proteins L4 and L22 that lead to decreased affinity for macrolides; and third, the overexpression of chromosomal multidrug efflux pumps [16, 17].

The objectives of the present study were (1) to determine fluoroquinolones, macrolides, and β-lactams susceptibility profiles of six fluoroquinolone-resistant *H. influenzae* clinical isolates identified at Geneva University Hospitals; (2) to analyze the whole-genome of these isolates; (3) to characterize their mechanisms of resistance to fluoroquinolones, macrolides, and imipenem; (4) to assess the extent of the efflux pump-mediated imipenem, erythromycin, and levofloxacin resistance by using carbonyl cyanide *m*-chlorophenylhydrazone (CCCP); and (5) to determine and assess a core genome multilocus sequence typing (cgMLST) scheme for the isolates analyzed in this study in comparison to 19 *H. influenzae* closed genomes available in NCBI in April 2018, the genomes of 4 *H. influenzae* strains monoresistant to ciprofloxacin isolated in Denmark, and the genomes of 8 *H. influenzae* strains heteroresistant to imipenem but fluoroquinolone susceptible isolated at Geneva University Hospitals.

Materials and methods

Bacterial isolates

The six fluoroquinolone-resistant *H. influenzae* isolates (FQR) examined in this study were identified in the bacteriology laboratory at Geneva University Hospitals between 2016 and 2018. These strains were selected primarily on the basis of quinolone-resistant phenotype. The two imipenem-resistant *H. influenzae* isolates (GE47 and GE88), analyzed in this study, were retrieved from a collection of 46 NTHi strains previously studied [6]. The sources of the six FQR isolates were as follows: one bronchial aspirate (FQR-1/2016), two sputa (FQR-3/2016; FQR-4/2017), one genital ulcer swab (FQR-6/2018), one endocervical swab (FQR-5/2017), and one nasopharyngeal aspirate (FQR-2/2016). The patients ranged in age from 27 to 68 years, with two male and four female subjects. All isolates were stored at − 80 °C in skim milk with 15% glycerol. *H. influenzae* isolates were cultured on chocolate agar supplemented with PolyViteX (bioMérieux) and incubated at 35 °C ± 2 for 18 to 24 h in a humid atmosphere containing 5% CO_2. The identification of the *H. influenzae* isolates was confirmed by using matrix-assisted desorption ionization-time of flight mass spectrometry (MALDI-TOF MS; Maldi Biotyper compass, Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

In vitro susceptibility testing was performed for the six FQR, GE47, and GE88 isolates using the *E* test method according to the manufacturer's instructions and disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods. Twelve antimicrobial agents were tested: ciprofloxacin (CIP), moxifloxacin (MXF), levofloxacin (LVX), ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), ceftriaxone

(CRO), ertapenem (ERT), imipenem (IMI), meropenem (MEM), azithromycin (AZM), and erythromycin (ER).

Effect of CCCP on minimum inhibitory concentrations and minimum bactericidal concentrations of imipenem, erythromycin, and levofloxacin

To assess the contribution of the efflux pump-mediated resistance on imipenem, erythromycin, and levofloxacin, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) levels were determined using broth microdilution assay in the absence or presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Stock solution of CCCP was prepared in dimethyl sulfoxide (DMSO). Final concentrations of CCCP used in broth microdilution assay were 0.78 mM, which is the highest concentration of CCCP that does not affect cell growth as determined previously [6]. The broth microdilution assay was performed according to EUCAST [18].

Whole-genome sequencing

The genomic DNA of the six FQR isolates was sequenced using Illumina MiSeq (150 bp paired-end reads) technology (Illumina, San Diego CA, USA). The Illumina sequence quality was assessed using the Fastqc program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and filtered using the Fastq-mcf programs (Eautils Eautils: http://code.google.com/p/ea-utils/). The assemblage of the genome reads was done by using the Edena v3 assembler. The National Center for Biotechnology Information annotation pipeline was used to determine genome annotations.

Core genome multi-locus sequence typing target genes

We defined a core genome multi-locus sequence typing (cgMLST) scheme with Ridom SeqSphere+ software version 5 (Ridom GmbH, Germany) by using 19 *H. influenzae* closed genomes available in NCBI in April 2018, the genomes of 4 *H. influenzae* strains monoresistant to ciprofloxacin isolated in Denmark [19], and the genomes of 8 *H. influenzae* strains heteroresistant to imipenem but fluoroquinolone susceptible isolated in Geneva [6]. We chose *H. influenza*e Rd KW20 strain as reference. We performed genome-wide gene-by-gene comparison using the cgMLST target definer function of SeqSphere+ with default parameters. This step was needed to filter out genes of Rd KW20 reference strain from the cgMLST scheme that presented: a length shorter than 50 bp ("minimum length filter"); no start codon at the beginning of their sequence ("start codon filter"); no stop codon or more than one stop codon at the end of their sequence ("stop codon filter"); fragments that occur in multiple copies within a genome (with more than 90% identity and > 100 bp overlap; "homologous gene filter"). We considered a "gene overlap filter" that analyzes overlapping regions > 4 bases between two genes. This step discards the shorter gene of the pairs sharing the overlap. The remaining genes were used for pairwise-comparisons performed with BLAST version 2.2.12 with the other *H. influenzae* genomes. More specifically, we kept all genes of *H. influenzae* Rd KW20 strain shared with the other genomes with a sequence identity ≥ 90 and 100% overlap. The final cgMLST scheme consisted of 1083 genes covering the 56.5% of the genomic sequence of *H. influenzae* Rd KW20 strain. We extracted the sequences of cgMLST genes by SeqSphere+ with default settings from (i) the 6 FQR isolates which the nucleotide sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena/) under the study accession number PRJEB26752, (ii) 8 isolates taken from a collection of 46 *H. influenzae* clinical isolates previously analyzed [6], (iii) 4 strains monoresistant to ciprofloxacin isolated in Denmark, and (iv) 19 NCBI reference genomes mentioned above, including the *H. influenzae* Rd KW20 strain. From each isolate, complete sequence of each gene was analyzed according to cgMLST scheme and a numerical allele type was assigned to that given locus. Allelic profile was therefore determined by combining alleles of all cgMLST loci for each strain. A minimum spanning tree (MST) was eventually inferred by neighbor joining method on the allelic profiles. Genes that were not present in all the analyzed samples ($n = 33$) were also included in the generation of MST.

Results

Antimicrobial susceptibility testing

Table 1 shows the susceptibilities of the eight *H. influenzae* clinical isolates analyzed in this study against fluoroquinolones, β-lactams, and macrolides. The isolates were assigned to three groups: high-level fluoroquinolone resistance only ($n = 5$), low-level fluoroquinolone resistance associated to high-level resistance to imipenem ($n = 1$), and two isolates that were fluoroquinolones susceptible but highly resistant to imipenem (MIC, > 32 mg/L). These last two isolates were previously studied [20]. Among the six fluoroquinolone-resistant isolates (FQR), five were β-lactamase-negative, and susceptible to all β-lactam antibiotics tested. The sixth isolate (FQR-6) was also β-lactamase-negative and had ciprofloxacin, moxifloxacin, levofloxacin MICs of 0.75, 1, and 0.75 mg/L respectively and was categorized as either susceptible (CLSI) or resistant (EUCAST) to fluoroquinolones. By disk diffusion (5 mg), all six isolates had fluoroquinolone inhibition zones smaller than 20 mm and were categorized as resistant by EUCAST breakpoints (Supplementary Fig. S1). Using

Table 1 Susceptibilities of eight *H. influenzae* clinical isolates to fluoroquinolones, β-lactams, and macrolides

Strain	Patient age (year)	Gender	Specimen	Serotype	MIC (μg/mL) by E-test										
					CIP	MXF	LVX	AMP	AMC	CXM	CRO	ERT	IMP	MEM	AZM	ER
FQR-1	63	M	Bronchial aspirate	B	> 32	> 32	> 32	0.38	0.38	0.38	0.004	0.023	2	0.064	8	12
FQR-2	58	F	Nasopharyngeal aspirate	NT	> 32	> 32	> 32	0.19	0.38	0.38	0.003	0.023	2	0.064	8	12
FQR-3	49	M	Sputum	NT	> 32	> 32	> 32	0.25	0.38	0.38	0.004	0.023	2	0.047	12	16
FQR-4	59	F	Sputum	NT	32	24	32	0.19	0.38	0.38	0.003	0.023	2	0.064	8	12
FQR-5	27	F	Cervicovaginal smear	NT	> 32	32	> 32	0.25	0.75	0.5	0.003	0.016	2	0.064	12	12
FQR-6	57	F	Genital ulcer smear	NT	0.75	1	0.75	0.19	0.38	0.5	0.004	0.023	> 32	0.047	8	8
FQS-1 (GE47)	75	M	Sputum	NT	0.032	0.023	0.023	2	1.5	3	0.023	0.094	> 32	0.19	12	8
FQS-2 (GE88)	75	M	Sputum	NT	0.064	0.032	0.023	0.19	0.125	0.75	0.032	0.25	> 32	0.75	24	12

NT nontypeable, *FQR* fluoroquinolones-resistant isolate, *FQS* fluoroquinolones susceptible isolate
Ciprofloxacin (CIP), Moxifloxacin (MXF), Levofloxacin (LVX), Ampicillin (AMP), Amoxicillin-clavulanic acid (AMC), Cefuroxime (CXM), Ceftriaxone (CRO), Ertapenem (ERT), Imipenem (IMI), Meropenem (MEM), Azithromycin (AZM), Erythromycin (ER)

the EUCAST epidemiological cut-offs (ECOFFs), the MICs for azithromycin were greater than the ECOFF (MIC range, 8–24 mg/L) but for erythromycin, the MICs were smaller or equal to the ECOFF (MIC range, 8–16 mg/L). By the *E* test method, the FQR-6 isolate showed the presence of distinct colonies growing within the inhibition zone of the imipenem (Supplementary Fig. S2). These distinct colonies indicate the presence of imipenem heteroresistance. The same phenomenon was also documented for the GE47 and GE88 isolates [20].

Mutations in the quinolone resistance-determining regions

In 1996, Georgiou et al. reported that some key mutations identified in GyrA (Ser-84 to Leu or Tyr and Asp-88 to Asn or Tyr) and ParC (Ser-84 to Ile and Glu-88 to Lys) were associated with high-level resistance to ciprofloxacin [14]. Further studies confirmed these mutations and reported new ones involved in fluoroquinolones resistance (e.g., mutations in ParE and GyrB). Table 2 depicts the amino acid substitutions in each quinolone resistance-determining region (QRDR) for the six FQR isolates in comparison to a fluoroquinolone-sensitive reference strain, Rd KW20. Isolates with two substitutions in GyrA, one in ParC, and one in ParE had ciprofloxacin, moxifloxacin, and levofloxacin MICs of 24 to > 32 mg/L. Isolate with two substitutions in GyrA, and one in ParC had ciprofloxacin, moxifloxacin, and levofloxacin MICs of > 32 mg/L. In contrast, isolate with only one substitution in GyrA (Ser-84 to Leu), one in ParC, and one in ParE displayed lower MICS (ciprofloxacin, moxifloxacin, and levofloxacin MICs of 0.75 to 1.0 mg/L). This suggests that the substitution in GyrA at position 88 (Asp-88 to Gly) is related to high-level resistance to fluoroquinolones. In the present study, no substitutions were identified in GyrB. In addition to resistance mutations previously seen in *H. influenzae*, we identified other changes in GyrA, GyrB, ParC, and ParE which appeared not associated to the resistance phenotype (Supplementary Table S1). The amino acid substitution Asp420Asn in ParE has been previously reported [13, 21, 22], while its role in fluoroquinolones resistance has not been documented by genetic transformation.

Mutations in penicillin binding proteins and AcrAB-TolC components

We analyzed the presence of the genes encoding for the TEM-1 and ROB-1 β-lactamases, the amino acid substitutions in the penicillin binding proteins involved in β-lactam resistance, as well as the sequence of the acrR regulatory gene controlling the efflux pump AcrAB-TolC. No β-lactamases were detected in all the six FQR isolates. As shown in Table 3, we identified several changes in PBP1a, PBP2, PBP3, PBP4, PBP5, and

Table 2 Amino acid substitutions in each quinolone resistance-determining region (QRDR) for the six FQR isolates in comparison to a fluoroquinolone-sensitive reference strain, Rd KW20

Strain ID	Amino acid substitutions in each quinolone resistance-determining region (QRDR)			
	GyrA		ParC	ParE
Rd KW20	Ser-84	Asp-88	Ser-84	Asp-420
FQR-1	Leu	Gly	Ile	–
FQR-2	Leu	Gly	Ile	Asn
FQR-3	Leu	Gly	Ile	Asn
FQR-4	Leu	Gly	Ile	Asn
FOR-5	Leu	Gly	Ile	Asn
FQR-6	Leu	–	Ile	Asn

"–" indicates no amino acid substitution

PBP7 which seem not implicated in β-lactams resistance. The FQR-6 was highly resistant to imipenem (MIC > 32 mg/L) but we did not find any amino acid substitution surrounding the three highly conserved amino acid motifs in PBP3 (STVK (Ser327-Thr-Val-Lys), SSN (Ser379-Ser-Asn), and KTG (Lys-512-Thr-Gly)). An analysis of the deduced amino acid sequences of the other PBPs from the six FQR isolates revealed considerable polymorphism in comparison with the *H. influenzae* strain Rd KW20 sequence, irrespective of the imipenem susceptibility phenotype (Table 3). Seven amino acid substitutions in *dacA* (S13G, A19T, M27I, G30A, G32M, Q157K, A214P) were found specifically in FRQ-6 isolate, but only two of these (A19T and A214P) were common to imipenem heteroresistance stains previously studied [6]. The amino acid substitution in *dacB* (R111S), found only in FQR-6 isolate, was previously identified in imipenem susceptible strains (imipenem MIC = 2 mg/L) [6]. Two amino acid substitutions in *pbp2* (N389D and A434T) and in *ponA* (D363E and N802S) were found only in FQR-6 but these mutations were not common to imipenem heteroresistance stains previously studied. Furthermore, the affinity patterns of PBP varied contingent upon the structures of the β-lactam antibiotics. As defined previously by using Bocillin-FL assays, the relative order of the affinities for imipenem were PBP3 >>> > PBP7 > PBP1a = PBP1b > PBP2 > PBP4 > > PBP5 [6]. Our findings are in agreement with a previous reports displaying important allelic variation among the genes encoding for PBP1a, PBP1b, PBP2, PBP4, PBP5, and PBP7 from different *H. influenzae* isolates and indicating that there is no evidence between the amino acid substitutions identified in these PBPs and β-lactams resistance [23–25]. The PBP3 mutations found in FQR-6 (P31S, L165S, A239E) were not fully responsible for imipenem resistance since it is commonly found in imipenem susceptible strains. In fact, as reported by Cerquetti et al. [23] and Osaki et al. [25], the imipenem MICs were greater for the *H. influenzae* clinical isolates than for the recombinants which carries a mutated *ftsI* gene, or by site-directed mutagenesis. In our previous study, we found evidence of association between AcrAB-TolC and imipenem resistance by using carbonyl cyanide m-chlorophenylhydrazone, which was confirmed in this study. These results are in agreement with the data reported by Kitaoka et al. [26].

Moreover, the FQR-6 isolate did not harbor a nonsense mutation in the negative regulator gene *acrR* that leads to the overexpression of the chromosomal efflux pump AcrAB-TolC. Previous studies have shown that the sensitivity of the efflux pump inhibitors was defined by their steric effects implicating the amino acids around the inhibitor-binding pit [27, 28]. This suggests that the amino acid substitution in the efflux pump can affect its activity. As shown in Table 3, we identified several changes in AcrA and TolC. However, these mutations are frequently seen in imipenem susceptible strains. We did not find any amino acid substitution in AcrB in comparison to a reference strain, Rd KW20.

Detection of macrolide resistance genes and amino acid substitution in the ribosomal proteins L4

Of the six FQR isolates tested, all possessed the *ermB* gene and none had the *mefA* gene. Additionally, one amino acid substitution on ribosomal proteins L4 was detected at position 121 (Thr-121 to Ser) for FQR-2, FQR-4, and FQR-6 isolates in comparison to a reference strain, Rd KW20. All the six FQR isolates did not harbor a nonsense mutation in *acrR* gene, which likely contributes to the macrolide resistance. Moreover, we did not find any amino acid substitution in AcrB which is related to azithromycin resistance as reported previously [28].

Effect of CCCP on the MICs and MBCs of imipenem, erythromycin, and levofloxacin

The effect of the broad-spectrum efflux pump inhibitor CCCP on imipenem MIC and MBC was assessed for the FQR-6 isolate and the two imipenem-resistant *H. influenzae* isolates (GE47 and GE88). All three isolates had imipenem MICs by

Table 3 Amino acid substitutions in penicillin binding proteins and AcrAB-TolC components

Strain ID	*acrA* gene (AcrA), Amino acid substitution for :														
Rd KW20	I-36	G-48	S-52	A-82	M-83	A-91	V-92	V-163	S-165	I-191	D-269	V-296	R-333	V-361	I-390
FQR-1	.	E	.	.	L	T	I	L	N	.	N	.	.	A	V
FQR-2	M	E	P	V	.	.	.	.	.	V	.	.	.	A	V
FQR-3	.	E	.	.	L	T	I	L	N	.	N	.	.	A	V
FQR-4	M	E	P	V	.	.	.	.	.	V	.	.	.	A	V
FQR-5	.	E	.	.	L	T	I	L	N	.	N	.	.	A	V
FQR-6	M	E	P	V	.	.	.	.	.	V	.	I	H	A	.

Strain ID	*acrR gene* (AcrR), Amino acid substitution for :									
Rd KW20	S-14	R-22	N-26	Q-27	L-31	L-33	T-77	I-121	H-131	Q-134
FQR-1	L	K	D	R	H	I	S	V	D	K
FQR-2	.	.	.	.	H	.	.	.	.	.
FQR-3	L	K	D	R	H	I	S	V	D	K
FQR-4	.	.	.	.	H	.	.	.	.	.
FQR-5	L	K	D	R	H	I	S	V	D	K
FQR-6	.	.	.	.	H	.	.	.	.	.

Strain ID	*dacA* gene (PBP5), Amino acid substitution for :										
Rd KW20	S-13	A-19	M-27	G-30	T-32	Q-157	N-205	A-214	L-312	V-343	F-377
FQR-1	.	.	.	.	.	.	D	.	.	A	.
FQR-2	.	.	.	.	.	.	D	.	F	A	L
FQR-3	.	.	.	.	.	.	D	.	.	A	.
FQR-4	.	.	.	.	.	.	D	.	F	A	L
FQR-5	.	.	.	.	.	.	D	.	.	A	.
FQR-6	G	T	I	A	M	K	D	P	F	A	L

Strain ID	*dacB* gene (PBP4), Amino acid substitution for :											
Rd KW2	V-43	F-45	D-58	S-67	D-84	N-101	N-107	R-111	A-172	E-262	Q-270	L-476
FQR-1	.	.	.	.	G	S	H	.	.	A	.	.
FQR-2	A	V	N	P	G	.	.	.	S	A	K	I
FQR-3	.	.	.	.	G	S	H	.	.	A	.	.
FQR-4	A	V	N	P	G	.	.	.	S	A	K	I
FQR-5	.	.	.	.	G	S	H	.	.	A	.	.
FQR-6	A	V	.	P	G	.	.	S	.	A	K	.

Strain ID	*ftsI* gene (PBP3), Amino acid substitution for :																		
Rd KW20	P-31	C-56	N-75	L-124	A-131	E-135	S-152	L-165	S-166	L-219	T-228	A-239	D-350	A-437	V-547	N-569	A-586	S-594	A-595	E-603
FQR-1	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
FQR-2	S	G	S	I	S	Q	A	S	N	M	I	E	N	S	I	S	S	T	T	D
FQR-3	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
FQR-4	S	G	S	I	S	Q	A	S	N	M	I	E	N	S	I	S	S	T	T	D
FQR-5	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
FQR-6	S	.	.	.	.	.	.	S	.	.	.	E	.	.	.	.	.	.	.	.

Strain ID	*pbp2* gene (PBP2), Amino acid substitution for :													
Rd KW20	Q-59	N-126	R-132	L-321	N-389	A-434	I-437	L-508	N-513	A-518	T-569	A-570	D-589	K-592
FQR-1	R	H	L	P	.	.	M	I	S	T	A	V	E	R
FQR-2	R	H	.	P	.	.	M	I	S	T	A	V	E	R
FQR-3	R	H	L	P	.	.	M	I	S	T	A	V	E	R
FQR-4	R	H	.	P	.	.	M	I	S	T	A	V	E	R
FQR-5	R	H	L	P	.	.	M	I	S	T	A	V	E	R
FQR-6	R	H	L	P	D	T	M	I	S	T	A	V	E	R

Strain ID	*ponA* gene (PBP1a), Amino acid substitution for :																										
Rd KW20	N-9	H-28	K-40	S-88	D-92	S-133	S-187	A-298	G-319	G-356	V-358	D-363	H-397	A-411	M-487	I-513	A-564	D-588	I-591	N-626	D-633	M-657	T-782	S-793	N-802	A-822
FQR-1	.	.	R	N	.	P	A	.	S	E	A	.	R	.	.	M	.	.	.	S	.	F	M	P	.	V
FQR-2	S	Y	.	.	E	P	A	D	.	.	.	.	R	S	L	M	S	E	V	S	E	.	M	P	.	V
FQR-3	.	.	R	N	.	P	A	.	S	E	A	.	R	.	.	M	.	.	.	S	.	F	M	P	.	V
FQR-4	S	Y	.	.	E	P	A	D	.	.	.	.	R	S	L	M	S	E	V	S	E	.	M	P	.	V
FQR-5	.	.	R	N	.	P	A	.	S	E	A	.	R	.	.	M	.	.	.	S	.	F	M	P	.	V
FQR-6	.	.	R	.	.	.	.	.	.	.	.	E	R	S	.	M	.	.	.	S	.	F	.	P	S	V

Strain ID	*pbp7 gene* (PBP7), Amino acid substitution for :										
Rd KW20	A-16	H-35	S-37	K-123	R-171	S-176	Q-185	Q-212	R-225	K-289	K-292
FQR-1	L	Y	P	N	L	.	H	R	L	.	R
FQR-2	V	.	.	.	.	.	H	.	.	.	.
FQR-3	L	Y	P	N	L	.	H	R	L	.	R
FQR-4	V	.	.	.	.	.	H	.	.	.	.
FQR-5	L	Y	P	N	L	.	H	R	L	.	R
FQR-6	.	.	.	N	.	N	H	.	L	Q	.

Strain ID	*tolC gene* (TolC), Amino acid substitution for :		
Rd KW2	G-15	G-64	V-201
FQR-1	S	E	A
FQR-2	S	E	A
FQR-3	S	E	A
FQR-4	S	E	A
FQR-5	S	E	A
FQR-6	S	E	A

E test method greater than 32 mg/L by taking into account the isolated colonies present in the inhibition ellipses at up to 32 mg/L (Supplementary Fig. S2). The imipenem MICs for the same isolates, when determined by the standard broth dilution method that uses a lower inoculum (5×10^5 CFU/ml), were considerably lower: 2.0 mg/L for FQR-6 and 8.0 mg/L for GE47 and GE88. This suggests the presence of imipenem-resistant subpopulations in the cultures of FQR-6 with a small fraction of cells capable to grow in a medium containing high concentrations of imipenem. As shown in Fig. 1, CCCP decreased the MIC of imipenem by twofold in FQR-6. Interestingly, a significant difference was seen for the GE47

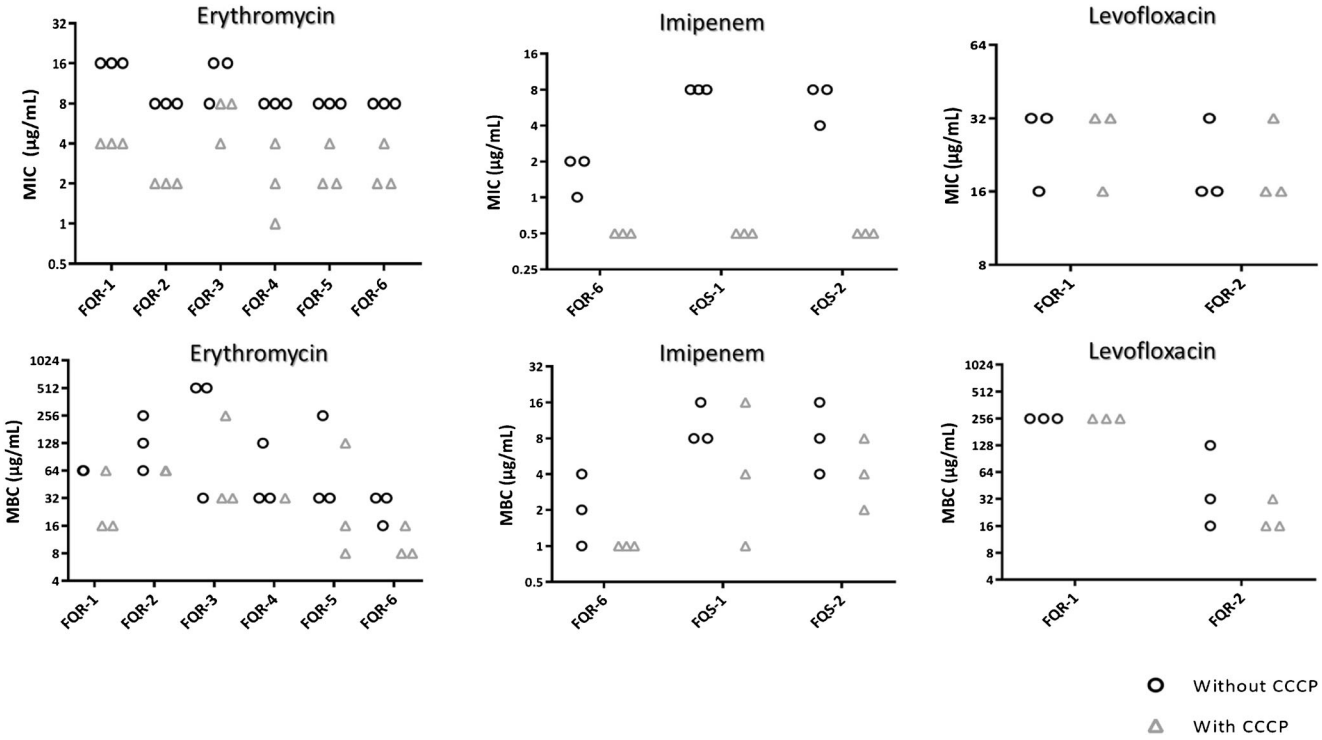


Fig. 1 Effect of broad-spectrum efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on MIC and MBC levels of erythromycin, imipenem, and levofloxacin in *H. influenzae* clinical isolates. We used highest concentration of CCCP (0.78 mM) that did not affect cell growth as defined previously. FQS-1 = GE47, FQS-2 = GE88. All the experiments were done on three independent biological replicates. *MBC* minimum bactericidal concentration, *MIC* minimum inhibitory concentration

and GE88 strains, which the CCCP decreased the MIC of imipenem by fourfold. As depicted in Supplementary Fig. S2, in comparison to FQR-6, GE47 and GE88 strains had an important fraction of imipenem-resistant subpopulations with imipenem MICs greater than 32 mg/L, which explains the CCCP effect difference seen between FQR-6 and GE47 and GE48.

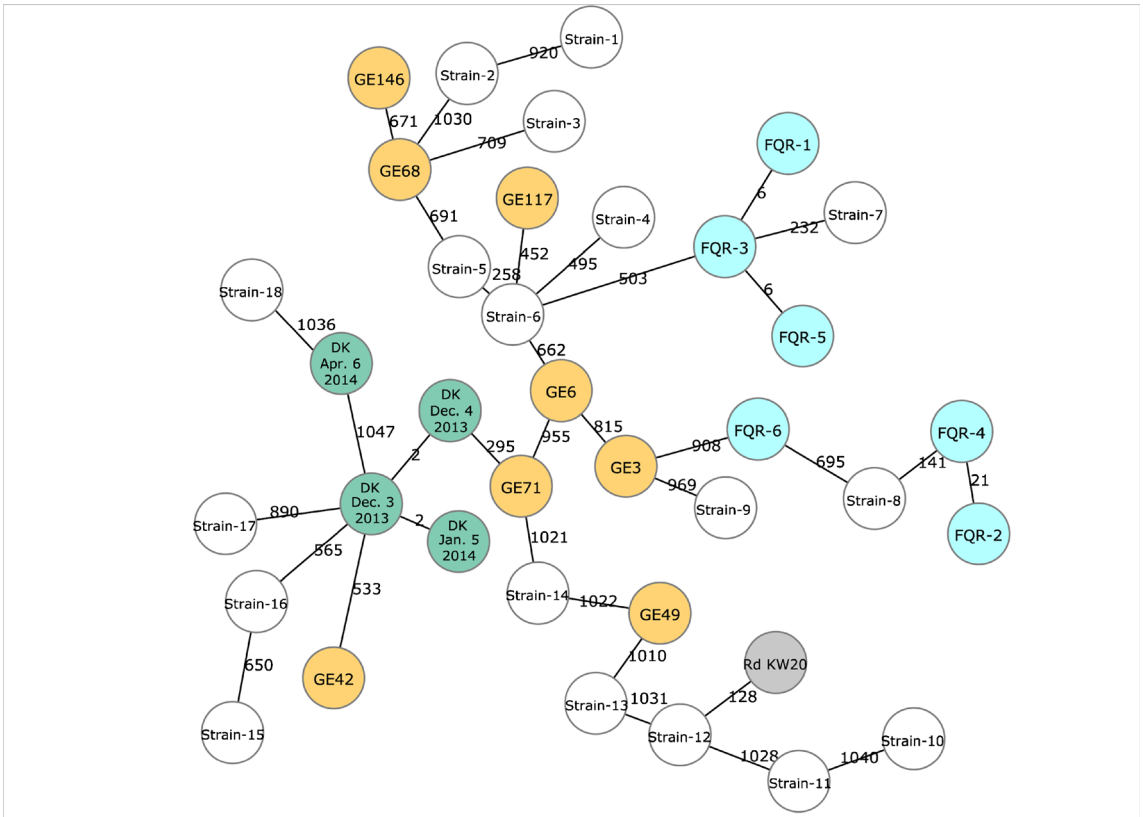
The effect of the broad-spectrum efflux pump inhibitor CCCP on erythromycin MIC and MBC was determined for all the FQR isolates. CCCP decreased the MIC of erythromycin by twofold. Small and non-significant differences were observed in the MBC of erythromycin. In contrast, CCCP did not reduce the MIC and MBC of levofloxacin.

Minimum spanning tree based on the core genome MLST

We investigated the genetic relatedness by whole-genome sequencing between the six FQR isolates. By performing pairwise comparisons of core genome multilocus sequence typing (cgMLST) allelic patterns, we found that the six FQR isolates were clustered in two groups of closely related isolates: the former including FQR-1, FQR-3, and FQR-5; the latter constituted by FQR-2 and FQR-4 (Fig. 2). FQR-6 was found to be apart from the other FQR isolates. The number of different loci within FQR-1_FQR-3_FQR-5 cluster was 6, while FQR-2 and FQR-4 differed for 21 loci. FQR-1_FQR-3_FQR-5 and FQR-2_FQR-4 clusters were distant among each other and compared to the other strains analyzed including the four strains monoresistant to ciprofloxacin isolated in Denmark. In addition, FQR-1, FQR-3, and FQR-5 had the same mutations patters for all the PBPs and AcrAB-TolC.

Discussion

In the present project, we defined that the six fluoroquinolone-resistant *H. influenzae* harbored amino-acids substitutions at Ser84 and Asp88 in GyrA and at Ser84 in ParC, and at Asp420 in ParE. As reported previously, these amino acids changes induce resistance by hampering the binding of the drugs to subunit A [29]. Furthermore, the degree of resistance appears to be closely linked to the number of these amino acids substitutions [22]. We identified other amino acids substitutions at different positions in GyrA, GyrB, ParC, and ParE which seem not linked to the fluoroquinolone resistance phenotype. Different reports have outlined the mechanism of quinolone resistance in *Pseudomonas aeruginosa*, *Staphylococcus aureus* and other clinical pathogens [30, 31]. High quinolone resistance in *S. aureus* occurs by stepwise amino acids substitutions, first in ParC, then in GyrA, and then in ParC again [32]. In contrast, for *H. influenzae*, the



Strain ID	NCBI Reference Sequence	Strain ID	NCBI Reference Sequence	Strain ID	NCBI Reference Sequence	Strain ID	NCBI Reference Sequence
Rd KW20	NC_000907	Strain-10	NZ_LN831035	GE3	LVZC00000000	DK-Apr. 6, 2014	not available
Strain-1	NC_016809	Strain-11	CP009610	GE6	LVZD00000000	DK-Dec. 3, 2013	not available
Strain-2	NZ_CP017811	Strain-12	NC_022356	GE42	LVZE00000000	DK-Jan. 5, 2014	not available
Strain-3	NZ_CP020411	Strain-13	NZ_CP007470	GE49	LVZF00000000	DK-Dec. 4, 2013	not available
Strain-4	NC_017451	Strain-14	NC_014922	GE68	LVZG00000000		
Strain-5	NZ_CP007805	Strain-15	NC_014920	GE71	LVZH00000000		
Strain-6	NC_017452	Strain-16	NC_007146	GE117	LVZI00000000		
Strain-7	CP000671	Strain-17	CP000672	GE146	LVZJ00000000		
Strain-8	NZ_CP007471	Strain-18	NZ_CP008740				
Strain-9	NZ_CP007472						

Fig. 2 Minimum spanning tree (MST) based on 1083 cgMLST genes from 33 samples: 6 genomes from this study (FQR-1, FQR-2, FQR-3, FQR-4, FQR-5, FQR-6); 8 genomes from our previously study (GE3, GE6, GE42, GE49, GE68, GE71, GE117, and GE146); 4 genomes (DK-Apr. 6, 2014, DK-Dec. 3, 2013, DK-Jan. 5, 2014, and DK-Dec. 4, 2013) from Denmark (Fuursted et al., 2016), 18 *H. influenzae* genomes (strain-1 to strain-18), and Rd KW20 strain downloaded from NCBI via SeqSphere+. MST was built using a modified version of Kruskal's algorithm (Kruskal 1956). Circles indicate samples; numbers on the lines represent the number of loci that change from one strain to another

order of emergence of changes in GyrA and ParC does not influence the quinolone MICs [22]. The GyrB and ParE subunits have ATPase activity; amino acid substitutions in these subunits seem to be involved in quinolone resistance [15]. The amino acid substitution (Asp420 to Asn) identified in *H. influenzae* ParE has a close similarity with the resistance-associated mutation sites in *S. aureus* ParE (Asp432 to His) and in *S. pneumoniae* ParE (Asp435 to Asn) [33, 34]. Nowadays, the prevalence of fluoroquinolone resistance in *H. influenzae* is still low. The average annual FQR isolates at Geneva University Hospitals is less than 2%, which can explain the low number of fluoroquinolone-resistant *H. influenzae* isolates studied here. However, as documented with β-lactams, it is more than likely that the increased usage

of fluoroquinolones in different countries may lead to an increase in fluoroquinolone resistance. Therefore, the surveillance of these strains seems to be necessary.

RND-type multidrug efflux pump AcrAB-TolC constitutes an important contributor to development of multidrug resistance in different bacteria. AcrAB-TolC efflux pump, in which AcrB forms a trimer that functions as a complex with the outer membrane channel TolC and the membrane fusion protein AcrA, is steered by the proton motive force [35]. This chromosomal multidrug efflux pump is negatively regulated by AcrR. To assess the extent of the efflux pump-mediated multidrug resistance, efflux pump inhibitors are widely used. One of these inhibitors is CCCP, which uncouples electron transport from ATP synthesis by dissipating the H+ gradient. In several studies, CCCP has been reported to increase the susceptibility of different multidrug resistant bacteria, including *H. influenzae* [6, 36]. Beta-lactams (e.g., imipenem) are known to induce cell lysis and death by acting on the bacterial cell wall. Thus, the cell wall and cell membrane with embedded efflux pumps constitute an important drug target. One of the objectives of this study was to determinate, by using CCCP, the role of the efflux pump AcrAB-TolC in imipenem resistance of *H. influenzae* isolates with imipenem MICs greater than 32 mg/L. In agreement with our previous study, CCCP decreased the MIC of imipenem by greater than or equal to twofold in FQR-6, GE47, and GE88 isolates, confirming that the efflux pump AcrAB-TolC constitutes a substantial contributor to the development of imipenem resistance in *H. influenzae*.

Shoji Seyama et al. proposed that the resistance to azithromycin in *H. influenzae* emerged from stepwise mutations in the *acr* region; the first step was the acquisition of a nonsense mutation in *acrR*, followed by an amino acid substitution at position 327 (Arg327Ser) in AcrB [28, 37]. For the six FQR isolates, no mutations were found in AcrR and AcrB, but they harbored *ermB* gene plus one amino acid substitution on ribosomal proteins L4 at position 121. Moreover, the susceptibility of these isolates to erythromycin was enhanced after treating cells with CCCP, confirming the role of efflux pump AcrAB-TolC.

Nowadays, WGS has been considered as an ultimate typing tool to assess bacteria outbreaks. cgMLST analyses enabled clustering of FQR1, FQR3, and FQR5 isolates with < 10 loci difference and unambiguous separation from other isolates including the four highly clonal *H. influenzae* strains monoresistant to ciprofloxacin isolated in Denmark. Thus, the cgMLST scheme exhibit higher discriminatory power comparing to other typing method for *H. influenzae*.

The present study has some limitations: a relatively small number of clinical isolates analyzed, and the heterogeneity of imipenem resistance expression was not defined by population analysis profile methods.

Conclusion

The present work investigated the mechanisms of quinolones, macrolides, and β-lactams resistance in *H. influenzae*. We confirmed that specific amino acid substitutions in GyrA and ParC are implicated in quinolone resistance. In addition of the fact that the degree of resistance is linked to the number of these amino acid substitutions. FQR-6 isolates had a single amino acid substitution in GyrA and ParC, and showed a lower increased MIC. Hence, this isolate may constitute the initial stage of quinolone resistance. We confirmed also that drug efflux is one of the important mechanisms accounting for imipenem and erythromycin resistance in *H. influenzae*.

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Funding This study was performed by using internal funding.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Molecular characterization of fluoroquinolones, macrolides and imipenem resistance in *Haemophilus influenzae*: analysis of the mutations in QRDRs and assessment of the extent of the AcrAB-TolC mediated resistance

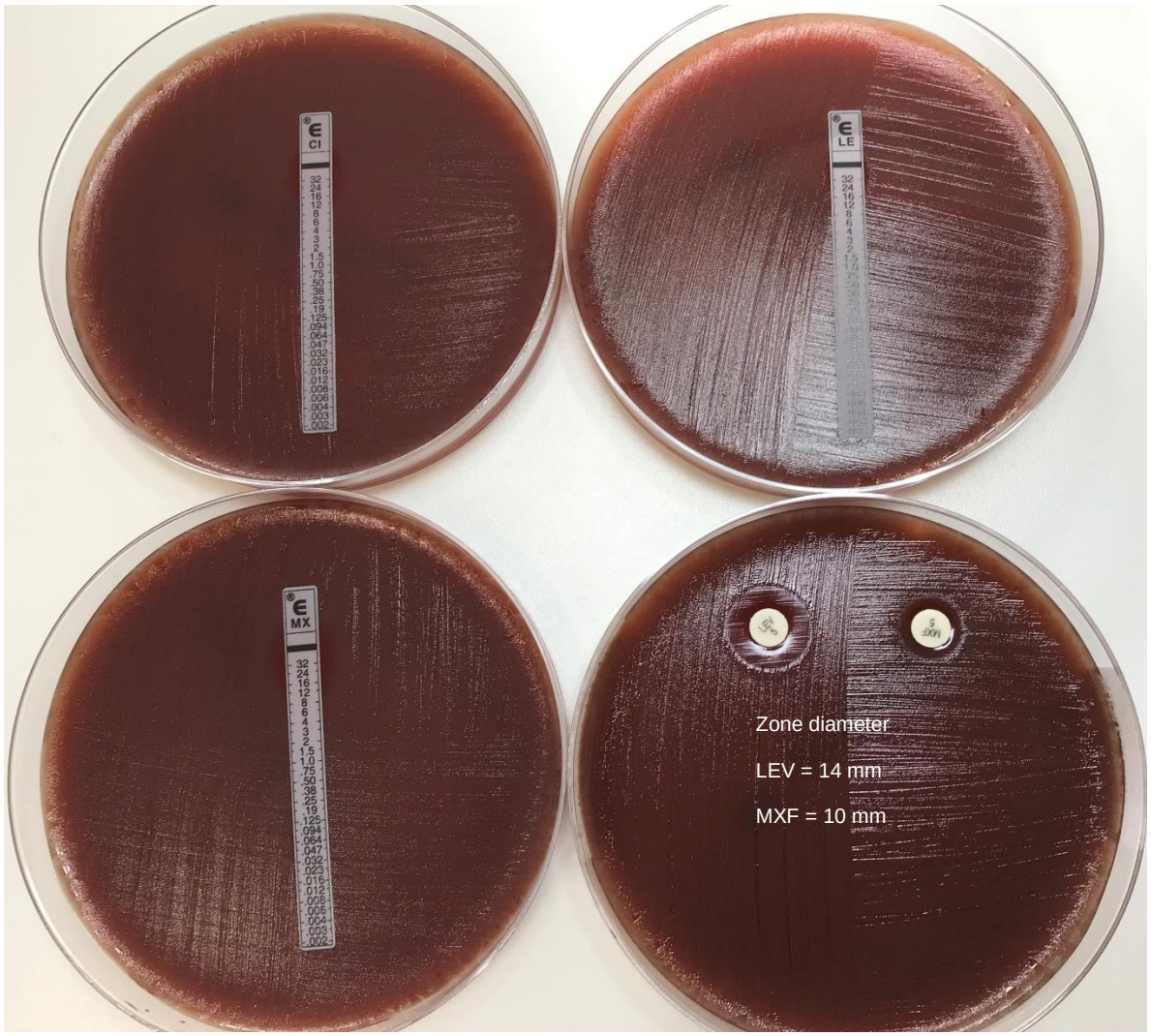
Abdessalam Cherkaoui[1*], Nadia Gaïa[2], Damien Baud[2], Stefano Leo[2], Adrien Fischer[1], Etienne Ruppe[3], Patrice François[2], Jacques Schrenzel[1,2]

[1]Bacteriology Laboratory, Department of Genetics, Laboratory Medicine and Pathology, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

[2]Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

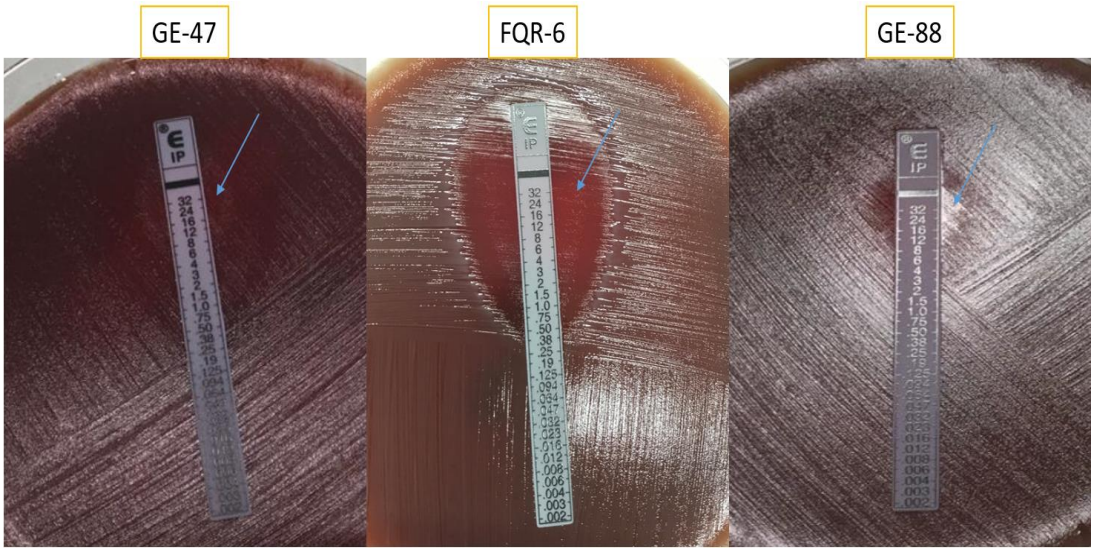
[3]AP-HP, Hôpital Bichat, Laboratoire de Bactériologie, INSERM, IAME, UMR 1137 and Université Paris Diderot, IAME, UMR 1137, Sorbonne Paris Cité, F-75018, Paris, France

Supplementary materials



Supplementary Figure S1: Levofloxacin and moxifloxacin disk diffusion susceptibility and minimum inhibitory concentration (MIC) determination using E-test method for fluoroquinolones resistant *H. influenzae* isolate (**FQR-4**)

Ciprofloxacin (CI), Moxifloxacin (MX and MXF), Levofloxacin (LE and LEV)
EUCAST zone diameter breakpoint:
Levofloxacin (S ≥ 30 mm)
Moxifloxacin (S ≥ 28 mm)



Supplementary Figure S2: Minimum inhibitory concentration (MIC) determination using imipenem E-test strip for FQR-6, GE-47 and GE-88 isolates. The blue arrows indicate the imipenem resistant subpopulations.

The photos of GE-47 and GE-88 isolates were previously published

Strain ID	GyrA, Amino acid substitution for :										
Rd KW20	**S-84**	**D-88**	**K-201**	**H-212**	**A-353**	**S-409**	**A-427**	**G-442**	**E-433**	**I-472**	**A-521**	**D-686**
FQR-1	L	G	N	·	.	A	V	.	.	V	T	.
FQR-2	L	G	N	·	S	A	V	.	D	V	.	N
FQR-3	L	G	N	·	.	A	V	.	.	V	T	.
FQR-4	L	G	N	·	S	A	V	.	D	V	.	N
FQR-5	L	G	N	·	.	A	V	.	.	V	T	.
FQR-6	L	.	N	Y	S	A	V	D	D	V	.	.

Strain ID	GyrB, Amino acid substitution for :							
Rd KW20	**G-80**	**H-218**	**T-573**	**A-606**	**Q-610**	**I-620**	**E-625**	**I-711**
FQR-1	R	.	A	V	·	.	.	T
FQR-2	R	.	A	V	·	.	.	T
FQR-3	R	.	A	V	·	.	.	T
FQR-4	R	.	A	V	·	.	.	T
FQR-5	R	.	A	V	·	.	.	T
FQR-6	R	Y	A	.	K	V	D	.

Strain ID	ParC, Amino acid substitution for :														
Rd KW20	**K-20**	**S-84**	**N-138**	**V-197**	**G-206**	**E-276**	**D-356**	**E-395**	**A-409**	**V-478**	**L-561**	**A-621**	**Y-705**	**A-723**	**T-731**	**P-747**
FQR-1	E	I	S	.	R	D	.	.	V	S	I	T	.	T	.	.
FQR-2	E	I	S	.	.	D	T	.	.	S	.	T	H	T	R	L
FQR-3	E	I	S	.	R	D	.	.	V	S	I	T	.	T	.	.
FQR-4	E	I	S	.	.	D	T	.	.	S	.	T	H	T	R	L
FQR-5	E	I	S	.	R	D	.	.	V	S	I	T	.	T	.	.
FQR-6	E	I	S	I	.	.	A	D	.	S	.	T	.	.	.	.

Strain ID	ParE, Amino acid substitution for :									
Rd KW20	**I-136**	**S-145**	**I-152**	**I-164**	**A-192**	**K-235**	**A-245**	**D-420**	**N-542**	**S-599**
FQR-1	V	N	V	T	.	E	T	.	S	.
FQR-2	V	.	V	T	.	E	T	N	.	A
FQR-3	V	N	V	T	.	E	T	N	S	.
FQR-4	V	.	V	T	.	E	T	N	.	A
FQR-5	V	N	V	T	.	E	T	N	S	.
FQR-6	V	.	V	T	G	E	T	N	S	A

Supplementary Table S1: Amino acid substitutions in each Quinolone Resistance-Determining Regions (QRDRs) and the whole protein sequences.

13. Discussion and perspectives

The important role played by routine Hib vaccination is tangible and should be considered as a major milestone in public health. But the epidemiology of invasive *H. influenzae* infections has undergone major changes in the recent years. NTHi cause invasive (and non-invasive) infections in both, children and older adults. The steady increase of NTHi colonization among frail patients and the presence of coexisting medical conditions (such as chronic respiratory disease) are considered as predisposing factors for invasive NTHi infections. For example, in patients with cystic fibrosis, NTHi belong to the early colonizers of the lungs, suggesting that NTHi might favour bacterial pathogenesis by impairing host cell function and facilitating infections by more virulent pathogens such as *Pseudomonas aeruginosa* (40). The constant increase in the incidence of invasive infections caused by NTHi, associated to the globally widespread BLNAR and BLPACR, emphasizes the importance of a continuous monitoring of β-lactam susceptibility testing. In *H. influenzae*, β-lactamase-negative and β-lactamase-positive strains were recognized as originating from different genotypes (gBLNAR and gBLPACR), which are characterized by mutations in the *ftsI* gene encoding the PBP3 protein. The link between different key mutations in PBP3 and the increase in MICs for various β-lactam antibiotics is now well established. PBP3 in *H. influenzae* is a homologue of PBP2 in *Neisseria* species, sharing 36% amino acid sequence identity (112). It has been proposed that the mosaic structure of PBP2 in *N. gonorrhoeae* and *N. meningitidis* results from the horizontal genetic exchange of the *penA* gene, encoding PBP2, between commensal *Neisseria* species, such as *N. cinerea,* and *N. flavescens* (113). The same scenario was reported in *H. influenzae*. Mosaic structures of the *ftsI* gene were found in clinical isolates of *H. influenzae,* suggesting homologous recombination of the *ftsI* gene between *H. influenzae* and commensal *Haemophilus* species such as *Haemophilus haemolyticus* (112). Hence, it

is more than likely that evolutionary changes of gBLNAR and gBLPACR occurred through homologous recombination from commensal *Haemophilus* species rather than through point mutations in the *ftsI* gene.

The oral cephalosporins are extensively used for the treatment of upper respiratory tract infections in children. BLNAR and BLPACR strains harboring mutations in PBP3 are often resistant to oral cephalosporins such as cefuroxime. The use of these antibiotics with poor activities against these strains not only leads to treatment failure but also enables the selection of the strains harboring the mutated *ftsI* gene. It was previously assumed that carbapenem resistance had not affected NTHi; but we do have now evidence that this is not the case (114). Thus, it is likely that therapeutic failures will be associated with such imipenem resistant strains. Unfortunately, this postulate was confirmed few weeks ago in the intensive care unit of Geneva University Hospitals by documenting the first case of imipenem failure for pneumonia caused by NTHi in a male patient. In this case, the NTHi isolate was resistant to imipenem (MIC = 6 mg/L). Understanding the complexity of the resistance mechanisms should lead to the development of more reliable tests for the routine detection of such strains in clinical microbiology laboratories. Especially as shown in this thesis work, the appearance of imipenem hetero-resistance in NTHi results from the combination between altered PBP3, slowed drug influx and direct efflux regulation. Dissecting such pathways under the heat stress conditions enabled us to identify the heat stress-regulated genes and processes implicated in the improved NTHi susceptibility to imipenem under heat stress conditions. Moreover, we established that drug efflux through AcrAB-TolC must be considered as one of the most important mechanisms involved in *H. influenzae* resistance to imipenem. Even though the broad spectrum β-lactam antibiotics (e.g. imipenem) remain the most commonly prescribed drugs, the information concerning the resistance rate of NTHi to imipenem remains insufficient

and might further be complicated by its association to heterogeneous expression of imipenem resistance. Accordingly, the establishment of standardized methodologies and criteria to detect imipenem heteroresistance in routine clinical microbiology laboratories becomes of paramount importance. In addition, the involvement of other resistance mechanisms (e.g. extended-spectrum β-lactamases, carbapenemases) cannot be excluded and should therefore be routinely monitored.

The possible dissemination of multidrug-resistant NTHi in healthcare settings in Switzerland and abroad constitute a significant threat to modern medicine. Thus, the surveillance represents one of the core actions in the fight against antimicrobial resistance and therapeutic failures. With respect to that subject, inter-institutional and regional collaborations should be established in order to detect and prevent the dissemination of multidrug-resistant NTHI and in some cases specific NTHI clones as documented previously in Denmark (*H. influenzae* clone monoresistant to ciprofloxacin).

In Switzerland, the surveillance of multidrug-resistant on a national level already exists for different micro-organisms (e.g., methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant Enterococci, extended-spectrum beta-lactamases produced by *Enterobacteriaceae,* and carbapenemase-producing *Enterobacteriaceae*) through different organizations:

- The Swiss Centre for Antibiotic Resistance (ANRESIS) which is a wide-ranging and representative surveillance system collecting susceptibility profiles of some relevant bacterial strains isolated in different routine clinical microbiology laboratories across the country.
- The Federal Office of Public Health (FOPH): The purpose of mandatory reporting is to monitor the incidence of invasive organisms (e.g., *H. influenzae*) to evaluate potential vaccine recommendations. As no immediate precautionary

measure is required in the neighborhood of patients, the reporting period is one week.

- The National Emerging Resistance Unit (NARA) represents the national reference center of antibiotic resistance. Its mission is to detect and characterize emerging resistance to antimicrobials.
- The Swiss Antibiogram Committee (SAC), operating under the auspices of the Swiss Society for Microbiology (SSM), aims at testing, promoting and explaining the best practices in antibiotic susceptibility testing for Swiss clinical microbiological laboratories.

Regarding the *H. influenzae*, the existing surveillance systems have some drawbacks:

i. Nowadays, elderly patients (≥65 years of age) affected by invasive *H. influenzae* diseases, caused predominately by NTHi strains, have higher case-fatality rates than children. This information is not visible from ANRESIS that displays only aggregated data.

ii. The lack of homogenous antibiotic susceptibility testing methods in the different routine clinical microbiology laboratories affects proper detection and reporting. In addition, standardized methodologies and criteria to routinely detect imipenem heteroresistance in clinical microbiology laboratories are not yet established. This could be one of the future tasks of the SAC.

iii. Upcoming surveillance system would consider the use of next-generation sequencing (NGS) to characterise invasive *H. influenzae* and establish outbreak dynamics and transmission pathways. A centralized laboratory performing NGS-based typing is currently missing in the Swiss strategy of the FOPH.

iv. A dedicated national reference center on *H. influenzae* is currently missing in Switzerland and could be of paramount importance to perform the phenotypic characterization of the isolates and to consolidate information on molecular

resistance mechanisms as well as on the molecular epidemiology.

13.1. National *H. influenzae* control project

Based on the above information and the results highlighted in this thesis work, I would plan designing a national *H. influenzae* control project with the purpose to improve our knowledge on the epidemiology of invasive *H. influenzae* clinical isolates, to thwart the potential transmission across different healthcare institutions, and to determine targeted infection control interventions for identifying and preventing the dissemination of multidrug-resistant NTHi (and in some cases other specific *H. influenzae*) clones.

13.1.1 Objectives

This national *H. influenzae* control project has several unique features:

i. to identify populations at risk for invasive *H. influenzae diseases*;
ii. to improve and homogenize the antibiotic susceptibility testing methods used for *H. influenzae*;
iii. to assess the heterogeneous resistance of imipenem by using the population analysis method;
iv. to introduce the systematic detection of imipenem heteroresistance;
v. to use Next-Generation Sequencing (NGS) to describe in details the major mechanisms of antibiotic resistance and to assess a core genome multilocus sequence typing (cgMLST) scheme for *H. influenzae* strains isolated in laboratories/healthcare institutions across Switzerland;
vi. To develop a rapid antibiotic susceptibility testing method for invasive *H. influenzae* clinical strains by using WASPLab automation (automated inoculation, and automated incubation combined with timely-defined high-resolution digital imaging). A rapid antibiotic susceptibility testing method will allow the rapid prevention of therapeutic failures in the cases where the empirical antibiotic therapy was inadequate.

13.1.2. Materials and methods

a. Clinical strains of interest

In the post-vaccine era, NTHi was identified as the principle cause of increased invasive *H. influenzae* infections in elderly persons and replaced Hib as the pathogen of primary concern. In addition, several studies in various post-vaccine populations have witnessed a steadily increase of antibiotic resistance in NTHi. Thus, NTHi clinical strains are a high-priority in this project. In order to facilitate progressive capacity building, I propose a stepwise approach and recommend to sequentially implement this national *H. influenzae* control project. For the first step, I plan to analyze only invasive *H. influenzae* strains by using the mandatory reporting setup by Federal Office of Public Health. For the second step, I will extend the analysis to NTHi strains isolated from upper and lower respiratory specimens (e.g. broncho-alveolar lavages and bronchial aspirations). For the last step, I will include the NTHi strains isolated from gynecological specimens.

b. Project concept

Rule-1: University hospitals and private routine clinical microbiology laboratories, which voluntarily participate in this project, will directly send *H. influenzae* isolates to the bacteriology laboratory at Geneva University Hospitals with basic and anonymized epidemiological data (date and place of collection, infection type, age and gender of the patient, and antibiotic susceptibility profile). Isolates should be shipped by using ESwab within no longer than 7 days after detection.

Rule-2: All the experiments will be performed at Geneva University Hospitals. Results will be communicated to the submitting institution and all NGS data will be available to all institutions participating in the project.

Rule-3: In case of a confirmed clone (i.e. match between isolates from the same institution), the institution concerned will be informed as well as the Federal Office of

Public Health. The same approach in case of clustering of strains from different institutions.

c. Antimicrobial susceptibility testing

The antibiotic susceptibility testing will be performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods. The following antibiotics will be tested: penicillin, ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, ceftriaxone, imipenem, meropenem, ciprofloxacin, levofloxacin, and co-trimoxazole. Isolates that display an imipenem MICs >2 mg/mL according to the E-test method will be further analyzed using the population analysis method.

d. Alterations in PBPs and acrR gene will be investigated by NGS

This project will benefit from all the relevant methods used in this thesis work. The genomic DNA will be sequenced using Illumina MiSeq (150 bp paired-end reads) technology (Illumina, San Diego CA, USA). The Illumina sequence quality will be evaluated using the Fastqc program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and filtered using the Fastq-mcf programs (Eautils: http://code.google.com/p/ea-utils/). The genome sequences will be assembled using the Edena v3 assembler. The genome annotation will be performed using the National Center for Biotechnology Information annotation pipeline.

e. Core genome multi-locus sequence typing target genes

The core genome multi-locus sequence typing (cgMLST) scheme will be defined by using Ridom SeqSphere+ software version 5 (Ridom GmbH, Germany). *H. influenzae* Rd KW20 will be chosen as the reference strain.

13.1.3. Epidemiological data

For all clinical isolates, some epidemiologic data such as sample type, age of the patient, date and place of specimen collection (i.e. name of hospital, clinic, and laboratory) will be provided by the participating institution. These epidemiologic data

will be used to identify the potential clustering within the same institution or across different institutions. In addition, they will be required to assess the distribution of invasive NTHi according to the age group of the patients.

13.1.4. Importance of this project

To our knowledge, no other research group is working on *H. influenzae* in Switzerland. In addition, this project is innovative and can be the starting point for the creation of a reference center on *H. influenzae* in Switzerland as is already the case for *Neisseria meningitidis* or *Streptococcus pneumoniae*.

To date, an epidemiological surveillance for *H. influenzae* by genotypic analysis and accurate antibiotic susceptibility testing methods including the assessment of the heterogeneous resistance of imipenem has not been established in Switzerland and these analyses are not mandatory. Furthermore, we do have currently no idea about the frequency of treatment failure caused by imipenem heteroresistant strains.

I am convinced that this project fills an important gap in the surveillance and control of *H. influenzae* in Switzerland. The experience and knowledge acquired during my thesis work applied to a public health question should constitute a sound basis for this project.

13.1.5. Budget

a. Volume

We expect to analyse approximatively 150 strains within the first year, fulfilling the predefined features and permitting capacity building of this project. A rule to cap the number of strains to 150 will be elaborated if the number of collected strains would be significantly larger.

b. Costs

The expected project costs for the upcoming three years will be defined after submission of this project to Federal Office of Public Health and Geneva University Hospitals.

c. Timeline

The pilot phase of this project will last approximatively 6 months (from July to December 2020). A first evaluation of the pilot phase will be scheduled for March 2021. On the basis of experience acquired during the pilot phase, the operating procedures of the project will be adjusted. At the end of the project an exhaustive report will be written to evaluate the real impact of this project and the potential creation of the reference centre of *H. influnezae* at Geneva University Hospitals.

13.2. Rapid antibiotic susceptibility testing method

13.2.1. Disk diffusion susceptibility method

Traditional antimicrobial susceptibility (AST) testing for *H. influenzae* can take 48-72 hours before yielding a result which can be explained by the lack of automation of the test. Unfortunately, the long time needed to perform the AST may lead in infected patients to detrimental outcomes.

13.2.2. Procedure for *H. influenzae* AST by disk diffusion susceptibility method (traditional procedure)

The inoculum suspension is prepared by selecting several colonies from overnight growth (16-24 hours of incubation) on chocolate agar plates and suspending the colonies in sterile saline to the density of a 0.5 McFarland standard. The inoculum is spread over the entire surface of the Müller-Hinton agar plate supplemented with 5% defibrinated horse blood and 20 mg/mL β-NAD (MH-F) and the plates are incubated in a humid atmosphere containing 5% CO_2 at 35 ± 1°C for 18 ± 2 hours. Thereby, the AST results are only available after 18 hours.

13.2.3. Rapid AST directly from blood culture bottles

Last year, EUCAST came up with a list of recommendations for short incubation (4, 6 and 8 hours) AST directly from positive blood culture bottles for *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis* and *Enterococcus faecium.* However, no recommendations were provided for *H. influenzae* despite the steady increase of invasive infections caused by NTHi among frail patients.

13.2.4. The EUCAST rapid AST procedure directly from positive blood culture bottles

(source: http://www.eucast.org/rapid_ast_in_blood_cultures/)

- Direct inoculation of disk diffusion Müller-Hinton agar plates using 100 - 150 µL directly from a positive blood culture bottle.
- Reduced incubation time from 18 ± 2 hours to 4, 6 and 8 hours. The disk diffusion Müller-Hinton agar plates should be interpreted by using the breakpoints adapted to each incubation time.
- The interpretation of rapid AST results will be done after the identification of bacteria species.
- The zone diameters will be read only when an obvious zone edge can be determined. Or else, Müller-Hinton agar plates should be re-incubated and read after 6 or 8 hours.

13.3. *H. influenzae* rapid AST project

13.3.1. Objectives

This project will be divided into two parts:

1. Development of *H. influenzae* rapid AST directly from bacterial colonies.
2. Development of *H. influenzae* rapid AST directly from positive blood culture bottles.

13.3.2. Study design for *H. influenzae* rapid AST directly from bacterial colonies

I plan to adapt for the *H. influenzae* rapid AST project the procedures described previously (115).

In order to identify the shortest incubation times for disk diffusion MH-F plates with optimal analytical performances, I will perform time-series image acquisitions on the WASPLab several hours before and up to the traditional incubation duration specific for *H. influenzae* AST. I plan to compare the results obtained by WASPLab automation (automated inoculation, and automated incubation combined with timely-defined high-resolution digital imaging) against conventional incubation and manual diagnostic, which represents the routine method used in our laboratory. AST analysis for each workflow will be performed by trained clinical microbiologists, blinded of the results obtained by the other method. Results will be compared and optimal imaging times will be defined in the derivation cohort. The assessment between the two methods will be performed on an independent validation cohort using routine clinical *H. influenzae* strains collected in the bacteriology laboratory at Geneva University Hospitals. In the derivation set, the incubation period of disk diffusion MH-F plates on the WASPLab will be assessed at eight incubation time points (4, 6, 8, 10, 12, 14, 16, and 18h). For each incubation time assessed on the WASPLab, several high-resolution digital images will be taken under different light and exposure conditions according to the manufacturer's instructions. For the independent validation set, I will perform the same analysis as for the derivation set but only on the incubation time points and imaging conditions that were selected for their optimal analytical performances. The zone diameters will be read only when an obvious zone edge can be determined. I expect to analyse approximatively 300 *H. influenzae* clinical strains collected in bacteriology laboratory at Geneva University Hospitals.

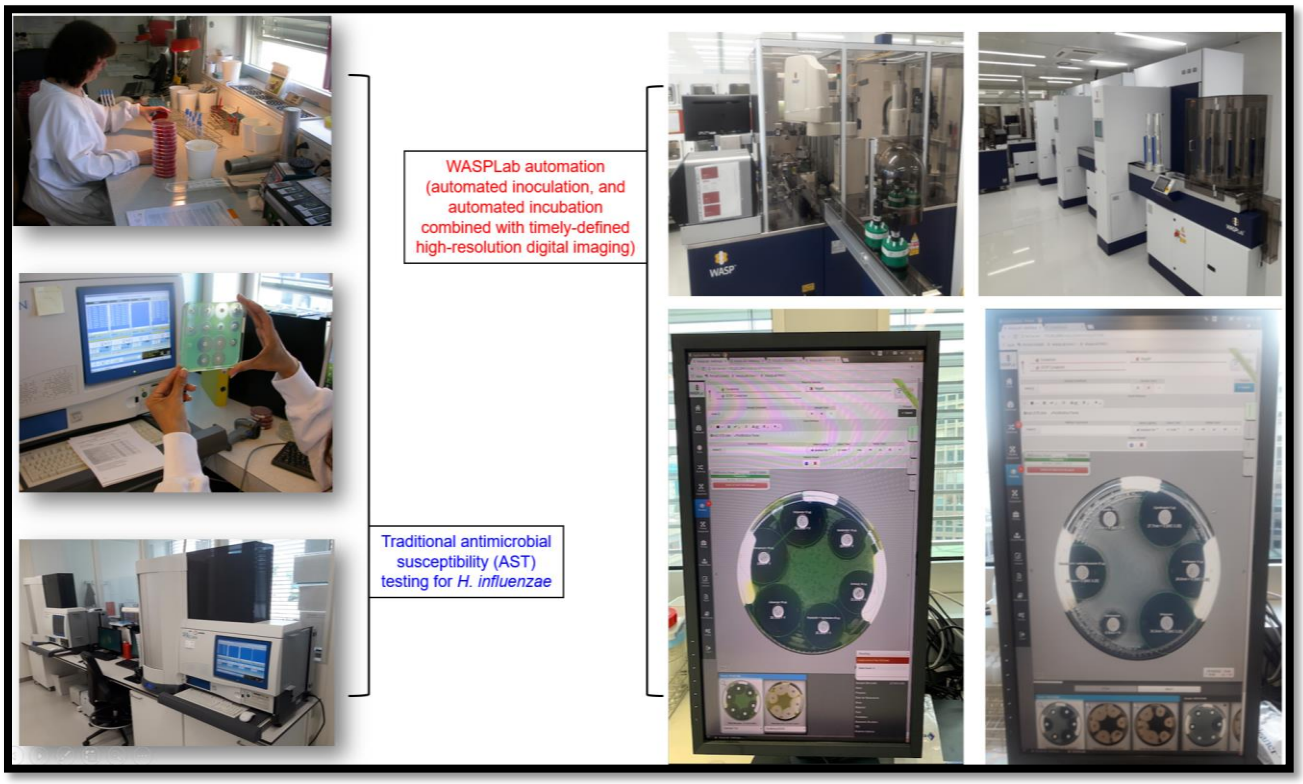


Figure-1: WASPLab automation (automated inoculation, and automated incubation combined with timely-defined high-resolution digital imaging) and traditional AST testing

13.3.3. Study design for *H. influenzae* rapid AST directly from positive blood culture

The evaluation of the *H. influenzae* rapid AST directly from positive blood culture will be performed by spiking 200 *H. influenzae* strains associated with bloodstream infections in 200 negative BD BACTEC™ Plus Aerobic blood culture bottles, in order to mimic positive blood cultures. The inoculum suspensions will be prepared by selecting several colonies from overnight growth on chocolate agar and suspending the colonies in sterile saline to the density of a 0.5 McFarland standard, corresponding to approximately $3x10^8$ CFU/mL. Five hundred microliters of different inoculum suspensions will be injected in negative BD BACTEC™ Plus Aerobic blood culture bottles. Inoculated bottles will be immediately placed in the BD BACTEC™ FX system. Individual blood culture bottles will be removed from the automated blood culture system when growth was detected. One 4mL aliquot were aspirated from each positive bottle with sterile airway needle. The aliquot will be used in WASPLab to perform the

AST according to EUCAST rapid AST procedure directly from positive blood culture bottle. The incubation period of disk diffusion MH-F plates on the WASPLab will be assessed at three incubation time points (4, 6, and 8h). For each incubation time evaluated on the WASPLab, several high-resolution digital images will be taken under different light and exposure conditions according to manufacturer's instructions. The zone diameters will be read only when an obvious zone edge can be defined.

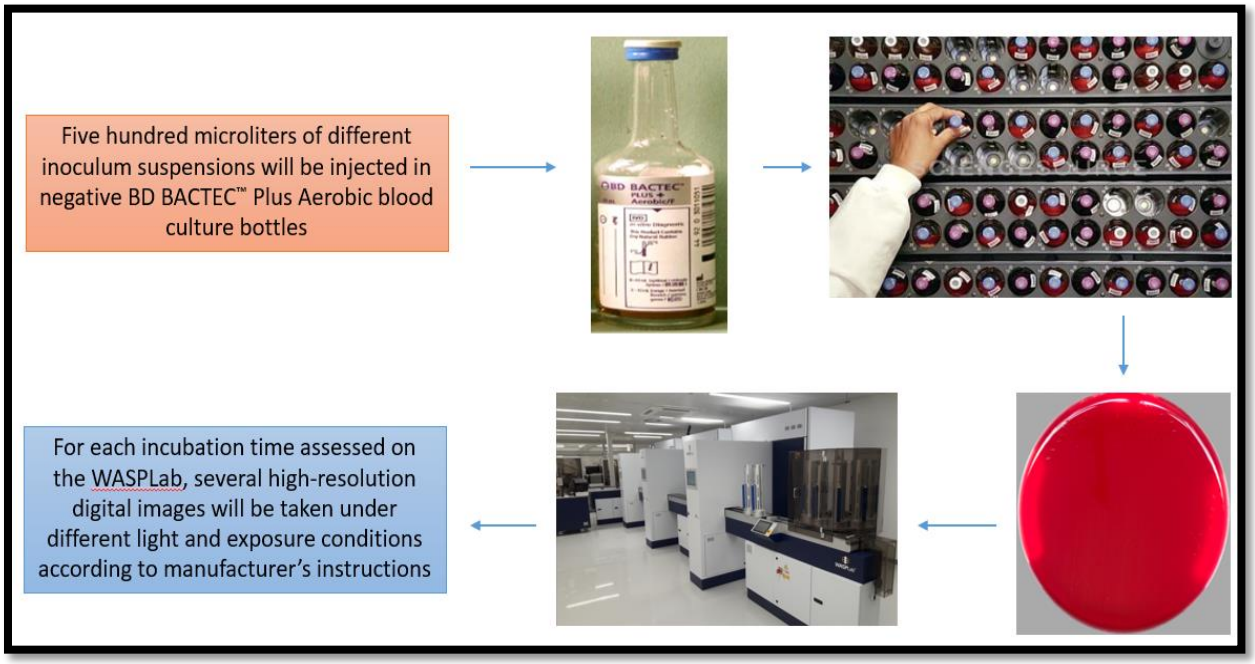


Figure-2: The workflow of *H. influenzae* rapid AST directly from positive blood culture

13.3.4. Importance of the *H. influenzae* rapid AST project

As mentioned previously, in the post-vaccine era, NTHi was recognized as the main cause of increased invasive *H. influenzae* infection like sepsis and meningitis in elderly persons. This increase is coupled to steadily increase of antibiotic resistance in NTHi. This project will enable the antimicrobial susceptibility results in hours instead of days and will allow effective antibiotic stewardship interventions. In addition, this project will apply knowledge acquired during my thesis work to techniques and tools that address critical medical needs linked to *H. influenzae* (translational research).

14. Conclusions

In spite of the many studies showing interesting results, the mechanisms responsible for the heterogeneous expression of resistance have not been fully established. In *S. aureus* for example the *mecA* gene, which encodes a low-affinity PBP2a, is involved in methicillin resistance; nonetheless, as reported previously the autolysins and the presence of multiple unrelated regulatory mechanisms influence the resistance level and the heterogeneous resistance phenotype (116). Likewise, mutations in the *ftsI* gene may be the initial condition for imipenem resistance in *H. influenzae*, but as documented in this thesis work, additional levels of regulation in the efflux system and the drug influx contribute to the heterogeneous expression of resistance. Finally, NTHi small colony variants (SCVs) were shown to arise under imipenem stress. Thus, the mechanisms of SCVs persistence and resistance, as well as the population dynamics of NTHi, will also need to be thoroughly studied, by adding tools that are currently lacking in routine clinical microbiology laboratories.

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Research article

Copan WASPLab automation significantly reduces incubation times and allows earlier culture readings

Abdessalam Cherkaoui, Gesuele Renzi, Nicolas Vuilleumier, Jacques Schrenzel

Authors' contribution statements

Abdessalam CHERKAOUI designed the study and all the experiments. He carried out the experiments, analysed the data, and wrote the manuscript

G. Renzi carried out the experiments and analysed the data

N. Vuilleumier helped in interpreting the results and revised the manuscript

J. Schrenzel supervised the research and revised the manuscript

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Original article

Copan WASPLab automation significantly reduces incubation times and allows earlier culture readings

A. Cherkaoui [1,*], G. Renzi [1], N. Vuilleumier [2,3], J. Schrenzel [1,4]

[1)] Bacteriology Laboratory, Division of Laboratory Medicine, Department of Diagnostics, Geneva University Hospitals, Geneva, Switzerland
[2)] Division of Laboratory Medicine, Department of Diagnostics, Geneva University Hospitals, Geneva, Switzerland
[3)] Division of Laboratory Medicine, Department of Medical Specialities, Faculty of Medicine, Geneva, Switzerland
[4)] Genomic Research Laboratory, Division of Infectious Diseases, Department of Medical Specialities, Faculty of Medicine, Geneva, Switzerland

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ABSTRACT

Objective: The aim was to evaluate whether laboratory automation (inoculation and automated incubation combined with timely defined high-resolution digital imaging) may help reduce the time required to obtain reliable culture analysis results.
Methods: We compared the results obtained by WASPLab automation against WASP-based automated inoculation coupled to conventional incubation and manual diagnostic on 1294 clinical samples (483 for the derivation set and 811 for the independent validation set) that included urine, genital tract and non-sterile site specimens, as well as ESwabs for screening of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), extended-spectrum beta-lactamases (ESBLs) and carbapenemase-producing Enterobacteriaceae (CPE). We used sequential routine specimens referred to the bacteriology laboratory at Geneva University Hospitals between October 2018 and March 2019.
Results: The detection sensitivity of MRSA and MSSA at 18 hr on WASPLab was 100% (95% confidence interval [CI], 94.48–100.00%). The detection sensitivity of ESBL and CPE at 16 hr on WASPLab was 100% (95% confidence interval [CI], 94.87% to 100.00%). For urine specimens, the similarity was 79% (295/375) between 18 hr and 24 hr of incubation on WASPLab. For genital tract and non-sterile site specimens, the similarity between 16 hr and 28 hr of incubation on WASPLab were 26% (72/281) and 77% (123/159) respectively. Thus, 28 hr was defined as the final incubation time on WASPLab for genital tract and non-sterile site specimens.
Conclusions: The results of this study show that WASPLab automation enables a reduction of the culture reading time for all specimens tested without affecting performances. Implementing the established and duly validated incubation times will allow appropriate laboratory workflows for improved efficiency to be built. **A. Cherkaoui, Clin Microbiol Infect 2019;▪:1**

Introduction

Over the last decade, laboratory automation has improved productivity, traceability and quality in clinical chemistry, molecular biology, immunology and haematology laboratories [1,2]. In addition, automation has significantly reduced the time required to obtain the analysis results (i.e. the turn-around time, TAT) [3]. The diversity of clinical specimens and container types, the complexity of the analytical procedures and the variety of the diagnostic methods constituted major hurdles that impaired automation in the clinical microbiology laboratory. However, within less than a decade, the introduction of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for the identification of bacteria, mycobacteria and fungi has fundamentally modified the well-established diagnostic methods in routine microbiology to the point where MALDI-TOF has become the reference standard for microbial identification [4–6]. Nowadays, two automated instrument systems are currently available for clinical specimen streaking; inoculated media are loaded on

* Corresponding author. A. Cherkaoui, Bacteriology Laboratory, Division of Laboratory Medicine, Department of Diagnostics, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland.
E-mail address: abdessalam.cherkaoui@hcuge.ch (A. Cherkaoui).

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conveyors for transfer between instruments and automated incubators where cultures are read with high-resolution digital imaging at pre-defined times. Work Cell Automation (WCA) and Total Lab Automation (TLA) has been developed by BD Kiestra (Drachten, The Netherlands), while WASPLab has been developed by Copan WASP srl, (Brescia, Italy). These systems are built around fundamental techniques used in the clinical microbiology laboratory, namely growing bacterial colonies on agar media plates. New features are now available such as the pre-sorted segregation of agar plates based on colony counts with growth and no growth discrimination, as well as automated image analysis to interpret chromogenic media plate results (e.g. WASPLab software is capable of reading chromogenic plates to detect methicillin-resistant *Staphylococcus aureus* (MRSA) with high sensitivity) [7,8]. Additionally, both automation system manufacturers claim that bigger technological breakthroughs are coming soon.

Rapid microbiology diagnosis associated with antimicrobial stewardship has a perceptible effect on patient management and care [9]. One of the most important issues in clinical microbiology is therefore the rapid identification of critical antibiotic-resistant pathogens that impose infection control procedures, such as MRSA, vancomycin-resistant *Enterococcus* (VRE), extended-spectrum beta-lactamases (ESBL) and carbapenemase-producing Enterobacteriaceae (CPE). Moreover, the need for rapid microbiology results in defining infections that can be better managed, using narrow spectrum drugs or early oral administration, has spurred the development of products and concepts which are integrated in automated instrument systems.

In this study, we assessed whether the use of WASPLab automation (automated inoculation, and automated incubation combined with timely defined high-resolution digital imaging) may help reduce the time required to obtain reliable culture results.

Material and methods

Setting

This study was conducted at Geneva University Hospitals, a Swiss tertiary care centre with 1920 beds, and about 63 000 yearly admissions. The hours of operation of our clinical bacteriology laboratory are from 7.30 to 22.00 from Monday to Friday, 7.30 to 17.00 on Saturday and 7.30 to 13.00 on Sunday in addition to the on-call service until 22.00 during the weekend.

Study design

In order to identify the shortest incubation times for agar media plates with optimal analytical performances, we performed time-series image acquisitions on WASPLab several hours before and up to the traditional incubation duration specific for each specimen type. We analysed selective chromogenic plates for the screening-ESwabs for MRSA, methicillin-sensitive *Staphylococcus aureus* (MSSA), extended-spectrum beta-lactamases (ESBLs) and carbapenemase-producing Enterobacteriaceae (CPE). We also assessed urine, and genital tract and non-sterile site specimens to differentiate the presence of pathogens from that of a normal flora. We compared the results obtained by WASPLab against WASP-based inoculation coupled to conventional incubation and manual diagnostic, which represents the routine method used in our laboratory. Culture media analysis for each workflow was performed by trained clinical microbiologists blinded to the results obtained by the other method. Results were compared and optimal imaging times were defined in the derivation cohort. The assessment between the two methods was then performed on an independent validation cohort, again using routine clinical samples sequentially referred to the bacteriology laboratory at Geneva University Hospitals between October 2018 and March 2019. In the derivation set, the incubation period on WASPLab was assessed at different incubation time points covering the full traditional incubation period, specific for each analysis and specimen type included in this study. For each incubation time assessed on WASPLab, several high-resolution digital images were taken under different light and exposure conditions according to the manufacturer's instructions, and analysed by the first author (a clinical microbiologist) and one expert medical laboratory technologist. Both had been trained by COPAN's application specialist. For the independent validation set, we performed the same analysis as for the derivation set but only on the incubation time points and imaging conditions that were selected for their optimal analytical performances.

Conventional diagnostic work-up

The identification of bacterial and yeast colonies was performed by matrix-assisted laser desorption ionization time-of-flight mass

Table 1
The incubation protocols, the culture media used for each specimen type, and the number of specimens included in the derivation set and in the independent validation set

Clinical specimen types	WASP coupled to conventional incubation and manual diagnostic		WASPLab	
	Culure media type	Routine incubation period	Number of samples included in the derivation set	Number of samples included in the independant validation set
Urine specimens	CHROMID® CPS® Elite (BioMérieux, Geneva, Switzerland)	18 hr to 24 hr and 48 hr	109	266
Genital tract specimens	Blood agar, chocolate agar, CNA agar, and MacConkey agar	24 hr and 48 hr	92	189
Non-sterile site specimens	Blood agar, chocolate agar, CNA agar, and MacConkey agar	24 hr, 48 hr and 72 hr	50	109
Nasal and inguinal/perineal screening-ESwabs for MRSA and MSSA	CHROMID® MRSA (BioMérieux) and SaSelect Medium (BioRad)	18 hr to 24 hr and 48 hr	148	181
Rectal screening-ESwabs for ESBL-producer and CPE	CHROMID® ESBL (BioMérieux) coupled to CHROMID® OXA-48 (BioMérieux)	18 hr to 24 h and 48 hr	84	66
		Total	483	811

CPE, carbapenemase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; ESBL, extended-spectrum beta-lactamases; CNA agar, colistin-nalidixic Acid agar.

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Table 2
Results of the derivation and validation sets for nasal and inguinal/perineal screening ESwabs for Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-sensitive *Staphylococcus aureus* (MSSA)

Derivation dataset					
Nasal and inguinal/perineal screening ESwabs for MRSA and MSSA	WASP coupled to conventional incubation and manual diagnostic		WASPLab		
	Incubation time points		Incubation time points		
Pathogenes type (no. of samples / semi-quantification)	18 hr to 24 hr	48 hr	18 hr	22 hr	48 hr
MRSA negative samples (114)	—	—	—	—	—
MRSA (12 / + to +++)	+	+	+	+	+
MSSA negative samples (18)	—	—	—	—	—
MSSA (2 / + to +++)	+	+	+	+	+
MSSA (2 / +)	—	—	+	+	+
Total = 148					

Independent validation dataset			
Nasal and inguinal/perineal screening-ESwabs for MRSA and MSSA	WASP coupled to conventional incubation and manual diagnostic		WASPLab
	Incubation time points		Incubation time
Pathogenes type (no. of samples / semi-quantification)	18 hr to 24 hr	48 hr	18 hr
MRSA negative samples (123)	—	—	—
MRSA (24 / + to +++)	+	+	+
MSSA negative samples (9)	—	—	—
MSSA (24 / + to +++)	+	+	+
MSSA (1 / +)	—	—	+
Total = 181			

—, negative; +, positive.

spectrometry (MALDI-TOF MS; compass, Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions. The presence of ESBL was confirmed by double-disc synergy tests (DDST20 and DDST30). The presence of a carbapenemase was confirmed by the Eazyplex® SuperBug CRE system (Amplex Biosystems GmbH, Giessen, Germany). Confirmation of MRSA and MSSA strains was performed by a previously published qPCR assay targeting *femA* and *mecA* [10]. Table 1 details the incubation protocols, the agar media plates used for each sample type and the number of samples included in the derivation set and in the independent validation set.

Results

Nasal and inguinal/perineal screening-ESwabs for MRSA and MSSA

Derivation dataset

As depicted in Table 2, all the 14 positive clinical specimens (12 MRSA and two MSSA) had already been detected at 18 hr, with the specific colour on CHROMID® MRSA (BioMérieux, Geneva, Switzerland) or SaSelect medium for MSSA (BioRad, Fribourg, Switzerland) when the chromogenic media were incubated on

Table 3
Results of the derivation and validation sets for rectal screening-ESwabs for ESBL-producer and CPE

Derivation dataset						
Rectal screening-ESwabs for ESBL-producer and CPE	WASP coupled to conventional incubation and manual diagnostic		WASPLab			
	Incubation time points		Incubation time points			
Pathogenes type (no. of samples / semi-quantification)	18 hr to 24 hr	48 hr	16 hr	18 hr	22 hr	48 hr
ESBL and CPE negative samples (52)	—	—	—	—	—	—
Escherichia coli ESBL (23 / + to +++)	+	+	+	+	+	+
Klebsiella pneumoniae ESBL (5 / + to +++)	+	+	+	+	+	+
Salmonella Typhimurium ESBL (1 / +++)	+	+	+	+	+	+
Escherichia coli OXA-48 (1 / +++)	+	+	+	+	+	+
Klebsiella pneumoniae OXA-48 (1 / +++)	+	+	+	+	+	+
Klebsiella pneumoniae NDM (1 / +++)	+	+	+	+	+	+
Total = 84						

Independent validation dataset			
Rectal screening-ESwabs for ESBL-producer and CPE	WASP coupled to conventional incubation and manual diagnostic		WASPLab
	Incubation time points		Incubation time
Pathogenes type (N° of samples / semi-quantification)	18h - 24h	48h	16h
ESBL and CPE negative samples (28)	—	—	—
Escherichia coli ESBL (18 / + to +++)	+	+	+
Klebsiella pneumoniae ESBL (11 / + to +++)	+	+	+
Enterobacter cloacae complex ESBL (1 / +++)	+	+	+
Escherichia coli OXA-48 (1 / +++)	+	+	+
Klebsiella pneumoniae OXA-48 (4 / ++ to +++)	+	+	+
Klebsiella pneumoniae OXA-181 (2 / +++)	+	+	+
Escherichia coli NDM (1 / +++)	+	+	+
Total = 66			

ESBL, extended-spectrum beta-lactamases; CPE, carbapenemase-producing *Enterobacteriaceae*
—, negative; +, positive

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WASPLab. No difference was observed between 18 hr, 22 hr and 48 hr for both the presence and the semi-quantification of MSSA or MRSA. Among the 148 specimens used for the derivation set, 89% (132/148) were negative using the two compared methods. In two cases, the specimens were validated as MSSA negative by the manual method at either 18 hr, 24 hr or 48 hr. In contrast, when SaSelect medium was incubated on WASPLab, these two cases showed a few colonies had already been detected with the specific colour at 18 hr. MSSA was confirmed positive by qPCR (yielding only a *femA* positive signal).

Independent validation dataset

The cut-off point for MRSA and MSSA screening ESwabs on WASPLab was defined as 18 hr using the derivation set. This incubation time point was validated on another 181 clinical samples, of which 13% (24/181) were MRSA positive and 13% (24/181) were MSSA positive. All the positive specimens were detected at 18 hr on WASPLab, except one sample validated as MSSA negative using the manual method but a few colonies were detected at 18 hr on WASPLab with the specific colour on the SaSelect medium (Table 2). This discrepancy can be explained by the fact that the SaSelect

Table 4
Results of the derivation and validation sets for bacteriological examination of urine specimens

Derivation dataset							
Bacteriological examination of urine specimens	WASP coupled to conventional incubation and manual diagnostic		WASPLab				
	Incubation time points		Incubation time points				
Pathogenes type and flora (no. of samples / Quantification)	18 hr to 24 hr	48 hr	16 hr	18 hr	22 hr	24 hr	26 hr up to 48 hr
Negative samples (21)	—	—	—	—	—	—	—
Escherichia coli (21 / 100 to >100000 CFU/mL)	+	+	+	+	+	+	+
Proteus mirabilis (2 / 100 000 CFU/mL)	+	+	+	+	+	+	+
Klebsiella pneumoniae (1 / 100 000 CFU/mL)	+	+	+	+	+	+	+
Pseudomonas aeruginosa (1 / 100 000 CFU/mL)	+	+	+	+	+	+	+
Candida albicans (9 / 1000 to >100 000 CFU/mL)	—	+	—	—	—	+	+
Candida glabrata (3 / 1000 to >100 000 CFU/mL)	—	+	—	—	—	+	+
Cyberlindnera fabianii (1 / 100 000 CFU/mL)	—	+	—	—	—	+	+
Enterococcus faecalis (5 / 1000 to 100 000 CFU/mL)	+	+	—	+	+	+	+
Streptococcus agalactiae (3 / 10 000 to >100 000 CFU/mL)	—	+	—	+	+	+	+
Mixt flora (9 / 1000 to >100000 CFU/mL)	+	+	+	+	+	+	+
Gram positive flora (18 / 100 to 1000 CFU/mL)	—	+	—	—	+	+	+
Gram positive flora (8 / 10 000 to >100 000 CFU/mL)	+	+	—	+	+	+	+
Lactobacillus sp. (5 / 10000 to >100 000 CFU/mL)	—	+	—	+	+	+	+
Staphylococcus epidermidis (2 / 10 000 to 100000 CFU/mL)	+	+	—	+	+	+	+
Total = 109							

Independent validation dataset				
Bacteriological examination of urine specimens	WASP coupled to conventional incubation and manual diagnostic		WASPLab	
	Incubation time points		Incubation time points	
Pathogenes type and flora (no. of samples / Quantification)	18 hr to 24 hr	48 hr	18 hr	24 hr
Negative samples (30)	—	—	—	—
Escherichia coli (57 / 100 to >100 000 CFU/mL)	+	+	+	+
Klebsiella pneumoniae (24 / 100 to >100 000 CFU/mL)	+	+	+	+
Klebsiella oxytoca (2 / 1000 CFU/mL)	+	+	+	+
Klebsiella aerogenes (4 / 1000 to >100 000 CFU/mL)	+	+	+	+
Proteus mirabilis (9 / 100 to 100 000 CFU/mL)	+	+	+	+
Morganella morganii (1 / 100 000 CFU/mL)	+	+	+	+
Citrobacter koseri (3 / 1000 to >100 000 CFU/mL)	+	+	+	+
Acinetobacter sp. (1 / 1000 CFU/mL)	+	+	+	+
Achromobacter xylosoxidans (1 / 10000 CFU/mL)	+	+	+	+
Pseudomonas aeruginosa (9 / 100 to >100 000 CFU/mL)	+	+	+	+
Candida albicans (8 / 100 to 10 000 CFU/mL)	—	+	—	+
Candida glabrata (2 / 1000 and >100 000 CFU/mL)	—	+	—	+
Methicillin-resistant *Staphylococcus aureus* (2 / 100 000 CFU/mL)	+	+	+	+
Methicillin sensitive *Staphylococcus aureus* (2 / 1000 CFU/mL)	+	+	+	+
Staphylococcus epidermidis (2 / 100 000 CFU/mL)	+	+	+	+
Staphylococcus hominis (2 / 100 and 10 000 CFU/mL)	+	+	+	+
Enterococcus faecalis (28 / 1000 to >100 000 CFU/mL)	+	+	+	+
Enterococcus faecium (2 / 10 000 CFU/mL)	+	+	+	+
Streptococcus agalactiae (6 / 100 to 10 000 CFU/mL)	—	+	+	+
Aerococcus urinae (3 / 10 000 to >100 000 CFU/mL)	—	+	—	+
Mixt flora (13 / 1000 to >100 000 CFU/mL)	+	+	+	+
Gram positive flora (36 / 100 to 1000 CFU/mL)	—	+	—	+
Gram positive flora (13 / 10 000 to >100 000 CFU/mL)	+	+	+	+
Lactobacillus sp. (4 / 10 000 to >100 000 CFU/mL)	—	+	+	+
Lactobacillus johnsonii (1 / 10 000 CFU/mL)	—	+	+	+
Corynebacterium sp. (1 / 1000 CFU/mL)	—	+	+	+
Total = 266				

—, negative; +, positive

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medium is highly sensitive to light exposure, favouring WASPLab, which allows immediate incubation after streaking.

The detection sensitivity of MRSA and MSSA at 18 hr on WASPLab compared with the manual method for the 329 specimens included in the derivation and the validation sets was 100% (95% CI 94.48–100.00%).

Rectal screening ESwabs for ESBL producer and CPE

Derivation dataset

The incubation period was assessed at 16 hr, 18 hr, 22 h and 48 hr on WASPLab. Among the 84 clinical samples included in the derivation set, 62% (52/84) were ESBL and CPE negative using the two compared methods. At 16 hr on WASPLab, 29 samples were detected ESBL positive and three CPE positive (Table 3), which reached optimal detection sensitivity (100%/95% CI 89.11–100.00%) compared with the manual method.

Independent validation dataset

We selected 16 hr as the defined incubation period on WASPLab. Sixty-six independent clinical specimens were included in the validation set, of which 28 samples were ESBL and CPE negative, 30 specimens were ESBL positive and eight specimens were CPE positive (Table 3). For the derivation set, all the 38 ESBL- and CPE-positive specimens were detected at 16 hr on WASPLab.

The detection sensitivity of ESBL and CPE at 16 hr on WASPLab compared with the manual method for the 150 specimens included in the derivation and the validation sets was 100% (95% CI, 94.87–100.00%).

Bacteriological examination of urine specimens

Derivation dataset

Among the 109 urine samples included in the derivation set, 19% (21/109) were negative by the two compared methods. The incubation period for urine samples was explored on WASPLab at 11 different incubation time points (from 16 hr up to 48 hr). In 24 samples, the common Enterobacteriaceae uropathogens and *Pseudomonas aeruginosa* were detected on CHROMID® CPS® Elite at 16 hr. In 13 samples, it would have been necessary to wait until 24 hr of incubation on CHROMID® CPS® Elite to have sufficient growth for *Candida* and *Cyberlindnera fabianii* detection. The optimal incubation time to have sufficient growth of Gram positive bacteria, including uncommon uropathogens and flora, was determined as 18 hr (Table 4).

Independent validation dataset

We defined on WASPLab for CHROMID® CPS® Elite an intermediate incubation time at 18 hr and final incubation period at 24 hr. At 18 hr on WASPLab, among the 266 specimens included in the independent validation set, 38% (100/266) were positive for common Enterobacteriaceae uropathogens, 24% (63/266) were positive for various Gram-positive bacteria including uncommon uropathogens and flora, and 4% (11/266) were positive for *Pseudomonas aeruginosa*, *Acinetobacter* sp. or *Achromobacter xylosoxidans*. In contrast, 24 hr was necessary for CHROMID® CPS® Elite in order to identify *Candida albicans, Candida glabrata* and *Aerococcus urinae* (Table 4). Therefore, 24 hr is validated as the cut-off point providing optimal analytical performances.

Table 5
Results of the derivation and validation sets for bacteriological examination of genital tract specimens

Derivation dataset							
Bacteriological examination of genital tract specimens	WASP coupled to conventional incubation and manual diagnostic		WASPLab				
	Incubation time points		Incubation time points				
Pathogenes type and flora (no. of samples / semi-quantification)	24 hr	48 hr	16 hr	18 hr	20 hr	28 hr	30 hr up to 48 hr
Vaginal flora (57 / + to +++)	—	+	—	+	+	+	+
Gardnerella vaginalis (1 / +++)	—	+	—	—	+	+	+
Methicillin-sensitive *Staphylococcus aureus* (3 / + to +++)	+	+	+	+	+	+	+
Methicillin-resistant *Staphylococcus aureus* (1 / +++)	+	+	+	+	+	+	+
Enterobacteriaceae (6 / + to +++)	+	+	+	+	+	+	+
Streptococcus agalactiae (4 / + to +++)	+	+	+	+	+	+	+
Enterococcus faecalis (5 / + to +++)	+	+	+	+	+	+	+
Candida albicans (14 / + to +++)	—	+	—	—	—	+	+
Candida glabrata (1 / +)	—	+	—	—	—	+	+
Total = 92							

Independent validation dataset				
Bacteriological examination of genital tract specimens	WASP coupled to conventional incubation and manual diagnostic		WASPLab	
	Incubation time		Incubation time points	
Pathogenes type and flora (no. of samples / semi-quantification)	24 hr	48 hr	16 hr	28 hr
Vaginal flora (86 / + to +++)	—	+	—	+
Gardnerella vaginalis (14 / +++)	—	+	—	+
Methicillin-sensitive *Staphylococcus aureus* (9 / + to +++)	+	+	+	+
Enterobacteriaceae (28 / + to +++)	+	+	+	+
Streptococcus agalactiae (9 / + to +++)	+	+	+	+
Streptococcus pyogenes (1 / +++)	+	+	+	+
Enterococcus faecalis (6 / + to +++)	+	+	+	+
Candida krusei (1 / +++)	—	+	—	+
Candida dubliniensis (1 / ++)	—	+	—	+
Candida albicans (30 / + to +++)	—	+	—	+
Candida glabrata (4 / +)	—	+	—	+
Total = 189				

—, negative; +, positive

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Bacteriological examination of genital tract specimens

Derivation dataset

The incubation period on WASPLab was divided into several incubation time points (from 16 hr up to 48 hr). Among the 92 samples analysed in the derivation set, 62% (57/92) were positive with only vaginal flora as early as 18 hr. Three per cent (3/92) were positive for MSSA, 1% (1/92) for MRSA, 7% (6/92) for *Enterobacteriaceae*, 4% (4/92) for *S. agalactiae* and 5% (5/92) for *E. faecalis*. All these micro-organisms showed sufficient growth at 16 hr. One sample was positive for *Gardnerella vaginalis* with sufficient growth at 20 hr. However, the incubation period had to be prolonged up to 28 hr to permit sufficient growth for *Candida* spp. (Table 5).

Independent validation dataset

We defined an intermediate incubation time on WASPLab of 16 hr and the final incubation period of 28 hr for the 189 specimens included in the validation set. Except for *Candida* spp. and *Gardnerella vaginalis*, all potential pathogens were identified at 16 hr, indicating the importance of this intermediate incubation period, to provide early diagnostic information. However, we had to extend the incubation period to 28 hr in order to permit the reliable identification of *Candida* spp. and other pathogens like *Gardnerella vaginalis* (Table 5).

Bacteriological examination of non-sterile site specimens

Non-sterile site specimens included in this study were conjunctival ESwab (6%, 10/159), ear ESwab (16%, 25/159) and superficial ESwab specimens (78%, 124/159).

Derivation dataset

The incubation period on WASPLab was divided into several incubation time points (from 16 hr up to 72 hr). At 16 hr, among the

Table 6
Results of the derivation and validation sets for bacteriological examination of non-sterile site specimens

Derivation dataset							
Bacteriological examination of non-sterile site specimens	WASP coupled to conventional incubation and manual diagnostic			WASPLab			
	Incubation time points			Incubation time points			
Pathogenes type and flora (N° of samples / semi-quantification)	24 hr	48 hr	72 hr	16 hr	18 hr	20 hr	28 hr up to 72 hr
Negative samples (6)	—	—	—	—	—	—	—
Mixt flora (4 / + to +++)	+	+	+	+	+	+	+
Gram positive flora (6 / + to +++)	—	+	+	—	+	+	+
Methicillin-sensitive *Staphylococcus aureus* (12 / + to +++)	+	+	+	+	+	+	+
Staphylococcus epidermidis (3 / +++)	+	+	+	+	+	+	+
Staphylococcus lugdunensis (1 / +++)	+	+	+	+	+	+	+
Escherichia coli (2 / + to ++)	+	+	+	+	+	+	+
Klebsiella pneumoniae (1 / +)	+	+	+	+	+	+	+
Klebsiella aerogenes (3 / +++)	+	+	+	+	+	+	+
Acinetobacter baumannii (1 / +++)	+	+	+	+	+	+	+
Pseudomonas aeruginosa (5 / +++)	+	+	+	+	+	+	+
Streptococcus pyogenes (1 / +++)	+	+	+	+	+	+	+
Streptococcus agalactiae (2 / ++)	+	+	+	+	+	+	+
Enterococcus faecalis (1 / +)	+	+	+	+	+	+	+
Candida glabrata (2 / +)	—	+	+	—	—	—	+
Total = 50							

Independent validation dataset					
Bacteriological examination of non-sterile site specimens	WASP coupled to conventional incubation and manual diagnostic			WASPLab	
	Incubation time points			Incubation time points	
Pathogenes type and flora (N° of samples / semi-quantification)	24h	48h	72h	16h	28h
Negative samples (6)	—	—	—	—	—
Mixt flora (7 / + to +++)	+	+	+	+	+
Gram positive flora (22 / + to +++)	+	+	+	—	+
Methicillin-sensitive *Staphylococcus aureus* (18 / + to +++)	+	+	+	+	+
Staphylococcus lugdunensis (1 / ++)	+	+	+	+	+
Staphylococcus epidermidis (5 / ++)	+	+	+	+	+
Staphylococcus warneri (4 / +++)	+	+	+	+	+
Escherichia coli (6 / ++ to +++)	+	+	+	+	+
Klebsiella pneumoniae (8 / + to ++)	+	+	+	+	+
Klebsiella aerogenes (2 / +++)	+	+	+	+	+
Citrobacter koseri (1 / +++)	+	+	+	+	+
Morganella morganii (1 / +++)	+	+	+	+	+
Serratia marcescens (1 / ++)	+	+	+	+	+
Proteus mirabilis (2 / +++)	+	+	+	+	+
Stenotrophomonas maltophilia (1 / ++)	+	+	+	+	+
Pseudomonas aeruginosa (7 / +++)	+	+	+	+	+
Streptococcus pyogenes (1 / +++)	+	+	+	+	+
Streptococcus agalactiae (2 / +++)	+	+	+	+	+
Streptococcus dysgalactiae (2 / ++)	+	+	+	+	+
Enterococcus faecalis (6 / +)	+	+	+	+	+
Candida glabrata (1 / +++)	—	+	+	—	+
Candida albicans (5 / + to +++)	—	+	+	—	+
Total = 109					

Non-sterile site specimens included in this study were conjunctival-ESwab (6%, 10/159), ear-ESwab (16%, 25/159), and superficial-ESwab specimens (78%, 124/159). —, negative; +, positive

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50 specimens included in the derivation set, 12% (6/50) were negative, 24% (12/50) were positive for MSSA, 16% (8/50) were positive for other common Gram-positive pathogens, 12% (6/50) were positive for *Enterobacteriaceae* and 12% (6/50) were positive for *P. aeruginosa* and *Acinetobacter baumannii* (Table 6). Two samples were positive for *Candida glabrata* with sufficient growth for reliable identification at 28 hr.

Independent validation dataset

As for the genital tract, we have chosen an intermediate incubation time at 16 hr and a final incubation period at 28 hr. In the validation set we included 109 specimens. As depicted in Table 6, a panel of 10 potential pathogens was identified with sufficient growth at the intermediate incubation time without any difference with the final traditional incubation period. The detection of *Candida glabrata* was validated at 28 hr.

Discussion

The purpose of this study was to assess if the use of WASPLab, and in particular that of the automated incubators coupled to digital imaging, permitted to shorten the time required for obtaining reliable culture analysis results. As summarized in Table 7, the use of WASPLab allows reducing the length of the incubation time for urine, genital tract and non-sterile site specimens, as well as that for screening for MRSA, MSSA, ESBL and CPE without affecting the analytical performances.

In a recent study, Bielli *et al.* [11] found a similarity of 93% when comparing the urine specimens tested by WASPLab at 16 hr and 24 hr of incubation. In contrast, our derivation study found only 50% (55/109) similarity between those incubation times. Additionally, for all the urine specimens tested the similarity was 79% (295/375) between 18 hr and 24 hr on WASPLab by using CHROMID® CPS® Elite. The selection of a 24 hr incubation time as the final incubation period for urine specimens was dictated by the willingness to improve the detection yield of urine specimens that are potentially contaminated prior to culture, which contributes to limit overdiagnosis of urinary tract infections. For genital tract specimens, the similarity was 31% (72/230) for detecting bacteria between 16 hr and 28 hr of incubation on WASPLab. This similarity decreases to 26% (72/281) when we include *Candida* spp., whose slower growth requires more time to be detected. For non-sterile site specimens, the similarity was 77% (123/159) between 16 hr and 28 hr of incubation on WASPLab. Finally, a unique 16 hr incubation time is sufficient for the screening-ESwabs for ESBL-producer and CPE, and 18 hr incubation time for the screening-ESwabs for MRSA, which enables rapid identification of critical antibiotic-resistant pathogens for adjusting infection control procedures.

Clinical microbiology laboratories rely upon highly trained and skilled personnel to process a substantial amount of clinical specimens with various complex procedures, a range of sampling devices and a variety of diagnostic methods. The advent of new technologies emphasizes the need to automate the repetitive tasks that do not require specific skills of trained medical microbiology technologists [1,3,12]. The impact of automation to improve laboratory workflows and efficiency in clinical microbiology laboratories was highlighted in different recent publications [13–18]. As demonstrated in this study, the time required to obtain culture results is reduced by automated incubation combined with high-resolution digital imaging. To realize the maximum time gain of reduced incubation times, the clinical microbiology laboratory would need to broaden its operating hours, and ideally shift to a 24/7 model. This schedule would ensure that the images of the early incubation times can be interpreted without any delay. Hours of operating should be adjusted, in each centre, to ideally match laboratory resources with the decisions that can actually be taken by the medical staff working during night or weekend shifts. Defined and duly validated incubation times will allow building appropriate laboratory workflows for improved efficiency.

One limitation of this study pertains to the relatively small number of some bacterial species analysed as well as the overall number of samples studied, yet the specimens analysed in this study provide a range of pathogens and flora conditions.

Table 7
Definitive incubation protocoles based on the derivation and validation studies

Clinical samples type	WASPLab		
	Incubation time		
	Picture at T0	Intermediate incubation time, hr	Final incubation time, hr
Urine specimens	Yes	18	24
Genital tract specimens	Yes	16	28
Non-sterile site specimens	Yes	16	28
Nasal and inguinal/perineal screening-ESwabs for MRSA and MSSA	Yes	No	18
Rectal screening-ESwabs for ESBL-producer and CPE	Yes	No	16

CPE, carbapenemase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; ESBL, extended-spectrum beta-lactamases.

Conclusion

The important benefits of the use of automated incubators combined with digital imaging is that they permit continuous and automatic monitoring of the cultured media plates, favouring optimal bacterial growth. The high-resolution digital images taken under different light and exposure conditions open the potential of customized reading times to improve the detection of the early growth. Shortening the turn-around times could positively improve the patient's outcome. This implies providing earlier medically actionable results to the treating physician (e.g. switches from empiric to targeted drug regimens). In this study, the automation was found to reduce the incubation times for all specimens tested without compromising the analytical performances. Using defined and duly validated incubation times will allow building appropriate laboratory workflows, balancing laboratory resources and medical needs in each centre, for improved efficiency. Further studies are now needed to investigate the real impact of reduced time to results on the early adjustments of antimicrobial regimen.

Transparency declaration

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. This study was performed by using internal funding only.

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Research article

A comparison of Sensititre™ Anaerobe MIC plate with ATB ANA® test for the routine susceptibility testing of common anaerobe pathogens

Abdessalam Cherkaoui, Adrien Fischer, Nouria Azam, Arnaud Riat, and Jacques Schrenzel

Authors' contribution statements

Abdessalam CHERKAOUI designed the study and all experiments. He analysed the data and wrote the manuscript.

A. Fischer and N. Azam carried out the experiments

A. Riat provided help and support for the experiments

J. Schrenzel supervised the research and revised the manuscript

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ORIGINAL ARTICLE

A comparison of Sensititre™ Anaerobe MIC plate with ATB ANA® test for the routine susceptibility testing of common anaerobe pathogens

Abdessalam Cherkaoui[1] · Adrien Fischer[1] · Nouria Azam[1] · Arnaud Riat[1] · Jacques Schrenzel[1,2]

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Abstract

The accuracy of the Thermo Scientific™ Sensititre™ Anaerobe MIC plate was assessed against the ATB ANA® test (bioMérieux) on 56 clinically relevant anaerobic strains collected at Geneva University Hospitals. The overall categorical agreement between both methods reached 95%. The Sensititre™ Anaerobe MIC plate had excellent accuracy for most antibiotics tested. When the Sensititre™ Anaerobe MIC plate disagreed with ATB ANA® test, the gradient strip method resolved the antimicrobial susceptibility categories of all the antibiotics tested, except for piperacillin, piperacillin-tazobactam, and penicillin, in favor of the Sensititre™ Anaerobe MIC plate (58% [21 out of 36]). Several very major errors were observed for piperacillin (12.5% [7 out of 56]), piperacillin-tazobactam (12.5% [7 out of 56]), and penicillin (2% [1 out of 56]). The gradient strip method revealed that the categorical differences for piperacillin, piperacillin-tazobactam, and penicillin were at least partly explained by heterogeneity in resistance expression. The Sensititre™ Anaerobe MIC plate offers therefore a useful alternative to the ATB ANA® test for the routine antimicrobial susceptibility testing of anaerobes in clinical microbiology laboratories.

Keywords Sensititre broth dilution · ATB ANA test · Anaerobes · Gradient strip method · Heteroresistance

Introduction

Nowadays, in the vast majority of clinical microbiology laboratories, antimicrobial susceptibility testing (AST) is no longer routinely performed for common anaerobe pathogens. A variety of reasons were highlighted to explain this attitude: the relatively slow growth of these organisms; the polymicrobial nature of infections involving anaerobic bacteria that usually respond to debridement and/or drainage; and in some instances, the poor correlation between in vitro susceptibility results and clinical responses. The latter can be explained, at least partially, by the inappropriate isolation, identification, and/or antimicrobial susceptibility testing of anaerobes from patients with mixed infections [1, 2]. Nonetheless, different reports provide robust evidence indicating an association between antibiotic resistance in anaerobes and negative clinical outcomes [3, 4]. Moreover, the antimicrobial resistance is now reported among anaerobe bacterial pathogens that were hitherto considered to be highly susceptible [5]. Accurate identification of anaerobic bacteria and AST constitute important factors contributing to patient outcome. Biochemical tests are still commonly used in most routine clinical laboratories for the identification of anaerobic bacteria. Nonetheless, many reports highlighted a high rate of misidentification by such methods [6, 7]. The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was evaluated in different studies for the identification of anaerobes [8] and shown to significantly improve the identification of anaerobes with a rapid turnaround time. The need for accurate identification of anaerobes and AST increases with escalating levels of antimicrobial resistance [9, 10]. Timely and reliable identification and susceptibility reports from clinical microbiology laboratories should therefore facilitate antibiotic stewardship programs by enabling appropriate empirical as well as targeted therapy. Phenotypic AST

✉ Abdessalam Cherkaoui
abdessalam.cherkaoui@hcuge.ch

[1] Bacteriology Laboratory, Service of Laboratory Medicine, Department of Genetics, Laboratory Medicine and Pathology, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

[2] Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland

requires an agreement on breakpoints and a rigorous standardization of methods and materials. Phenotypic AST methods involve culturing a sample to obtain a pure isolate and testing, under defined bacterial and drug concentrations, to determine which antimicrobial agents kill or inhibit the bacterial growth. The most widely used AST methods for anaerobes include agar dilution, broth microdilution (with or without the use of an instrument for panel readings), spiral gradient endpoint technique, or gradient strip method (E-test (bioMerieux) and MIC Test Strip (Liofilchem)). Each method has strengths and weaknesses. Phenotypic AST remains necessary since there are many different mechanisms of resistance, which makes the switch to genotype-based approaches quite challenging for the time being.

The purpose of the present study was to investigate the agreement between the Thermo Scientific™ Sensititre™ Anaerobe MIC plate and the ATB ANA® test (bioMérieux) on a panel of common anaerobe pathogens routinely isolated at Geneva University Hospitals.

Materials and methods

Characteristics of patients and samples

All the 56 isolates included in this study were recovered from invasive infections. The sources of the isolates were 26 blood cultures, 6 peritoneal fluids, 8 tissue biopsies, 1 bronchoalveolar lavage, and 15 pus samples. One isolate was from a teenager (14 years old), 25 from adults (18–60 years old), and 30 from older patients (> 60 years old). Males accounted for 45% (25 out of 56) of the cases.

Strains and growth conditions

This study examined 56 non-repetitive clinically relevant obligatory anaerobic and aerotolerant anaerobic strains representing 15 different species collected from a variety of clinical sources at Geneva University Hospitals. Anaerobic Gram-negative Bacilli represented 70% ($n = 39$) of the clinical

Table 1 Susceptibilities of 36 *Bacteroides fragilis* group and 17 Gram-positive Anaerobic Cocci and Bacilli isolates to different antibiotics according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints

	MIC (μg/mL) determined by the Sensititre™ Anaerobe MIC plate			EUCAST clinical breakpoints (% of isolates)		
	MIC range	MIC 50	MIC 90	*S*	*I*	*R*
Bacteroides fragilis group ($n = 36$)						
Penicillin	4–> 8	> 8	> 8	0	0	100
Ampicillin	4–> 32	16	> 32	0	0	100
Piperacillin	< 4–> 128	16	> 128	53	0	47
Amoxicillin/clavulanic acid	< 0.25/0.12–16/8	1/0.5	8/4	80	17	3
Piperacilline/tazobactam	≤ 0.25/4–< 16/4	< 16/4	< 16/4	100	0	0
Imipenem	< 0.06–4	0.25	1	97	3	0
Meropenem	≤ 0.5–1	≤ 0.5	≤ 0.5	100	0	0
Clindamycine	≤ 0.25–> 64	1	> 64	72	0	28
Metronidazole	≤ 0.5–8	2	4	97	0	3
Chloramphenicol	≤ 2–16	4	8	97	0	3
Moxifloxacin*	< 0.12–> 8	1	8	47	0	53
Gram-positive Anaerobic Cocci** and Bacilli*** ($n = 17$)						
Penicillin	< 0.06–0.25	0.06	0.12	100	0	0
Ampicillin	< 0.25–0.5	< 0.25	0.25	100	0	0
Piperacillin	< 4	< 4	< 4	100	0	0
Amoxicillin/clavulanic acid	< 0.25/0.12–0.5/0.25	< 0.25/0.12	0.25/0.12	100	0	0
Piperacilline/tazobactam	0.5/4	0.5/4	0.5/4	100	0	0
Imipenem	< 0.06–0.5	< 0.06	< 0.06	100	0	0
Clindamycine	< 0.5–64	0.5	8	82	0	18
Metronidazole	< 0.25–32	4	32	53	0	47
Chloramphenicol	< 2–4	< 2	4	100	0	0
Moxifloxacin*	< 0.12–4	0.25	1	56	0	44
Vancomycin	< 2	< 2	< 2	100	0	0

*The moxifloxacin MICs were interpreted according to PK/PD EUCAST breakpoints

***Finegoldia magna* and *Parvimonas micra*

****Actinomyces* spp., *Propionibacterium* spp., and *Clostridium perfringens*

isolates included in this study, i.e., *Bacteroides fragilis* ($n = 18$), *Bacteroides thetaiotaomicron* ($n = 10$), *Bacteroides ovatus* ($n = 4$), *Bacteroides nordii* ($n = 1$), *Bacteroides uniformis* ($n = 2$), *Bacteroides buccae* ($n = 1$), and *Fusobacterium necrophorum* ($n = 3$). Gram-positive Anaerobic Bacilli and Cocci represented 30% ($n = 17$) of the analyzed strains, i.e., *Finegoldia magna* ($n = 3$), *Parvimonas micra* ($n = 2$), *Actinomyces oris* ($n = 1$), *Actinomyces meyeri* ($n = 1$), *Actinomyces neuii* ($n = 2$), *Propionibacterium avidum* ($n = 2$), *Cutibacterium* (formerly *Propionibacterium*) *acnes* ($n = 2$), and *Clostridium perfringens* ($n = 4$).

All strains were stored at − 80 °C in skim milk with 15% glycerol. Anaerobic strains were cultured on CDC agar (bioMérieux SA, Switzerland) and incubated at 37 °C for 24–48 h in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% N_2) generated with the micro-incubator M23C (Scholzen Microbiology Systems AG, Switzerland). The identification was determined using MALDI TOF/MS (Biotyper compass, Bruker Daltonics, Bremen, Germany), according to the manufacturers' instructions.

Antimicrobial susceptibility testing methods

Antimicrobial susceptibility testing for all the isolates included in this study was performed in parallel by using the Thermo Scientific™ Sensititre™ Anaerobe MIC plate (ANO2B or ANAERO3) and ATB ANA® test (bioMérieux SA, Switzerland) according to the manufacturers' instructions. Data interpretation was based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints.

Discordant results

The Sensititre™ Anaerobe MIC plate results were compared to the ATB ANA® test routinely performed in our laboratory. When both methods agreed, we considered the susceptibility category correct and no further determination was performed. When the methods gave discordant results, we performed the gradient strip method (E-test (bioMérieux) or MIC Test Strip (Liofilchem)) according to the manufacturers' instructions to address this uncertainty. No further molecular characterization to determine resistance mechanisms was attempted in this study.

Results

Antimicrobial susceptibility testing

Table 1 shows the MIC_{50}, MIC_{90}, and MIC range determined by the Sensititre™ Anaerobe MIC plate and the susceptibility categories for the 53 clinical isolates grouped into two different categories (*Bacteroides fragilis*

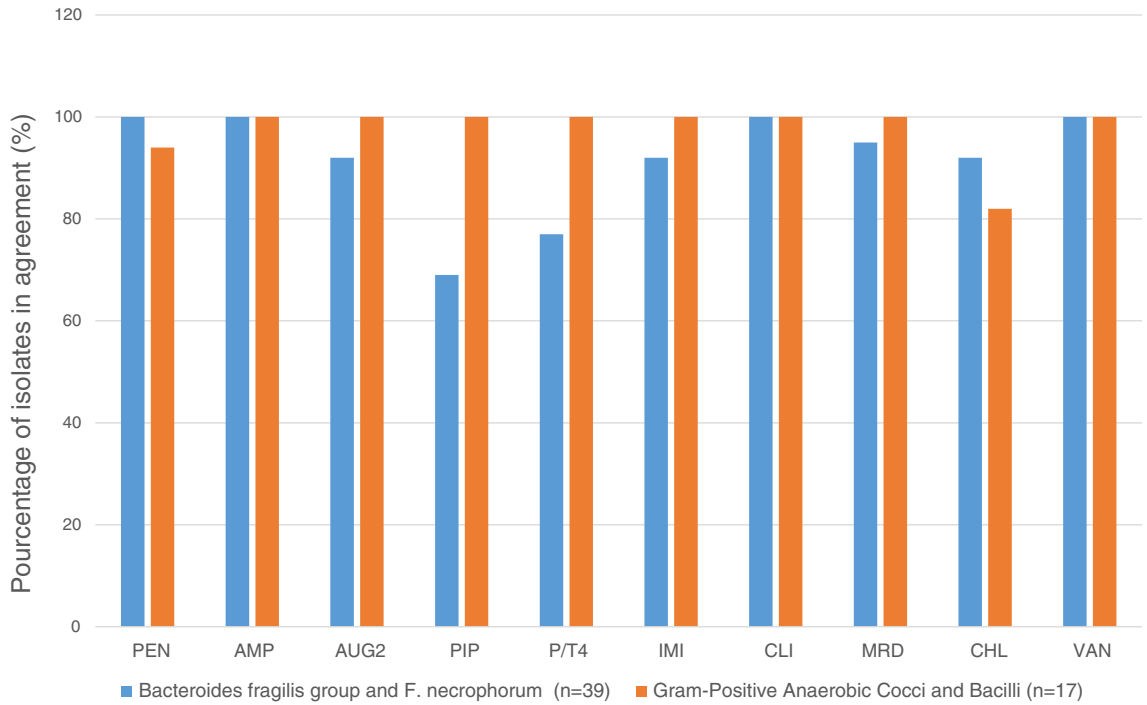

Fig. 1 Agreement between ThermoScientific™ Sensititre™ Anaerobe MIC plate and ATB ANA®bioMérieux penicillin (PEN), ampicillin (AMP), amoxicillin/clavulanicacid2: 1ratio (AUG2), piperacillin (PIP), piperacillin/tazobactamconstant4 (P/T4), imipenem (IMI), clindamycin (CLI), metronidazole (MRD), chloramphenicol (CHL), vancomycine (VAN)

group ($n = 36$), and Gram-positive Anaerobic Cocci and Bacilli ($n = 17$)). The three *F. necrophorum* isolates analyzed in this study were susceptible to all antibiotics tested, that for the sake of consistency, these three isolates were not depicted in Table 1. Metronidazole resistance was found in 3% (1 out of 39) and 47% (8 out of 17) of Gram-negative Anaerobic Bacilli (*B. fragilis* group and *F. necrophorum*) and Gram-positive organisms, respectively. In fact, *Propionibacterium* spp. and *Actinomyces* spp. are aerotolerant anaerobic bacteria and therefore generally resistant to the 5-nitroimidazole agents, including metronidazole ($MIC_{90} > 16$ μg/mL) which explains the high percentage of resistance to metronidazole among this group.

Table 2 Analysis of category differences interpretative results of Sensititre™ Anaerobe MIC plate, and ATB ANA® test for isolates in which there was not agreement

	Sensititre™ Anaerobe MIC plate	ATB ANA® bioMérieux	Gradient strip method
Bacteroides fragilis group			
Amoxicillin/clavulanic	0.5/0.25 (S)	R	0.125 (S)
	< 0.25/0.12 (S)	R	0.25 (S)
	< 0.25/0.12 (S)	R	0.25 (S)
Piperacillin	8 (S)	R	12 (S)
	< 16 (S)	R	64 (R)
	< 16 (S)	R	> 256 (R)
	< 4 (S)	R	24 (R)
	< 16 (S)	R	48 (R)
	< 16 (S)	R	64 (R)
	< 16 (S)	R	256 (R)
	< 16 (S)	R	64 (R)
	< 16 (S)	R	12 (S)
	< 16 (S)	R	12 (S)
	< 16 (S)	R	6 (S)
	< 16 (S)	R	3 (S)
Piperacillin/tazobactam	< 16/4 (S)	R	24 (R)
	< 16/4 (S)	R	24 (R)
	< 16/4 (S)	R	> 256 (R)
	< 16/4 (S)	I	24 (R)
	< 16/4 (S)	I	24 (R)
	< 16/4 (S)	R	24 (R)
	< 16/4 (S)	I	> 256 (R)
	< 16/4 (S)	R	1.5 (S)
	< 16/4 (S)	R	4 (S)
Imipenem	0.12 (S)	R	0.38 (S)
	1 (S)	I	0.25 (S)
	1 (S)	R	0.38 (S)
Metronidazole	2 (S)	R	0.19 (S)
	2 (S)	R	0.25 (S)
Chloramphenicol	4 (S)	R	4 (S)
	4 (S)	R	4 (S)
	4 (S)	R	4 (S)
Gram-positive Anaerobic Cocci and Bacilli			
Penicillin	0.25 (S)	I	> 256 (R)
Chloramphenicol	< 2 (S)	R	4 (S)
	4 (S)	R	4 (S)
	4 (S)	R	1.5 (S)

Sensitive (S), intermediate (I), or resistant (R)

Gradient strip method (E-test (bioMérieux) and MIC test strip (Liofilchem))

Agreement between Sensititre™ Anaerobe MIC plate and ATB ANA® test

Figure 1 shows that the Sensititre™ Anaerobe MIC plate had excellent accuracy for most antibiotics tested. However, a high number of very major errors were observed for piperacillin (12.5% [7 out of 56]), piperacillin-tazobactam (12.5% [7 out of 56]), and penicillin (2% [1 out of 56]). When the Sensititre™ Anaerobe MIC plate disagreed with ATB ANA® test, the gradient strip method resolved the antimicrobial susceptibility categories of all the antibiotics tested, except for piperacillin, piperacillin-tazobactam, and penicillin, in favor of the Sensititre™ Anaerobe MIC plate (58% [21 out of 36]), as depicted in Table 2. Moreover, the accuracy of the Sensititre™ Anaerobe MIC plate varied according to bacterial species and antimicrobial combinations. In particular, most of the clinical isolates in which there was no agreement between the two methods belong to *Bacteroides fragilis* group.

Discussion

Anaerobes constitute a predominant component of the normal human microbiota and are a frequent cause of bacterial infections of endogenous source [11, 12]. Rapid and comprehensive diagnostics would enable earlier use of the most appropriate and targeted drug regimen, thus improving patient outcomes and reducing overall healthcare costs and selection pressure. In spite of the fact that resistance trends have been investigated and reported generally through a large number of international surveillance programs, routine antimicrobial susceptibility testing of anaerobic pathogens remains uncommon. The continuous increase of antimicrobial resistance, which results of a mobilization of resistance genes [13–15], prompts for the implementation of the routine AST of anaerobic bacteria. We have shown here that the frequency of very major errors depends on the AST method, and the drugs-bugs combinations. Nonetheless, some antimicrobial combinations appear as typically problematic. For most drugs, such discrepancies are generally unknown, but obvious differences between piperacillin-tazobactam testing by broth microdilution, agar dilution, and E-test were already reported in several bacterial species [16].

Heteroresistance is commonly reported in a variety of microorganisms; this phenomenon contributes to recurrent or chronic infections because the presence of different antibiotic-resistant subpopulations can lead to treatment failure by selection of the resistant subpopulation [17–19]. Figure 2 shows the minimum inhibitory concentration as determined by a piperacillin E-test strip for *Bacteroides thetaiotaomicron*, piperacillin-tazobactam MIC test strip for *Bacteroides fragilis* and by a penicillin E-test strip for *Clostridium perfringens*. The arrows indicate the presence of distinct colonies growing within the inhibition zone, thus clearly demonstrating the presence of antibiotic-resistant subpopulations. This heteroresistance phenomenon was seen in all seven isolates in which there was a lack of agreement for piperacillin-tazobactam AST between both methods. Thus, the gradient strip method support that the categorical differences observed between Sensititre™ Anaerobe MIC plate and ATB ANA® test were at least partly due to heterogeneity of resistance expression. Further investigations are required to explain the exact mechanisms underlying such differences, especially since the resistant subpopulation may also implicate delayed growth.

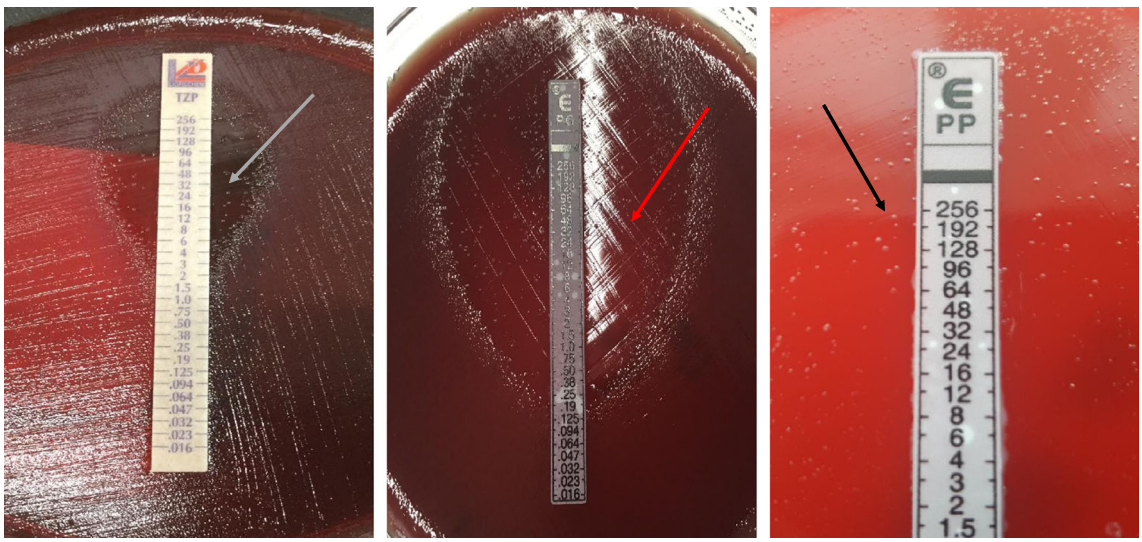

Fig. 2 Minimum inhibitory concentration (MIC) determination using a piperacillin-tazobactam MIC Test Strip for *Bacteroides fragilis*, penicillin E-test for *Clostridium perfringens,* and piperacillin E-test for *Bacteroides thetaiotaomicron.* The three strains were isolated from a blood culture at Geneva University Hospitals. The grey, red, and black arrows indicate respectively the piperacillin-tazobactam-, penicillin-, and piperacillin-resistant subpopulations

Conclusion

While the present study has provided much useful information about the accuracy of the two antimicrobial susceptibility testing methods for anaerobic bacteria, it has some limitations: we did not analyze the heterogeneity of resistance expression by population analysis profile methods, and this work studied a relatively small number of strains for some species. This is due to the fact that we included only isolates that were recovered from invasive infections which represent approximately 8% of all anaerobic isolates identified in our lab during the study period.

Despite the fact that the Sensititre™ Anaerobe MIC plate was unable to detect heteroresistant isolates, this method offers a convenient alternative to ATB ANA® test to improve the routine antimicrobial susceptibility testing of anaerobes in clinical microbiology laboratories.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Short communication

Comparison of analytical performances of the Roche Cobas 6800CT/NG assay with the Abbott m2000 Real Time CT/NG assay for detecting *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

Abdessalam Cherkaoui, Gesuele Renzi, Michèle Mombelli, Katia Jaton, Sabine Yerly, Nicolas Vuilleumier, and Jacques Schrenzel

Authors' contribution statements

Abdessalam CHERKAOUI designed the study and all experiments. He analysed the data and wrote the manuscript.

G. Renzi and **M. Mombelli** carried out the experiments

K. Jaton carried out the independent set of real-time PCR assays

S. Yerly and **N. Vuilleumier** helped in interpreting results and revised the manuscript

J. Schrenzel supervised the research and revised the manuscript

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MICROBIOLOGY SOCIETY

Comparison of analytical performances of the Roche Cobas 6800 CT/NG assay with the Abbott *m*2000 Real Time CT/NG assay for detecting *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

Abdessalam Cherkaoui,[1,*] Gesuele Renzi,[1] Michèle Mombelli,[1] Katia Jaton,[2] Sabine Yerly,[3] Nicolas Vuilleumier[4,5] and Jacques Schrenzel[1,6]

Abstract

The Roche Cobas 6800 CT/NG assay was compared to the Abbott *m*2000 Real Time CT/NG assay for detecting *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in 714 specimens referred to the bacteriology laboratory at Geneva University Hospitals, between November 2017 and March 2018, and in nine external quality controls for molecular diagnostics (seven from QCMD Glasgow and two from UK NEQAS). For *C. trachomatis,* the sensitivity of *C*6800 compared to *m*2000 was 100 % (95 % confidence interval [CI], 97.5 to 100 %), the specificity was 99.1 % (95 % CI, 98.0 to 99.7 %). For *N. gonorrhoeae,* the sensitivity of the *C*6800 compared to *m*2000 was 100 % (95 % CI, 90.5 to 100 %), whereas the specificity was 99.7 % (95 % CI, 98.9 to 99.9 %). The *C*6800 CT/NG assay appears to perform with great accuracy the detection of *C. trachomatis* and *N. gonorrhoeae.*

Neisseria gonorrhoeae and *Chlamydia trachomatis* are considered responsible for the most frequent bacterial sexually transmitted infections (STI) [1]. As the clinical presentations of signs and symptoms can be confused with those of other STIs, an accurate diagnosis is capital for appropriate treatment and effective subsequent control strategies for STIs [1]. For both asymptomatic and symptomatic individuals, the diagnosis of *N. gonorrhoeae* and *C. trachomatis* relies on nucleic acid amplification tests (NAATs) which are sensitive, specific, reproducible, and robust [2, 3]. Nowadays various automated systems for the detection of *C. trachomatis* and *N. gonorrhoeae* nucleic acid targets in urogenital samples are commercially available. Cobas 6800 CT/NG assay is a quantitative test that uses real-time polymerase chain reaction for the direct detection of *C. trachomatis* and *N. gonorrhoeae* in oropharyngeal and anorectal swab and different urogenital specimens (urine, vaginal and endocervical swab specimens). The recommendation of the Center for Disease Control and Prevention is to perform, at least once a year, a screening of *N. gonorrhoeae* and *C. trachomatis* in HIV-infected and at-risk men who have sex with men (MSM) by using oropharyngeal and rectal swab specimens [4]. Previous studies have reported that in MSM more than 80 % of the patients could be missed when only urogenital sites were screened [4–7]. Moreover, the study conducted by David Hardy *et al.*, showed good analytical performances for the Cobas 6800 CT/NG assay to detect both pathogens in oropharyngeal and anorectal swab specimens [8].

The objective of this study was to assess the analytical performances of the Cobas 6800 CT/NG assay (*C*6800) against the Abbott *m*2000 Real Time CT/NG (*m*2000) assay for the detection of *C. trachomatis* and *N. gonorrhoeae*. The evaluation of *C*6800 was performed retrospectively on 714 specimens referred to the bacteriology laboratory at Geneva University Hospitals, between November 2017 and March 2018, from general practice and sexual health wards; and prospectively on nine external quality controls for molecular diagnostics (seven from QCMD Glasgow and two from UK NEQAS). We have included all oropharyngeal and anorectal

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Author affiliations: [1]Bacteriology Laboratory, Division of Laboratory Medicine, Department of Genetics, Laboratory Medicine and Pathology, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland; [2]Institute of Microbiology, University of Lausanne & University Hospital Center, Lausanne, Switzerland; [3]Laboratory of Virology, Division of Laboratory Medicine, Department of Genetics, Laboratory Medicine and Pathology, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland; [4]Division of Laboratory Medicine, Department of Genetics, Laboratory Medicine and Pathology, Geneva University Hospitals, Geneva, Switzerland; [5]Laboratory Medicine Division, Department of Medical Specialities, Faculty of Medicine, Geneva, Switzerland; [6]Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland.
***Correspondence:** Abdessalam Cherkaoui, abdessalam.cherkaoui@hcuge.ch
Keywords: Cobas 6800; Abbott m2000; *Chlamydia trachomatis*; *Neisseria gonorrhoeae*.

Table 1. Specimens used for the evaluation of the analytical performances of the Cobas 6800 CT/NG assay against the Abbott *m*2000 Real Time CT/NG assay, for the detection of *C. trachomatis* and *N. gonorrhoeae*

Specimen type	No. of specimens tested including 9 external quality controls	*Chlamydia trachomatis*	*Neisseria gonorrhoeae*
Self- or clinician-collected vaginal swabs	463	**95**	**6**
First-catch urine samples	225	**46**	**18**
Oropharyngeal (throat) swab	16	**1**	**8**
Endocervical swab	2	**1**	0
Anal ulcer swab	10	**1**	**3**
Genital ulcer swab	1	0	0
Conjunctival swab	2	0	0
Urethral swab	4	**2**	**2**
Total	723	146	37

specimens referred to our routine laboratory during the specified study period.

Discordant specimens were sent to the Institute of Microbiology, University of Lausanne, Switzerland and retested by an independent set of real-time PCR assays previously published [9, 10]. Urine and swab specimens were collected using the Abbott multi-Collect Specimens Collection KIT. Specimens were transported in the multi-Collect Specimens Transport Tube. After *m*2000 analysis according to manufacturer's instructions, the multi-Collect Specimens Transport Tubes were stored at 4 °C until testing by *C*6800. For *C*6800 analysis, all the stored specimens were analysed by spiking 2 ml of urine and 600 µL of swabs specimens transported in the Abbott multi-Collect Specimens Transport Tubes into cobas PCR Media tube containing 4.3 ml of transport medium, and tested according to manufacturer's instructions. Among specimens (including the nine external quality controls) analysed in the *m*2000 and that met the criteria for supplementary testing by *C*6800, 19.6 % (142 of 723) were positive for *C. trachomatis,* 4.7 % (33 of 723) were positive for *N. gonorrhoeae,* while 0.6 % (4 of 723) were positive for both pathogens (Table 1).

Seventy-four percent (530 of 714) of the samples were from females, and 26 % (184 of 714) were from males. The median age of the patients at the time of testing was 31 years with a range from 12 to 80 years.

For *C. trachomatis,* the sensitivity of *C*6800 compared to *m*2000 was 100 %, the specificity was 99.1 %, the negative predictive value (NPV) was 100 %, and the positive predictive value (PPV) was 96.7 %. The kappa score for *C*6800 was 0.979 (95 % confidence interval [CI], 96.0 to 99.7 %). For *N. gonorrhoeae,* the sensitivity of *C*6800 compared to *m*2000 was 100 %, the specificity was 99.7 %, the NPV was 100 %, and the PPV was 94.9 %. The kappa score for *C*6800 was 0.972 (95 % CI, 93.4 to 100 %) (Table 2). One of six discrepant *C*6800 specimens (*m*2000 negative or indeterminate/ C6800 positive) tested by an independent real-time PCR assay at the Institute of Microbiology, University of Lausanne, was confirmed positive for *C. trachomatis*.

Taking into account all available bacteriological information, two discrepant specimens (*m*2000 negative or indeterminate/C6800 positive) were considered negative even though the two patients had, at the time of testing, another specimen positive with the same pathogen (Table 3).

The recent study conducted by Marlowe *et al.*, on over 12 000 urogenital and extragenital specimens in Germany and the United States, documents the accuracy of *C*6800 to detect *C. trachomatis and N. gonorrhoeae.* Among urogenital, oropharyngeal, and anorectal specimens, there were 125

Table 2. Comparison of Cobas 6800 CT/NG assay to Abbott *m*2000 Real Time CT/NG assay for the detection of *C. trachomatis and N. gonorrhoeae*

Test	No. of specimens tested	No. of specimens with indicated results								
C6800 system	**723**	***Chlamydia trachomatis***								
		M+/C+	M-/C-	M+/C-	M-/C+	% sensitivity (CI)	% specificity (CI)	% NPV (CI)	%PPV (CI)	Kappa (CI [%])
		146	572	0	5	100 (97.5–100)	99.1 (98–99.7)	100 (100)	96.7 (92.4–98.6)	0.979 (96–99.7)
C6800 system	723	***Neisseria gonorrhoeae***								
		M+/C+	M-/C-	M+/C-	M-/C+	% sensitivity (CI)	% specificity (CI)	% NPV (CI)	%PPV (CI)	Kappa (CI [%])
		37	684	0	2	100 (90.5–100)	99.7 (98.9–99.9)	100 (100)	94.9 (82.3–98.7)	0.972 (93.4–100)

M, *m*2000 (reference method) result; C, *C*6800 (comparative method) result; +, positive result; -, negative result; CI, 95 % confidence interval for overall agreement of the reference method and the comparative method; NPV, negative predictive value; PPV, positive predictive value.

Table 3. Discrepant *C*6800 specimens (*m*2000 negative or indeterminate/*C*6800 positive)

Sample	Specimen type	Abbott *m*2000 assay		Roche *C*6800 assay		Real-time PCR (CHUV)		Comments
		C. trachomatis	*N. gonorrhoeae*	*C. trachomatis* (cycle number)	*N. gonorrhoeae* (cycle number)	*C. trachomatis*	*N. gonorrhoeae*	
1	Oropharyngeal (throat) swab	NEG	NEG	NEG	**POS** (39.99)	NEG	NEG	The patient had urethral and urine samples positive for *N. gonorrhoeae* at the time of testing
2	Self- or clinician-collected vaginal swab	NEG	NEG	NEG	**POS** (39.35)	NEG	NEG	
3	First-catch urine sample	NEG	**POS**	**POS** (41.55)	**POS**	NEG	**POS** (1100 copies/mL)	
4	Oropharyngeal (throat) swab	NEG	**POS**	**POS** (37.99)	**POS**	**POS** (260 copies/mL)	**POS** (7900 copies/mL)	
5	First-catch urine sample	Indeterminate	NEG	**POS** (37.98)	NEG	NEG	NEG	The patient had cervical swab positive for *C. trachomatis* at the time of testing
6	First-catch urine sample	Indeterminate	NEG	**POS** (40.69)	NEG	NEG	NEG	
7	First-catch urine sample	Indeterminate	NEG	**POS** (34.13)	NEG	**POS** (257 copies/mL)	NEG	Quality Control for Molecular Diagnostics (QCMD) Glasgow/ CTDNA 18C1-05

Discordant specimens were sent to Institute of Microbiology, University of Lausanne, Switzerland (CHUV) and retested by an independent set of real-time PCR assays previously published. Indeterminate, *C. trachomatis* sample still with a cycle number beyond the assay cut-off after retesting. The results were assessed also with additional bacteriological information as described in the Comments

C. trachomatis and 42 *N. gonorrhoeae* discrepant specimens (*C*6800 positive and *C*4800 negative). Sequencing confirmed *C*6800 positive *C. trachomatis* results for 75 of 125 (60 %) specimens and positive *N. gonorrhoeae* results for 35 of 42 (83 %) specimens [3].

The discordant quality control (QCMD) Glasgow/CTDNA 18C1-05 (*m*2000 indeterminate/C6800 positive) was confirmed positive by the independent real-time PCR assay for *C. trachomatis* (257 copies per ml). After discordance analysis, the specificities for *C*6800 for *C. trachomatis* and *N. gonorrhoeae* increased to 99.5 % (95 % CI, 98.5 to 99.9 %) and 99.9 % (95 % CI, 99.2 to 100 %), respectively.

This study has one limitation related to the fact that the specimens included in the evaluation of *C*6800 were not placed directly into the manufacturer's transport media (cobas PCR Media tube containing transport medium) but were shipped in Abbott multi-Collect Specimens Collection KIT.

This study demonstrated that *C*6800 performed with great accuracy when detecting *C. trachomatis* and *N. gonorrhoeae*.

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Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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