

Emergence of clonally related multidrug resistant *Haemophilus influenzae* with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013

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Resistance to cephalosporins in *Haemophilus influenzae* is usually caused by characteristic alterations in penicillin-binding protein 3 (PBP3), encoded by the *ftsI* gene. Resistance to extended-spectrum cephalosporins is associated with high-level PBP3-mediated resistance (high-rPBP3), defined by the second stage S385T substitution in addition to a first stage substitution (R517H or N526K). The third stage L389F substitution is present in some high-rPBP3 strains. High-rPBP3 *H. influenzae* are considered rare outside Japan and Korea. In this study, 30 high-rPBP3 isolates from Norway, collected between 2006 and 2013, were examined by serotyping, multilocus sequence typing (MLST), *ftsI* sequencing, detection of beta-lactamase genes and minimum inhibitory concentration (MIC) determination. MICs were interpreted according to clinical breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Respiratory isolates predominated (proportion: 24/30). The 30 isolates included one serotype f isolate, while the remaining 29 lacked polysaccharide capsule genes. Resistance to extended-spectrum cephalosporins (cefixime, 29 isolates/30 isolates; cefepime, 28/30; cefotaxime, 26 /30; ceftaroline, 26/30; ceftriaxone, 14/30), beta-lactamase production (11/30) and co-resistance to non-beta-lactams (trimethoprim-sulfamethoxazole, 13/30; tetracycline, 4/30; chloramphenicol, 4/30; ciprofloxacin, 3/30) was frequent. The N526K substitution in PBP3 was present in 23 of 30 isolates; these included a blood isolate which represents the first invasive S385T+N526K isolate reported from Europe. The L389F substitution, present in 16 of 30 isolates, coincided with higher beta-lactam MICs. Non-susceptibility to meropenem

was frequent in S385T+L389F+N526K isolates (8/12). All 11 beta-lactamase positive isolates were TEM-1. Five clonal groups of two to 10 isolates with identical MLST-*ftsI* allelic profiles were observed, including the first reported high-rPBP3 clone with TEM-1 beta-lactamase and co-resistance to ciprofloxacin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole. Prior to this study, no multidrug resistant high-rPBP3 *H. influenzae* had been reported in Norway. Intensified surveillance of antimicrobial resistance is needed to guide empiric therapy.

Introduction

Haemophilus influenzae colonises the respiratory tract in humans and infection causes a wide range of conditions, including acute otitis media, arthritis, conjunctivitis, epiglottitis, meningitis, respiratory tract infections, septicaemia and sinusitis [1]. Encapsulated isolates of serotype b (Hib) have the highest potential for invasive disease but the introduction of Hib vaccines has dramatically reduced the burden of disease. Thus, the vast majority of infections, both invasive and non-invasive, are currently caused by nontypeable (i.e. lacking polysaccharide capsule) *H. influenzae* (NTHi) and non-b serotypes, mainly serotype f (Hif) [1-3].

Due to the global emergence of beta-lactamase (TEM-1 and ROB-1) producing strains in the 1970s, empiric therapy for severe disease caused by *H. influenzae* was altered from ampicillin to cephalosporins [4]. Development of resistance to extended-spectrum cephalosporins has forced a second shift in empiric treatment to carbapenems in some geographical areas [4]. The European Committee on Antimicrobial

Susceptibility Testing (EUCAST) categorises resistance to extended-spectrum cephalosporins in *H. influenzae* as an exceptional phenotype [5]. Considering such isolates ‘very rare or not yet reported’, EUCAST recommend that they are sent to a reference laboratory [6]. Notably, minimum inhibitory concentration (MIC) breakpoints for cephalosporins and *H. influenzae* from EUCAST are considerably lower than those from Clinical and Laboratory Standards Institute (CLSI): for instance, the susceptible minimum inhibitory concentration (MIC) breakpoint for cefotaxime from EUCAST is ≤ 0.12 mg/L while the corresponding CLSI breakpoint is ≤ 2 μ g/mL [7].

Resistance to cephalosporins is due to alterations in the transpeptidase domain of penicillin-binding protein 3 (PBP3), encoded by the *ftsI* gene, with phenotypic resistance profiles depending on amino acid substitution patterns. Several genotypic classification systems for PBP3-mediated resistance (rPBP3) have been suggested and the terminology is still developing [4,8-13]. Acquisition of one of the substitutions R517H or N526K represents the first stage of resistance development. The second stage substitution S385T is associated with increased resistance to cefotaxime and separates high-level resistant (high-rPBP3) isolates from those with low-level resistance (low-rPBP3) [4,12,13]. High-rPBP3 strains may be divided into group III (S385T+N526K) and group III-like (S385T+R517H) [9-11]. The additional L389F substitution is associated with further increased resistance levels in high-rPBP3 strains and may be considered the third stage in development of PBP3-mediated resistance [13].

High-rPBP3 isolates became frequent in Japan in the late 1990s [14] and in Korea during the 2000s [15]. A similar development in other geographical areas would compromise current empiric antimicrobial therapy in severe infections, which is largely based on third-generation cephalosporins. However, low-rPBP3 *H. influenzae*, lacking the S385T substitution and mainly susceptible to cefotaxime and ceftriaxone, still predominate in south-west and northern Europe [2,8,11,16]. In previous systematic surveys outside Asia where determination of PBP3 amino acid substitution patterns has been performed [2,3,8,11,16-19], very few group III isolates have been reported [1]; these include one invasive isolate from Canada [19], one otitis media isolate from France [16] and one upper respiratory tract isolate from Norway [8]. Group III-like isolates have been more frequently reported in Europe [1,3,8,11,16,18].

Since 2007, the Norwegian Working Group on Antibiotics (NWGA) has recommended that cefotaxime resistant *H. influenzae* isolates are sent to the reference laboratory for *ftsI* sequencing and susceptibility testing with broth microdilution. In Norway, this is carried out at Vestfold Hospital Trust, in collaboration with the Haemophilus Reference Laboratory at the Norwegian Institute of Public Health and the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance

at the University Hospital of North Norway. We present here susceptibility profiles, resistance genotypes and molecular strain characteristics of 30 high-rPBP3 isolates from Norway, defined by the S385T substitution, collected between May 2006 and July 2013.

Methods

Isolates

A total of 30 clinical high-rPBP3 (S385T positive) *H. influenzae* isolates from Norway, isolated at 10 different routine laboratories between May 2006 and July 2013, were included in the study. Of the 30 high-rPBP3 isolates, 27 (from 7 laboratories) with the S385T substitution were identified by partial *ftsI* sequencing of 39 routine isolates (from 10 laboratories) with cefotaxime MIC > 0.12 mg/L (gradient test); the remaining 12 isolates were categorised as group II low-rPBP3 (N526K positive, S385T negative) and had cefotaxime MIC = 0.25 mg/L (n=10) or 0.5 mg/L (n=2) by gradient test. We also included three isolates (isolates 4–6) from three additional laboratories, collected in 2007 and identified as high-rPBP3 through a previous surveillance study [8]. The 10 laboratories contributing the 30 isolates were located in mid Norway (Nord-Trøndelag county), the Oslo region (Akershus, Buskerud, Oslo and Vestfold counties), Western Norway (Sogn og Fjordane, Hordaland and Rogaland counties) and Southern Norway (Agder county).

Pure cultures were kept frozen at -70°C in Microbank vials (Pro-Laboratory Diagnostics, Ontario, Canada) pending further characterisation.

Demographic and clinical data

Information about sample types, demographic data and brief clinical information (provided by the clinician at the time of sampling) were acquired from the primary laboratories. Three recently hospitalised patients with X-ray confirmed pneumonia most likely caused by study isolates were selected as case reports. For these patients, relevant supplementary information about diagnosis, antimicrobial therapy and clinical outcome was extracted from the patient files.

Species identification and molecular characterisation

Species identification was carried out by API NH (bioMérieux, Marcy-l’Etoile, France), matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry with MALDI Biotyper (Bruker Daltonics GmbH, Bremen, Germany), and conventional methods, including absence of beta-haemolysis on blood agar. Amplification of the *fucK* gene when performing multi-locus sequence typing (MLST) (see below) was used as an additional criterion to separate *H. influenzae* from *H. haemolyticus*; all included isolates were *fucK* positive.

Biotypes [20] were determined using indole, urease and ornithine decarboxylase (ODC) reactions produced by API NH (bioMérieux).

Capsular serotyping was performed at the Norwegian Institute of Public Health according to previously described methodology [21] with modifications (under-scored) to the e1 (TTTGGTAACGAATGTAGTGGTAG) and e2 (ATAGCTTTACTGTATAAGCTTAG) primers (5' to 3').

MLST with sequencing of the seven housekeeping genes *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi* and *recA* was carried out at the Norwegian Institute of Public Health according to standard procedures [22]. Sequences were registered at <http://haemophilus.mlst.net>. Sequence types (ST) were assigned according to allelic profiles. STs were divided into clonal complexes (CC) (named according to founder) using eBURST Version 3 (<http://haemophilus.mlst.net/eburst>).

Pulsed-field gel electrophoresis (PFGE) of selected isolates was carried out at Unilabs Telelab (Skien, Norway) as previously described [17] and clonal relatedness was interpreted according to the Tenover criteria [23].

Beta-lactamase detection

All isolates were screened for beta-lactamase production and the presence of beta-lactamase genes (TEM-1 and ROB-1) as previously described using acidimetric agar [24] and polymerase chain reaction (PCR) [17].

Minimum inhibitory concentration determination

MICs to ampicillin, amoxicillin (ampicillin-sulbactam 2:1 ratio and amoxicillin-clavulanic acid 2:1 ratio for beta-lactamase positive isolates), cefuroxime, cefixime, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole were determined by broth microdilution according to CLSI methodology [25] at Vestfold Hospital Trust, using standard Sensititre plates (HPB1, STP6 and GNX3F; TREK Diagnostic Systems, Thermo Fisher Scientific, West Sussex, UK). Ceftaroline, azithromycin, clarithromycin, erythromycin, roxithromycin, telithromycin, doxycycline, minocycline and rifampicin MICs were determined at the EUCAST Development Laboratory, using custom Sensititre plates (SEFE3, TREK) with in-house prepared EUCAST MH-F broth (cation-adjusted Mueller-Hinton broth (BBL, BD Diagnostic Systems, Franklin Lakes, NJ) with 5% lysed horse blood and 20 mg/L beta-NAD) and 16 to 20 hours incubation in sealed panels in ambient air. *H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766 and *H. influenzae* ATCC 35056 (ampicillin-sulbactam and amoxicillin-clavulanic acid only) were incorporated for quality control purposes, with all MICs within accepted ranges. MICs were interpreted according to EUCAST clinical breakpoints [6]. MICs of all study isolates are included in the EUCAST MIC database (www.eucast.org/MIC_distributions).

ftsI/penicillin-binding protein 3 typing

PCR of the *ftsI* gene (transpeptidase domain) was performed as described previously [17]. Sequencing was performed at GATC Biotech AG (Konstanz, Germany). Sequences were analysed with the Lasergene software (DNASTAR, Madison, WI, US). An unweighted pair group method with arithmetic mean (UPGMA) phylogram (nucleotides 1010–1719) was created using ClustalW2 (www.ebi.ac.uk) and TreeDyn (www.phylogeny.fr). *H. influenzae* Rd KW20 (GenBank accession number: CU32793) was used as reference and *H. parainfluenzae* (European Molecular Biology Laboratory (EMBL) accession number: AB267856) as outgroup. The 710 bp fragments (hereafter denoted *ftsI* alleles) were assigned numbers (1–10) according to frequency of occurrence and used together with MLST allelic profiles for assignment to clonal groups (CG) (see below).

Deduced PBP3 amino acid sequences (338–573) were compared with *H. influenzae* Rd KW20 and sequence patterns (denoted PBP3 types) were assigned numbers (1–7) according to frequency of occurrence. The substitutions S385T, R517H and N526K were used for categorisation into PBP3 group III and group III-like [9–11]. For isolates with the third stage substitution L389F, the suffix '+' was added. The following group designations were thus used: group III (S385T, N526K); group III+ (S385T, L389F, N526K); group III-like (S385T, R517H); group III-like+ (S385T, L389F, R517H). The groups respectively correspond to PBP3 classes II, III, V and VI, proposed by Osaki et al. [13].

Clonal groups

According to a previously described approach to molecular typing of *H. influenzae* [4], CGs were defined as isolates with identical MLST-*ftsI* allelic profiles and numbered chronologically [1–5].

GyrA and *ParC* amino acid substitutions

For isolates with ciprofloxacin MIC > 0.06 mg/L and/or levofloxacin MIC > 0.03 mg/L, the quinolone-resistance determining regions (QRDR) of *gyrA* and *parC* were characterised by PCR methodology adapted from a previous report [26]. DNA sequencing of amplified products was carried out at GATC Biotech. Sequences were analysed with Lasergene. Deduced amino acid sequences of *GyrA* (64–177) and *ParC* (66–236) were compared with *H. influenzae* Rd KW20 (GenBank accession number: U32793).

Nucleotide sequences

The *ftsI* (HG983286–HG983315), *gyrA* (HG983316–HG983320) and *parC* (HG983321–HG983325) sequences from this study are available at the European Nucleotide Archive (www.ebi.ac.uk).

Ethics

The use of clinical data in this study is approved by the Regional Committees for Medical and Health Research Ethics in Norway (reference number 2014/411).

TABLE 1

Characterisation of *Haemophilus influenzae* with high-level penicillin-binding protein 3-mediated beta-lactam resistance (high-rPBP3), Norway, 2006–2013 (n=30)

Isolate ^a	Year/ region ^b		Category ^c / age group ^d		Site ^e	Clinical data ^f	Biotype ^g	MLST		<i>ftsI</i> ^h	Clonal group ⁱ	PBP3		Co-resistance ^l
								ST	CC			Type ^j	Group ^k	
1	2006	O	Hosp	1	Nose	ALL/AOM	III	155	155	3	1	4	III-like	–
2	2007	O	GP	1	Nose	URTI	IV	1197	395	1	2	1	III	β
3	2007	O	GP	5	Npx	No data	III	159	503	6	Single	5	III+	Ts
4	2007	O	OC	3	Nose	No data	IV	1197	395	1	2	1	III	–
5	2007	O	OC	1	Npx	No data	III	155	155	3	1	4	III-like	–
6	2007	M	Hosp	7	LRT	No data	III	155	155	3	1	4	III-like	–
7	2008	O	GP	3	Npx	No data	III	1197	395	1	2	1	III	–
8	2008	O	Hosp	1	Nose	URTI	IV	1197	395	1	2	1	III	–
9	2008	O	GP	1	Npx	No data	IV	1197	395	1	2	1	III	–
10	2008	O	GP	1	Nose	No data	IV	1197	395	1	2	1	III	–
11	2009	O	GP	5	Npx	Sinusitis	I	408	3	7	Single	6	III+	–
12	2009	W	OC	1	Ear	AOM	IV	1197	395	1	2	1	III	–
13	2009	W	GP	6	Nose	Sinusitis	III	1197	395	1	2	1	III	–
14	2009	W	GP	1	Ear	AOM	III	1197	395	1	2	1	III	–
15	2010	W	GP	1	Npx	AOM	III	1197	395	1	2	1	III	–
16	2011	O	GP	1	Eye	Conjunctivitis	II	422	422	4	3	3	III-like+	β,q,Ts
17	2011	O	GP	4	Nose	Sinusitis	II	142	142	8	Single	3	III-like+	–
18	2012	S	Hosp	8	Blood	MM/pneumonia	I	1287	None	9	Single	7	III	–
19	2012	W	OC	8	BAL	COPD/DC	II	160	160	2	Single	2	III+	Ts
20	2012	W	OC	1	Sputum	CF	III	148	245	10	Single	2	III+	–
21	2012	O	GP	1	Ear	AOM	I	124	124	2	Single	2	III+	Ts
22	2013	W	GP	8	Sputum	Pneumonia	IV	1282	503	4	Single	3	III-like+	β,C,T,Ts
23	2013	O ^m	Hosp	7	Nose	Pneumonia	II	422	422	4	3	3	III-like+	β,q,Ts
24	2013	W	Hosp	8	Eye	Dacryocystitis	III	159	503	5	4	2	III+	β,Q,C,T,Ts
25	2013	W	OC	1	Npx	PCD	III	159	503	5	4	2	III+	β,Q,C,T,Ts
26	2013	W	OC	4	Sputum	CF/pharyngitis	III	159	503	5	4	2	III+	β,Q,C,T,Ts
27	2013	W	Hosp	8	Sputum	CVD/pneumonia	IV	836	245	2	5	2	III+	β,Ts
28	2013	W	Hosp	8	Sputum	DC/pneumonia	III	836	245	2	5	2	III+	β,Ts
29	2013	W	Hosp	7	Sputum	COPD/pneumonia	IV	836	245	2	5	2	III+	β,Ts
30	2013	W	Hosp	8	Npx	Possible pneumonia	III	836	245	2	5	2	III+	β,Ts

CC: clonal complex; MLST: multilocus sequence typing; PBP3: penicillin-binding protein 3; ST: sequence type.

^a All isolates lacked polysaccharide capsule genes except for isolate 21 (serotype f).

^b M: mid Norway (Nord-Trøndelag); O: Oslo region (Oslo, Akershus, Buskerud, Vestfold); S: Southern Norway (Agder); W: Western Norway (Sogn og Fjordane, Hordaland, Rogaland).

^c GP: general practice; Hosp: hospitalised; OC: outpatient clinic.

^d 1: 0–9 years; 2: 10–19 years; 3: 20–29 years; 4: 30–39 years; 5: 40–49 years; 6: 50–59 years; 7: 60–69 years; 8: ≥70 years.

^e BAL: bronchioalveolar lavage; LRT: lower respiratory tract; Npx: nasopharynx.

^f ALL: acute lymphoblastic leukemia; AOM: acute otitis media; CF: cystic fibrosis; COPD: chronic obstructive pulmonary disease; CVD: cardiovascular disease; DC: disseminated cancer; MM: multimorbidity (hypogammaglobulinemia, chronic lymphocytic leukaemia, myasthenia gravis, COPD); PCD: primary ciliary dyskinesia; URTI: upper respiratory tract infection.

^g According to reactions (positive (+) or negative (-)) to indole, urease and ornithine decarboxylase (ODC) respectively. I: +/+/-; II: +/-/-; III: -/+/-; IV: -/+/- [18].

^h *ftsI* alleles (Figure 1).

ⁱ Isolates with identical MLST-*ftsI* allelic profiles (Figure 1). Isolates qualified as 'single' are those among the 30 analysed with a unique MLST-*ftsI* allele combination.

^j PBP3 type according to amino acid sequences (substitutions underscored) in positions 350, 357, 377, 385, 389, 502, 517, 526, 532, 547, 557, 562 and 569. 1: NNMTLRKTIYVS; 2: NNITFARKTIYLS; 3: NNITFAHNSIHVS; 4: NNITLAHNSIYVN; 5: NNITFTRKTVYVN; 6: NNITFTRKTVYLN; 7: DSMILTRKTIYVS.

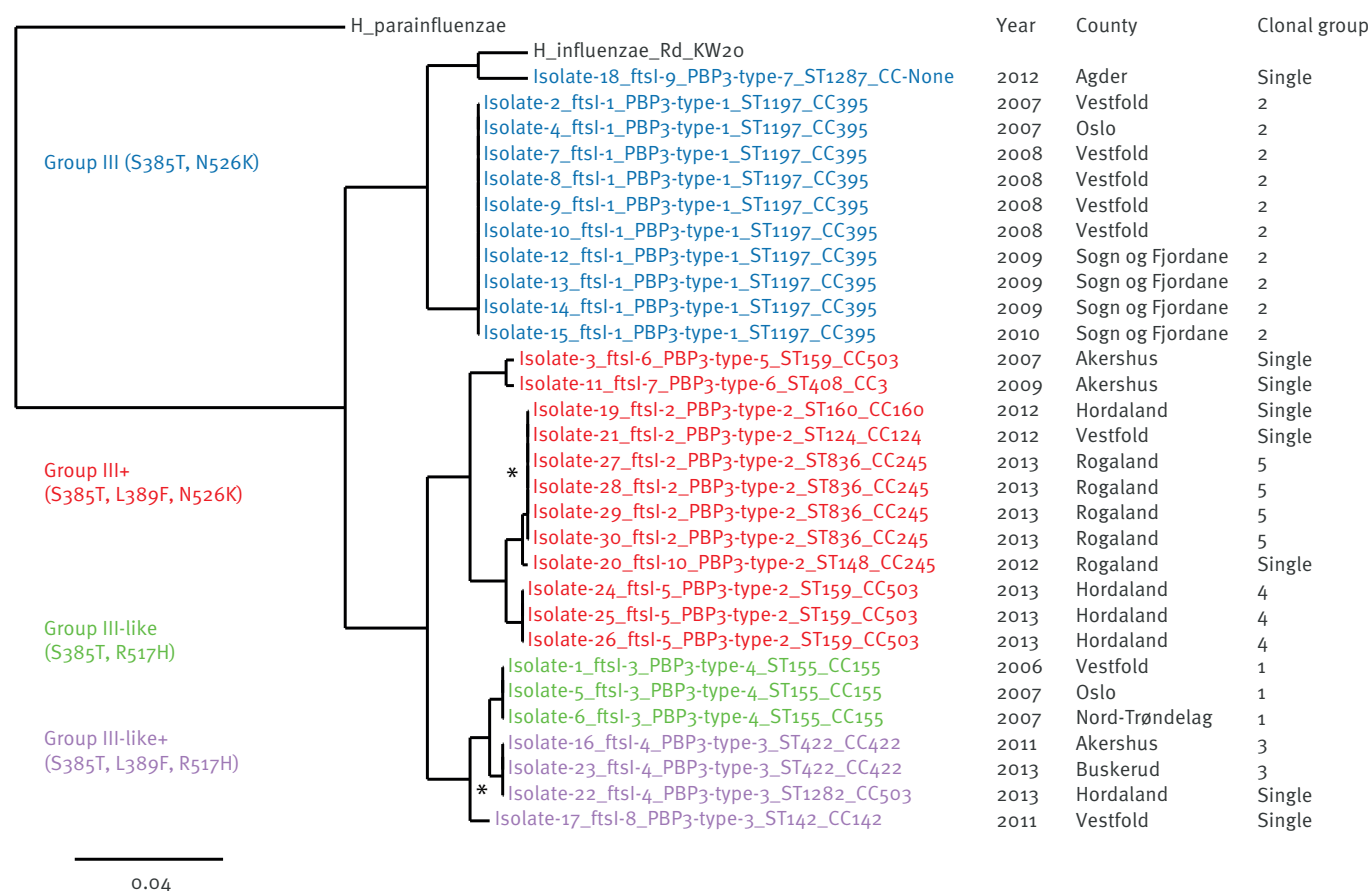
^k Group III: S385T and N526K; group III+: S385T, L389F and N526K; group III-like: S385T and R517H; group III-like+: S385T, L389F and R517H.

^l β: TEM-1 beta-lactamase; Q: quinolones; q: quinolones (low level resistant); C: chloramphenicol; T: tetracyclines; Ts: trimethoprim-sulfamethoxazole; –: none.

^m Recently travelled to Thailand.

FIGURE 1

Phylogram of *ftsI* DNA sequences from *Haemophilus influenzae* with high-level penicillin-binding protein 3-mediated beta-lactam resistance (high-rPBP3), Norway, 2006–2013 (n=30)



The phylogram was constructed using unweighted pair group method with arithmetic mean (UPGMA) and based on *ftsI* DNA sequences (transpeptidase domain, nucleotides 1010–1719) including *H. influenzae* Rd KW20 (GenBank accession number: U32793) as reference and *H. parainfluenzae* (European Molecular Biology Laboratory (EMBL) accession number: AB267856) as outgroup.

The scale is DNA divergence.

Labels indicate isolate numbers, *ftsI* alleles, penicillin-binding protein 3 (PBP3) types (Table 1), multilocus sequence typing sequence types (ST) and clonal complexes (CC).

Colours indicate PBP3 groups. Asterisks indicate *ftsI* alleles carried by unrelated STs.

Year/county of isolation and assignment to clonal groups (CG) to the right.

Results

Patient data

The median age of the 30 patients from whom the respective isolates were derived, was 29 years (range: 0–91). In the age group zero to nine years, most patients (proportion: 10/13) were below five years-old. The male/female ratio was 13/17. All but two patients lived in the Oslo region (14/30) or in Western Norway (14/30). Except for the patient with isolate 23, recently returning from Thailand, there was no known recent (< 3 months) travel history abroad (Table 1).

Ten isolates (10/30) were from hospitalised patients. The proportion of hospitalised patients was 4/21 in

the period between 2006 and 2012 compared to 6/9 in 2013. One of the 30 isolates was from blood; the rest were eye (2/30), ear (3/30) or respiratory isolates (24/30). Among the patients for whom clinical information was available (n=23), pneumonia (n=7) and acute otitis media (AOM) (n=5) were the most frequent infection types. At least seven of the 30 patients were predisposed for respiratory tract infections due to underlying chronic condition (Table 1).

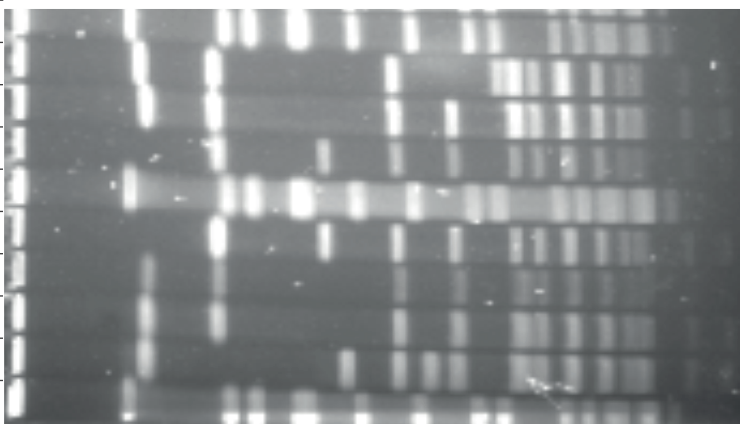
Strain characterisation

All isolates but one (Hif) lacked polysaccharide capsule genes and were categorised as nontypeable (NTHi). The isolates were biotypes I (proportion: 3/30), II (4/30), III

FIGURE 2

Pulsed-field gel electrophoresis (PFGE) band patterns and pulsotypes for *Haemophilus influenzae* isolates of clonal group 2, Norway, 2006–2013 (n=7)

Isolate	Year	County	Region	Pulsotype
Std				
2	2007	Ve	O	3
15	2010	SF	W	1
13	2009	SF	W	2
Std				
12	2009	SF	W	2
14	2009	SF	W	1
8	2008	Ve	O	1
4	2007	Os	O	4
Std				



O: Oslo region; Os: Oslo county; SF: Sogn og Fjordane county; Std: size standard (*Staphylococcus aureus* NCTC 8325); Ve: Vestfold county; W: Western Norway.

Labels indicate isolate numbers, year, county and region of isolation, and pulsotype.

The seven isolates depicted are described in Table 1. Isolate 2 is TEM-1 beta-lactamase positive, the remaining isolates are beta-lactamase negative.

(14/30) and IV (9/30). The Hif isolate and the blood isolate were both biotype I.

Twelve STs were represented, including ST1282 and ST1287 not previously registered in the MLST database (Table 1). Eleven of the 12 STs belonged to nine CCs; the last (ST1287) could not be assigned to a CC. ST1197 (CC395) was the most frequent ST (10/30). Two STs (ST1197 and ST836) encompassed both biotype III (ODC negative) and IV isolates (ODC positive).

ftsI/PBP3 genotypes

Ten *ftsI* alleles, encoding seven PBP3 substitution types and carried by one to 10 isolates, were observed (Table 1). Two *ftsI* alleles (*ftsI*-2 and *ftsI*-4) were present in unrelated STs (Figure 1).

The majority of isolates (proportion: 23/30) had the N526K first stage substitution and were thus categorised as group III (11/30) or group III+ (12/30). Seven isolates (7/30) had the R517H first stage substitution and were thus categorised as group III-like (3/30) or group III-like+ (4/30).

The third stage substitution L389F was present in 16 of 30 isolates (Table 1).

Clonal groups

Five clusters comprising two to 10 isolates with identical MLST-*ftsI* allelic profiles (clonal groups, CG) were identified, accounting for 22 of 30 isolates. Eight isolates of 30 had unique MLST-*ftsI* allelic profiles,

including the single Hif isolate and the single invasive isolate (Table 1).

CG2 (n=10 isolates) was found in two separate geographical regions (Oslo region and Western Norway) during a period of three years. To confirm genetic relationship, PFGE of seven isolates belonging to CG2 (one beta-lactamase positive and six beta-lactamase negative isolates) was performed, and four related pulsotypes (1–4) were observed (Figure 2). Pulsotypes 2 (n=2), 3 (n=1) and 4 (n=1) differed from pulsotype 1 (n=3) by two, two and three bands, respectively. Pulsotype 1 was observed in both geographical regions. No pulsotypes differed by more than five bands.

The three CG4 isolates were sampled during a period of four days from one hospitalised patient and two outpatients related to the same hospital. CG5 (n=4) included two patients from the same household (patients 27 and 28) and was restricted to one hospital department within a period of 16 days.

The clonal groups (and the single strains) differed considerably with respect to PBP3 resistance genotypes and co-resistance (Table 1). Notably, in contrast to CG1 and CG2, the third stage L389F substitution was present in CG3, CG4 and CG5. CG1 and CG2 had no co-resistance to non-beta-lactams, whereas CG4 was multidrug resistant.

TABLE 2

Antimicrobial susceptibility of *Haemophilus influenzae* isolates with high-level penicillin-binding protein 3-mediated beta-lactam resistance (high-rPBP3), Norway, 2006–2013 (n = 30)

Agents	MIC range (mg/L)	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	Breakpoints (S ≤/R >) ^a	R or I/R n (%) ^b
Ampicillin ^c	1 – ≥8	4	≥8	1/1	29 (97)
Amoxicillin ^d	4 – 32	8	8	2/2	30 (100)
Cefuroxime	2 – ≥16	8	≥16	1/2	1 (3) / 29 (97)
Cefotaxime	≤0.12 – ≥4	0.25	1	0.12/0.12	26 (87)
Ceftriaxone	≤0.03 – 2	0.12	0.5	0.12/0.12	14 (47)
Cefixime	0.12 – ≥2	0.5	≥2	0.12/0.12	29 (97)
Cefepime	0.25 – ≥4	1	2	0.25/0.25	28 (93)
Ceftaroline	0.03 – 0.25	0.12	0.25	0.03/0.03	26 (87)
Imipenem	≤0.5 – 2	1	1	2/2	0 (0)
Meropenem ^e	0.12 – 2	0.25	0.5	0.25/1 ^e	9 (30) / 1 (3)
Ciprofloxacin	≤0.06 – 2	≤0.06	0.25	0.5/0.5	3 (10)
Levofloxacin	≤0.03 – 1	≤0.03	0.25	1/1	0 (0)
Chloramphenicol	0.25 – ≥8	0.5	≥8	2/2	4 (13)
Rifampicin ^f	0.25 – 1	0.5	0.5	1/1 ^f	0 (0)
Tetracycline	0.5 – ≥8	0.5	≥8	1/2	0 (0) / 4 (13)
Doxycycline	0.25 – 2	0.25	2	1/2	4 (13) / 0 (0)
Minocycline	0.12 – 0.5	0.25	0.5	1/2	0 (0) / 0 (0)
Trimethoprim-sulfamethoxazole	0.12 – ≥4	0.5	≥4	0.5/1	0 (0) / 13 (43)
Erythromycin ^g	2 – 4	2	4	0.5/16 ^g	30 (100) / 0 (0)
Clarithromycin ^g	2 – 16	4	8	1/32 ^g	30 (100) / 0 (0)
Azithromycin ^g	≤0.25 – 1	0.5	0.5	0.12/4 ^g	30 (100) / 0 (0)
Roxithromycin ^g	2 – 16	4	8	1/16 ^g	30 (100) / 0 (0)
Telithromycin ^g	0.5 – 2	1	1	0.12/8 ^g	30 (100) / 0 (0)

I: intermediate; MIC: minimum inhibitory concentration; R: resistant; S: susceptible.

^a European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [6]. The I category is inferred from the S and R breakpoints.

^b Isolates categorised as R (when only S and R categories exist) or as I/R (when an I category is defined).

^c Beta-lactamase positive isolates (n = 11) tested in the presence of sulbactam (2:1 ratio).

^d Beta-lactamase positive isolates (n = 11) tested in the presence of clavulanic acid (2:1 ratio).

^e Meningitis breakpoints. All isolates were susceptible according to general breakpoints (2/2).

^f Prophylaxis breakpoints.

^g Macrolide breakpoints categorise the wild type population as intermediately susceptible.

Penicillin-binding protein 3-mediated resistance

All strains were categorised as resistant to at least one of the extended-spectrum cephalosporins cefotaxime, ceftriaxone, cefixime, cefepime and ceftaroline; proportions of resistant isolates varied from 14/30 for ceftriaxone to 29/30 for the oral agent cefixime (Table 2). In general, strains with the N526K first stage substitution had higher MICs to extended-spectrum cephalosporins (except cefixime) than R517H strains. Furthermore, the third stage L389F substitution was generally associated with higher MICs to extended-spectrum cephalosporins and meropenem. Notably, eight of 12 group III+ isolates (N526K, S385T and L389F) were non-susceptible to meropenem (Table 3).

Individual susceptibility profiles and resistance genotypes are shown in Figure 3. In contrast to other group III isolates, the single invasive ST1287 isolate (isolate number 18) was categorised as susceptible to ampicillin (MIC=1 mg/L), cefotaxime (MIC≤0.12 mg/L), ceftriaxone (MIC≤0.03 mg/L), cefixime (MIC=0.12 mg/L) and cefepime (MIC=0.25 mg/L). Conversely, the single ST124 Hif isolate (isolate number 21) generally expressed higher resistance levels to beta-lactams than other group III+ isolates.

Figure 3. Year-to-year overview of *Haemophilus influenzae* isolates with high-level penicillin-binding protein 3-mediated beta-lactam resistance (high-rPBP3), Norway, 2006–2013 (n=30)

TABLE 3

Penicillin-binding protein 3 (PBP3) groups and susceptibility to cephalosporins and carbapenems of *Haemophilus influenzae* isolates with high-level PBP3-mediated beta-lactam resistance (high-rPBP3), Norway, 2006–2013 (n=30)

Agents	Third stage high-rPBP3 ^a				Second stage high-rPBP3 ^b			
	Group III+ (n=12)		Group III-like+ (n=4)		Group III (n=11)		Group III-like (n=3)	
	MIC ₅₀ (mg/L)	R or I/R n (%) ^c	MIC ₅₀ (mg/L)	R or I/R n (%) ^c	MIC ₅₀ (mg/L)	R or I/R n (%) ^c	MIC ₅₀ (mg/L)	R or I/R n (%) ^c
Cefuroxime	8	1 (8) / 11 (92)	8	0 (0) / 4 (100)	8	0 (0) / 11 (100)	≥16	0 (0) / 3 (100)
Cefotaxime	1	12 (100)	0.5	4 (100)	0.25	9 (82)	≤0.12	1 (33)
Ceftriaxone	0.25	10 (83)	0.25	4 (100)	0.06	0 (0)	0.06	0 (0)
Cefixime	0.5	12 (100)	≥2	4 (100)	0.25	10 (91)	≥2	3 (100)
Cefepime	2	12 (100)	1	4 (100)	0.5	10 (91)	0.5	2 (67)
Ceftaroline	0.25	12 (100)	0.12	4 (100)	0.06	10 (91)	0.03	0 (0)
Imipenem	1	0 (0)	≤0.5	0 (0)	1	0 (0)	1	0 (0)
Meropenem	0.5	7 (58) / 1 (8) ^d	0.25	0 (0) / 0 (0) ^d	0.25	2 (18) / 0 (0) ^d	0.12	0 (0) / 0 (0) ^d

^a L389F present. Group III+: S385T, L389F and N526K; group III-like+: S385T, L389F and R517H.

^b L389F absent. Group III: S385T and N526K; group III-like: S385T and R517H.

^c According to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [6]. The exact breakpoints are presented in Table 2. Isolates categorised as I/R (when an I category is defined) or as R (when only S and R categories exist).

^d Meningitis breakpoints (0.25/1) used for categorisation. All isolates were susceptible according to general breakpoints (2/2).

Co-resistance

A total of 11 of 30 strains produced beta-lactamase and all were TEM-1 positive (Table 1). Co-resistance to trimethoprim-sulfamethoxazole (13/30) was frequent. Four of 30 isolates were resistant to chloramphenicol and tetracycline, including the three CG4 isolates and a single isolate belonging to the same clonal complex (CC503) as those forming the CG4. No isolates expressed increased resistance to macrolides or rifampicin (Table 3).

Five of 30 isolates had ciprofloxacin MIC > 0.06 mg/L and possessed amino acid substitutions in the QRDR of *GyrA* and/or *ParC*. Three isolates, all belonging to CG4, had substitutions in both proteins and were clinically resistant to ciprofloxacin. Two isolates, both CG3, had only one significant *GyrA* substitution and quinolone MICs within the susceptible category (Table 4).

Case reports

The clinical course was evaluated for three patients with well-defined serious infections most likely caused by one of the study isolates (patients and isolates 18, 27 and 28). Isolate 18 was cultured from the blood of a patient with X-ray confirmed pneumonia, chronic obstructive pulmonary disease (COPD), hypogammaglobulinemia, chronic lymphocytic leukaemia and myasthenia gravis (Table 1). The strain was categorised as susceptible to cefotaxime (MIC ≤ 0.12 mg/L, Figure 3). The patient responded well to initial parenteral therapy with cefotaxime (three days) followed by ciprofloxacin orally.

Isolates 27 and 28 belonged to CG5 and were beta-lactamase positive and resistant to ampicillin-sulbactam (MIC = 8 mg/L) and cefotaxime (MIC = 1 mg/L) according to EUCAST breakpoints [6]. Both were isolated from sputum in patients with X-ray confirmed pneumonia (C-reactive protein ≥ 160 mg/L; norm: < 5 mg/L) and significant co-morbidity. Patient 27 (cardiovascular disease) responded to therapy with cefotaxime (dosage 2 g three times a day) after initial treatment with benzylpenicillin. Patient 28 (disseminated cancer) responded to ciprofloxacin after initial treatment with piperacillin-tazobactam.

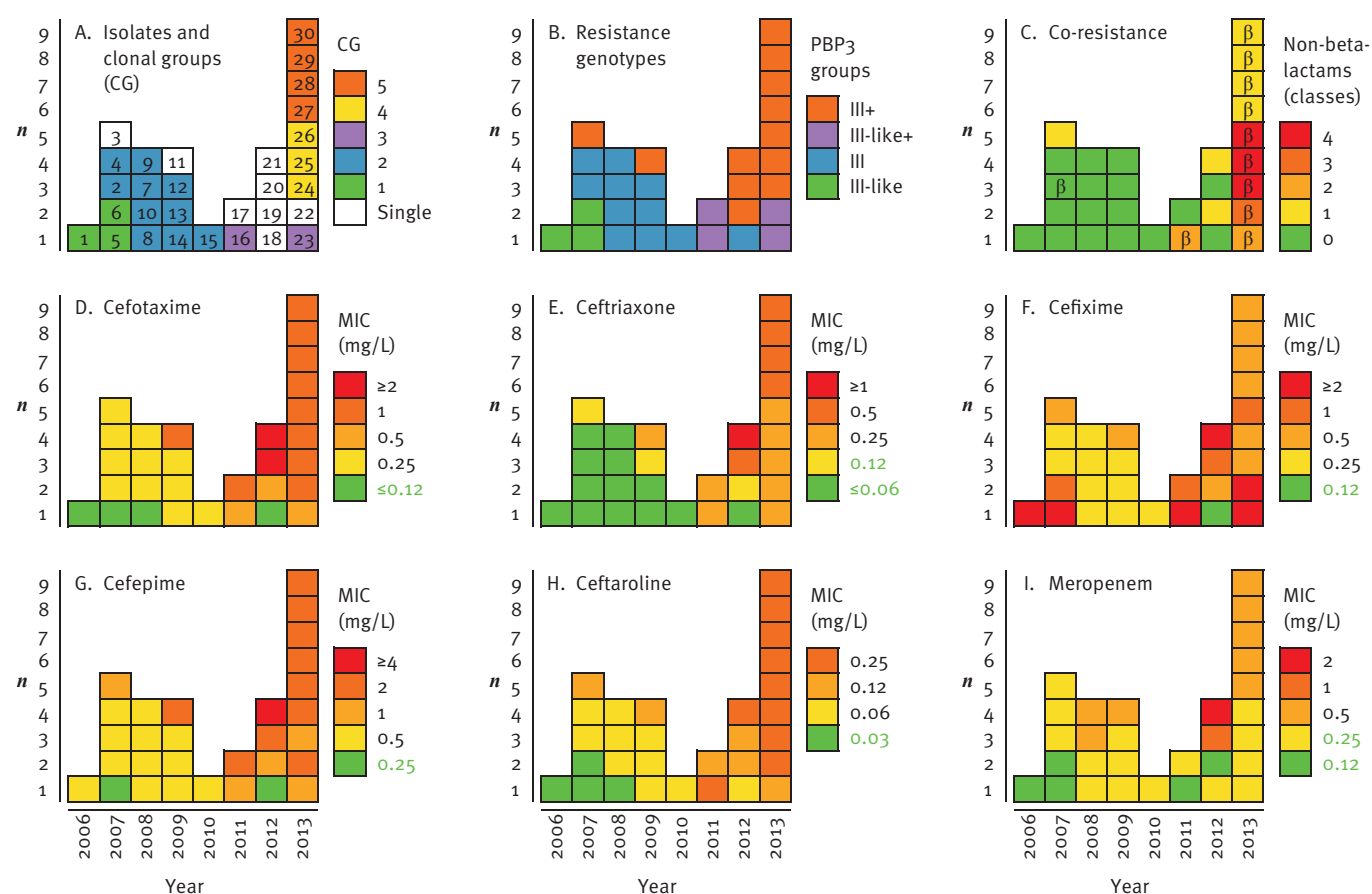
Discussion

We have characterised 30 clinical *H. influenzae* isolates from Norway with high-level PBP3-mediated beta-lactam resistance, including 23 isolates possessing both the S385T and N526K substitutions. Previously, isolates with this genotype (group III and group III+) have primarily been reported from Japan [14] and Korea [15].

The prevalence of low-rPBP3 in respiratory NTHi in Norway has increased gradually since 2001 and was estimated to 15% in 2007; 113 low-rPBP3 and three high-rPBP isolates (isolates 4–6 in this study) were reported among 795 surveillance isolates in 2007 [8]. When the prevalence of low-rPBP3 in Japan in the 1990s reached approximately the same level as in Norway in 2007, the prevalence of high-rPBP3 isolates increased from zero to 29% in few years [14]. Similar observations were reported from Korea during the 2000s [15]. NTHi was characterised as an emerging pathogen in a most

FIGURE 3

Year-to-year overview of *Haemophilus influenzae* isolates with high-level penicillin-binding protein 3-mediated beta-lactam resistance (high-rPBP3), Norway, 2006–2013 (n=30)



MIC: minimum inhibitory concentration; PBP3: penicillin-binding protein 3.

Colours indicate the following parameters: A, CGs (identical MLST-*ftsI* profiles, Table 1); B, PBP3 resistance genotypes (grouped according to substitutions in positions 385, 389, 517 and 526, Table 1); C, co-resistance to non-beta-lactam antimicrobial agents (number of classes, including low-level resistance to quinolones) and beta-lactamase (β); D–I, MICs (broth microdilution) to extended-spectrum cephalosporins and meropenem.

Green numbers on the MIC scale indicate values below the European Committee on Antimicrobial Susceptibility Testing (EUCAST) S-breakpoint [6]. Each box indicates the same isolate in all diagrams. Isolates collected the same year (columns) are sorted by PBP3 groups, clonal groups and cefotaxime MIC.

recent review article [1]. Emphasising the emergence and spread of strains with PBP3-mediated resistance, the authors recommended implementation of standardised surveillance protocols and typing methodologies.

We recently suggested MLST-*ftsI* typing as a tool for molecular surveillance of PBP3-mediated resistance in *H. influenzae* [8]. By this approach, DNA-based resistotyping using the transpeptidase region as an additional allele is combined with MLST allelic profiles. This sequence is readily available if PBP3 resistance genotyping is done by sequencing. In that study [8], MLST clonal complexes corresponded well to PFGE clusters. Addition of the *ftsI* allele to MLST allelic profiles increased the discriminatory power compared with MLST alone and MLST combined with PBP3 substitution patterns, without compromising consistency with PFGE-based grouping. The observation that CG2 in the

present study (and most MLST-*ftsI* types in the previous study) encompassed related pulsotypes supports the validity of the typing scheme although discriminatory power is inferior to PFGE. Increasing the length of the *ftsI* fragment could improve resolution but this would most likely be at the cost of reduced sensitivity for clone detection.

So far, the molecular epidemiology of high-rPBP3 strains is poorly described. However, two invasive group III-like isolates recently reported from Spain had MLST allelic profiles (ST155 and ST1118) identical or closely related to CG1 in the present study; the Spanish isolates and CG1 also had identical PBP3 substitution patterns as far as comparison is possible [3]. These observations suggest that CG1 and the two Spanish isolates may be representatives of a virulent high-rPBP3 NTHi clone distributed within Europe.

TABLE 4

GyrA/ParC amino acid substitutions in *Haemophilus influenzae* isolates with high-level penicillin-binding protein 3-mediated beta-lactam resistance (high-rPBP3) and raised minimum inhibitory concentrations to quinolones, Norway, 2006–2013 (n=5)^a

Isolate ^b	MICs (mg/L) and breakpoints (S≤/R>) ^c		GyrA ^d		ParC ^d		
	CIP (0.5/0.5)	LEV (1/1)	S84	D88	S84	N138	G206
16	0.25	0.25	L	–	–	–	R
23	0.25	0.25	L	–	–	–	R
24	2	1	L	N	I	S	–
25	2	1	L	N	I	S	–
26	2	1	L	N	I	S	–

CIP: ciprofloxacin; LEV: levofloxacin; MIC: minimum inhibitory concentration; –: no substitution.

^a The remaining 25 isolates had ciprofloxacin MICs≤0.06 mg/L and levofloxacin MICs≤0.03 mg/L.

^b Further characteristics are described in Table 1.

^c European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [6].

^d Amino acid substitutions in GyrA (64–177) and ParC (66–236).

Travel history suggested that one isolate in this study may have been imported from Thailand, although an isolate with identical MLST-*ftsI* profile was observed in Norway two years earlier (CG3).

In this study, CG4 and CG5 were respectively restricted to one hospital (both hospitals located in Western Norway) and sampled within short time periods. Two of the CG5 isolates were sampled from persons within the same household. Person-to-person transmission of nontypeable *H. influenzae* has been reported previously, including an outbreak with an amoxicillin-resistant strain in a respiratory ward [27] and intrafamilial transmission of rPBP3 strains [28]. These observations highlight the importance of hygiene measures in health institutions to prevent nosocomial spread.

The observation of identically mutated *ftsI* alleles in unrelated STs suggests horizontal gene transfer (HGT). Similar observations have been reported by us previously [8]. Thus, resistant strains may have developed in Norway by HGT of alleles conferring resistance; these alleles may have been imported at an earlier stage or have evolved locally. The observation of allele mosaicism in *H. influenzae* [29] supports this interpretation and strongly suggests that allele transfer has occurred via transformation and recombination with the chromosome.

By EUCAST clinical breakpoints [6], all isolates were categorised as resistant to at least one extended-spectrum cephalosporin. Clinically relevant differences in resistance profiles between PBP3 groups were observed. Isolates possessing the third stage substitution L389F were generally more resistant to cephalosporins than the remaining isolates, consistent

with the results of a previous investigation using site-directed mutagenesis and transformation to study the impact of PBP3 substitutions on beta-lactam susceptibility [13]. Notably, most L389F positive isolates in the present study were ceftriaxone resistant, while all isolates lacking this substitution were ceftriaxone susceptible. The three-stage classification system used in this study, separating between isolates with and without the L389F substitution, appears to reflect the step-wise development of PBP3-mediated resistance better than systems based on S385T and R517H/N526K only.

We evaluated the effectiveness of the antimicrobial therapy for three patients with X-ray confirmed pneumonia associated with study strains. Notably, one patient responded to therapy with high dosage of cefotaxime although the isolate (from sputum) was categorised as resistant (MIC=1 mg/L) according to EUCAST clinical breakpoints [6]. This is consistent with current EUCAST non-species related breakpoints for cefotaxime based on a dose of at least 2 g three times a day (R>2 mg/L) [6]. The isolate would have been categorised as susceptible to cefotaxime by CLSI breakpoints for *H. influenzae* (S≤2 µg/mL) [7].

The single ST1287 blood isolate from an immunocompromised patient with pneumonia is to our knowledge the first reported invasive group III high-rPBP3 isolate from Europe. This strain generally expressed lower MICs to beta-lactams than noninvasive group III study isolates and was categorised as cefotaxime susceptible (MIC≤0.12 mg/L). The patient responded well to cefotaxime therapy.

Conversely, the single Hif group III+ isolate was more resistant than nontypeable isolates with comparable genotypes. These observations suggest that

strain-associated mechanisms other than PBP3 substitutions may modify resistance levels in rPBP3 isolates. Increased efflux due to mutations in the *acrR* gene, encoding a repressor of the AcrAB efflux pump, is associated with high-level macrolide resistance in *H. influenzae* and may increase resistance to ampicillin in rPBP3 strains [30]. The *acrR* gene was not sequenced in the present study but all isolates expressed wild-type MICs to macrolides.

Co-resistance was more frequent than expected. The proportions of trimethoprim-sulfamethoxazole resistant and beta-lactamase positive isolates were twice and three times, respectively, the overall reported prevalences in respiratory *H. influenzae* isolates (n = 677) in a Norwegian nationwide surveillance study in 2011 [31]. Similarly, resistance rates for ciprofloxacin, tetracycline and chloramphenicol in this study were 10% (3/30), 13% (4/30) and 13% (4/30), respectively, compared with 0.1%, 0.4% and 0.7% in the 2011 surveillance report.

EUCAST define resistance to ciprofloxacin as an exceptional phenotype in *H. influenzae* and recommend that such isolates are referred to a reference laboratory [5,6]. Quinolone resistance in this species is usually due to QRDR substitutions in subunit A of topoisomerase II (*GyrA*) and subunit A of topoisomerase IV (*ParC*). Resistance levels depend largely on the number of substitutions in the positions 84 and 88 in both proteins [32]. Isolates with single substitutions are usually low-level resistant but other mechanisms such as the transferable plasmid-mediated acetyl transferase gene *aac(6')-Ib-cr* may increase resistance [33,34]. The QRDR substitution patterns and quinolone MICs observed in this study are consistent with previous reports [26,32]. The nalidixic acid 30 µg disk is superior to the ciprofloxacin 5 µg disk for detection of isolates with low-level quinolone resistance and may be used for screening [6,26].

Quinolone resistance in *H. influenzae* is associated with hypermutability [33]. It seems likely that hypermutability also increases the ability to acquire PBP3 substitutions by spontaneous mutations, favouring the development of strains with both resistance mechanisms. To our knowledge, CG4 represent the first ciprofloxacin-resistant high-rPBP3 isolates reported outside Japan, and the first clonal cluster ever with this particular combination of resistance mechanisms.

The CG4 isolates had TEM-1 beta-lactamase and were resistant to chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole. This resistance profile suggests the presence of a conjugative large plasmid [35], now recognised as an integrating and conjugative element (ICE) designated ICE*Hin1056* [36]. The resistance of CG4 might thus be the result of two independent factors: hypermutability, leading to PBP3 and QRDR substitutions, and the acquisition of ICE*Hin1056*. Hypermutable *H. influenzae* are prevalent in cystic

fibrosis (CF) patients [37]. One CG4 isolate was found in sputum from a CF patient; another isolate was from nasopharynx of a patient with primary ciliary dyskinesia (PCD). High antibiotic pressure in these patient categories may contribute to selection of multidrug resistant strains.

The ST of CG4 (ST159) is associated with increased virulence in chronic obstructive pulmonary disease due to an additional IgA1 protease, encoded by the *igaB* gene [38].

The emergence of clonally related high-rPBP3 isolates in Norway is a cause for concern. As previously reported from Japan and Korea, a shift from low-level to high-level rPBP3 might alter the epidemiological situation dramatically in few years [14,15]. A similar development in Europe would threaten current recommendations for empiric antimicrobial treatment in severe disease [1]. The situation urges for improved surveillance systems for antimicrobial resistance in *H. influenzae* in Europe.

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Conflict of interest

None declared.

Authors' contributions

DS conceived and coordinated the study; AS and BEK contributed to study design. DS, OBN and MS collected bacterial isolates and clinical information; DS performed or was responsible for testing and characterisation of isolates in collaboration with GK and EM (MIC determination); MS (MLST and capsular serotyping) and ILA (remaining molecular analyses). DS analysed and interpreted data and wrote the first draft. All authors participated in interpretation of results, critically revised the draft for intellectual content and approved the final article.

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