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Analysis of *Haemophilus* species in patients with respiratory tract infections in Yaoundé, Cameroon

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Objectives: To identify*Haemophilus* species and characterise the antimicrobial susceptibility of isolates from patients with respiratory tract infections (RTIs) in Cameroon.
Methods: Isolates (n = 95) were from patients with RTIs obtained from two hospitals in Yaoundé, Cameroon. Isolates were identified by biochemical assay, a polymerase chain reaction (PCR)-based method, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), and whole genome sequencing. Antibiotic minimum inhibitory concentrations were determined by E-test.
Results: Haemophilus influenzae (*H. influenzae*) was the most prevalent species, varying from 76.8 to 84.2% according to the different methods. The isolates were mainly non-typeable (n = 70, 96%). Three *H. influenzae* isolates were capsulated (b, e and f). The isolates were genetically diverse and 40 unique sequence types were identified, including 11 new ones. Resistance to ampicillin was observed among 52 of 94 (55.3%), and 14 of the 52 (26.9%) produced TEM-1 β-lactamase. PBP3 mutations occurred in 40 of 52 (76.9%) ampicillin-resistant isolates. Eleven isolates were chloramphenicol-resistant, with eight of 10 (80%) producing chloramphenicol acetyltransferase. Four *Haemophilus* isolates were rifampicin-resistant, with two mutations in *rpoB* gene. Five isolates were ciprofloxacin-resistant and harboured mutations in the quinolone-resistance-determining regions of *gyrA* and *parC* genes.
Conclusion: The*H. influenzae* isolates were highly diverse and showed high levels of antibiotic resistance. *H. influenzae* serotype b is still circulating in the post-vaccination era.
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Introduction

The genus *Haemophilus* is a member of the *Pasteurellaceae* family and is usually represented as non-motile, aerobic or facultative anaerobic Gram-negative coccobacillus (Winslow et al., 1917). The most commonly known species is *Haemophilus influenzae* (*H. influenzae*), which is classified into six serotypes (a–f) on the basis of a capsular polysaccharide as well as a non-encapsulated type (non-typeable). *Haemophilus haemolyticus* (*H. haemolyticus*), *Haemophilus parahaemolyticus* and *Haemophilus parainfluenzae* (*H. parainfluenzae*) are also species of the *Haemophilus* genus (Winslow et al., 1917). These species are among the early colonisers of the upper respiratory tract and can often cause respiratory tract infections (RTIs) in children and the elderly. Additionally, they are (particularly *H. influenzae*) major causes of severe invasive infections such as meningitis and bacteraemia. *H. influenzae* serotype b is the most virulent and was estimated to account for approximately 400,000 global deaths annually in 2007 (WHO, 2006).

Haemophilus infections in Cameroon were reported with a prevalence of: 20% in the upper respiratory tract among school children in Buea in 2008 (Ndip et al., 2008); 27.7% in bacterial meningitis in children in three hospitals located in Yaoundé, Dschang and Kousseri in 2012 (Gervaix et al., 2012); and 3.7% in

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non-tuberculosis purulent pleural effusion in adults in Yaoundé in 2012 (Pefura Yone et al., 2012). Considering antibiotic resistance, the isolates were frequently resistant to β-lactams (penicillin, 100%; ampicillin, 60%), sulfonamides (100%) and chloramphenicol (30%) (Ndip et al., 2008).

Vaccination against *H. influenzae* serotype b (Hib) was introduced in Cameroon in 2009 through the Expanded Program on Immunization and it is free of charge for children aged 0–11 months. Vaccine coverage varies from 22.8 to 93.3% according to location (Ateudjieu et al., 2020; Chiabi et al., 2017; Gervaix et al., 2012). Since the introduction of this vaccine in many countries, the burden of *Haemophilus*-related infections has been increasingly dominated by non-typeable *H. influenzae* (NTHi). For instance, no meningitis with Hib was recorded in the North of Cameroon after the introduction of the vaccine (Massenet and Tapindjin-Gake, 2010), showing the positive effect of the vaccine.

Vaccine failure has been observed in other countries (Lee et al., 2008; Purohit et al., 2014) and *Haemophilus* species as pathogens in RTIs after introduction of the Hib conjugated vaccine have not been well studied in Cameroon because most data have focused on phenotypic characterisation. Moreover, discriminating between NTHi and other *Haemophilus* species is challenging and misidentification of H. haemolyticus as NTHi has been reported (Pickering et al., 2014; Zhang et al., 2014) due to the high similarity in morphology and biochemical characteristics between them. Usually, NTHi is associated with RTIs that result in antibiotic prescription, and probabilistic antibiotic therapy can select resistant isolates, whilst *H. haemolyticus* is rarely associated with disease (Anderson et al., 2012). Correctly identifying *Haemophilus* species in infection is currently an expanding area of study because of the impact on diagnosis and treatment, and knowing that all these *Haemophilus* species present potential risk of triggering invasive and severe infections. Their colonisation begins in the upper airways and can spread throughout the respiratory tract, potentially leading to invasive infections (van Belkum et al., 2007).

This study aimed to describe the molecular epidemiology of *Haemophilus* species isolated from patients with RTIs in Yaoundé, Cameroon.

Methods

Patients and bacterial isolates

Haemophilus species isolates were recovered from hospitalised patients with RTIs who attended Jamot Hospital and Essos Hospital Centre in Yaoundé, Cameroon, from January 2017 to March 2018. Jamot Hospital is the referral hospital for management of respiratory diseases in Yaoundé and its surroundings. The Essos Hospital Centre is one of the referral hospitals for paediatrics and the main site for recruitment of children with severe RTIs in the city of Yaoundé as part of influenza surveillance. Patients were consecutively enrolled in the site studies. Patients who presented at least two of the following symptoms were considered as suffering from RTIs: fever, cough, dyspnoea, wheezing, chest pain or sore throat. Up to 100% of patients from Essos Hospital Centre suffered from upper RTIs whereas 89% of the patients from the Jamot Hospital suffered from lower RTIs. Age and sex of patients, history of Hib immunisation and prior antibiotic therapy were documented. The clinical samples from which *Haemophilus* isolates were detected were: nasopharyngeal swabs, pleural fluids, sputa, and bronchoalveolar lavage. The isolates were therefore mostly from non-invasive infections. Across the sites, more than half of the participants had taken antibiotics prior to admission (68.6%) and bacterial growth was associated with this parameter.

Bacterial growth, DNA preparation

Isolates were cultured onto polyvitex chocolate agar plates and incubated at 37 °C in 5% CO_2 for 18–24 h. DNA extraction for polymerase chain reaction (PCR) and next-generation sequencing were performed as previously described (Deghmane et al., 2019).

Bacterial identification

Haemophilus species were initially identified by colony morphology, Gram stain and requirement for growth factors (V-, X- and XV-factors). PCR of the *ompP2*, *bexA*, *fucK*, *iga*, and *hpd* genes was applied as previously described (Deghmane et al., 2019). Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) Biotyper, version 3.0 (Bruker Daltonics, Champs sur Marne, France) was performed as previously described (Hong et al., 2019). Genetic identification was performed by ribosomal multilocus sequence typing (rMLST) on pubmlst.org site. BLAST analysis for homology of the *rpoB* gene was performed on https://blast.ncbi.nlm.nih.gov for the identification of *Haemophilus* species.

Serotyping

A slide agglutination kit was used (ImmuLex *H. influenzae* type a–f antisera, MEDIFF, Aubagne, France). A PCR to detect the capsule-producing gene *bexA* and *cap* genes for determining capsular serotypes was also performed in all *H. influenzae* isolates (Falla et al., 1994).

Susceptibility testing

Disk diffusion method was used according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (EUCAST, http://www.eucast.org/) and minimal inhibitory concentrations (MICs) were determined by E-tests following the manufacturer's guidelines (bioMérieux SA, Marcy-l'Étoile, France). β-lactamase activity was screened for all isolates by the chromogenic nitrocefin test (nitrocefin disks, bioMérieux SA, Marcy-l'Étoile, France). The *H. influenzae* strains ATCC49247 and ATCC49766 were used as controls.

Whole genome sequencing (WGS) analysis

Illumina technology (NextSeq 500, Illumina) was used. Library preparation was performed as previously described. The Multilocus sequence typing (MLST) profiles for *H.* influenzae were extracted from the whole genome sequence through the website http://pubmlst.org/h*influenzae*/; allele numbers and sequence types (ST) were assigned. The relationship search between STs generated in this study and existing STs in the MLST global database was evaluated by eBURST analysis in Phyloviz 2.0 software (Francisco et al., 2009; Nascimento et al., 2017). Other tools were also used and available on the PubMLST database (genome comparator tools).

Whole genome sequence data were also analysed using a gene-by-gene approach using the annotated reference strain (Rd KW20) on the Bacterial Isolate Genome Sequence Database (BIGSdb) platform on PubMLST (Jolley and Maiden, 2010). SplitsTree4 (version 4.14.6) was used to visualise the resulting distance matrices as neighbour-net networks (Huson and Bryant, 2006). GrapeTree was also drawn on the basis of comparisons of allelic profiles for the isolates with complete MLST data. The IDs of *H. influenzae* isolates with complete MLST data are given in the Supplementary Table to allow retrieval of whole genome sequences in FASTA formats.

Multiple alignments of *rpoB*, *gyrA* and *ftsI* proteins were performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). For *ftsI* profiles not determined by Illumina sequencer, Sanger sequencing was applied. Neighbour-Net SplitsTree graphs were generated using SplitsTree4 to visualise trees of *Haemophilus* species from distance matrices.

Molecular mechanism of antibiotic resistance

From the sequencing data, mutations/alterations in genes encoding enzymes associated with antibiotic resistance were extracted from http://pubmlst.org/*hinfluenzae*/ on the basis of published data from the literature. The mutations associated with fluoroquinolone resistance were detected from the quinolone resistance-determining regions (QRDRs) of gyrA, *parC* and *parE* genes (Puig et al., 2014). Similarly, mutations associated to rifampicin were extracted from the *rpoB* gene (Chang et al., 2011). For chloramphenicol resistance, chloramphenicol acetyltransferase (*cat*) gene production was searched. For β-lactams, three approaches were considered: production of β-lactamases, mutations in the *ftsI* gene encoding penicillin-binding protein 3 (PBP3) or both mechanisms (Dabernat et al., 2002; Deghmane et al., 2019). ROB-1 or TEM-1 β-lactamases were determined by DNA sequence comparisons (Livrelli et al., 1991). Mutations in the *ftsI* gene encoding PBP3 were determined using http://pubmlst.org/*hinfluenzae*/ (Deghmane et al., 2019).

Data analysis

Data were analysed using the Statistical Package for Social Sciences software (version 22.0, SPSS Inc., Chicago, IL, USA). For isolate identification, the agreement between methods was estimated. The Chi-square test was used to compare categorical variables. Statistically significant differences were defined as those for which the probability of occurrence was <5%.

Results

Ninety-five apparent *Haemophilus* isolates from 440 patients (21.6%) suffering from RTIs were collected, among whom 59 (62.1%) were females. As shown in Table 1, 74 of the 95 (77.9%) *Haemophilus* isolates were from children. Hib immunisation status could be determined in 73.7% cases (70/95). Antibiotic treatment prior to bacterial culture was 54.7% (52/95).

Identification

On the basis of phenotypic growth requirement, 80 of the 95 (84.2%) tested isolates in this study were identified as *H. influenzae*, while the remaining 15 isolates were identified as *H. parainfluenzae*. The MALDI-TOF allowed to identification of 74 isolates as *H. influenzae* (Table 2). Molecular identification by WGS revealed 73 *H. influenzae* (76.8%) and 15 *Haemophilus haemolyticus* (15.8%), six *H. parainfluenzae* (6.3%) and one *Actinobacillus porcitonsillarum* (*A. porcitonsillarum*) (1.1%). WGS-based identification served as reference, and *rpoB* sequencing showed the best correlation with WGS data (99%). rMLST identified all *H. parainfluenzae* and *A. porcitonsillarum* as *Aggregatibacter segnis.* MALDI-TOF identified *A. porcitonsillarum* as *H. parahaemolyticus.* Identification agreement between *rpoB* BLAST and MALDI-TOF was 98.9% and that of rMLST and *rpoB* BLAST was 90.5%.

Serotyping

Of the 73 *H. influenzae* isolates, three encapsulated isolates (4.1%) were identified by agglutination test and PCR in female patients, with agreement of 100%. These serotypes were types b, e and f. The remaining isolates did not possess capsulation locus. A serotype b isolate occurred in a 12-year-old child who was not immunised against Hib. Serotypes e and f occurred in a 1-year-old (immunised) and 33-year-old (not immunised) patient, respectively.

Antibiotic resistance

AMC (amoxicillin/clavulanic acid) and CRO (ceftriaxone) were the most active antibiotics (100%) in all *Haemophilus* isolates. The main resistance rates included SXT (trimethoprim/sulfamethoxazole), API (ampicillin), TET (tetracycline), and CHL (chloramphenicol), with 91%, 55.3%, 10.6%, and 10.6%, respectively (Figure 1). The resistance to nalidixic acid and rifampicin was 4.2% for both. Of the ampicillin-resistant isolates, 14 produced β-lactamase, among which five (35.7%) isolates demonstrated multiple resistance to SXT25, TET30, API2, and CHL30. β-lactamase-positive rates were high in 11 of 14 children (78.6%).

Ampicillin resistance mechanisms

Of the 52 ampicillin-resistant *Haemophilus* isolates, there were 14 new *ftsI* alleles identified in this study. Forty *ftsI* alleles had mutations in the transpeptidase domain of the *ftsI* gene related to

Table 1
Characteristics of the study population.

Patient characteristics	*n* = 95	JH	EHC	*Hib* vaccine coverage (%)
Gender				
Male	36	10	26	28 (77.8)
Female	59	15	44	44 (74.6)
Median age (IQR)	3.2 (1.1–10.6)	37.7 (27.4–54.7)	2.4 (0.8–4.1)	/
Age group (years)				
<5	66	2	64	65 (98.5)
5–15	8	2	6	5 (62.5)
>15	21	21	0	0
Clinical samples				
Nasopharyngeal swab	73	3	70	/
Pleural fluid	2	2	0	/
Bronchioalveolar aspirate	5	5	0	/
Sputum	15	15	0	/
Antibiotic treatment	52	18	34	/

Abbreviations: /Not applicable; JHJamot Hospital; EHCEssos Hospital Centre.

Table 2
Identification of *Haemophilus* isolates following different methods.

	H. influenzae (%)	*H. haemolyticus* (%)	*H. parainfluenzae* (%)	*H. segnis* (%)	*Actinobacillus porcitonsillarum* (%)	*H. parahaemolyticus* (%)
Growth factors	80 (84.2)	0	15 (15.8)	0	0	0
PCR	73 (76.8)	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
MALDI-TOF	74 (77.9)	14 (14.7)	6 (6.3)	0	0	1 (1.1)
rMLST	73 (76.8)	14 (14.7)	0	8 (8.4)	0	0
rpoB BLAST	72 (75.8)	16 (16.8)	6 (6.3)	0	1 (1.1)	0
Whole genome BLAST	73 (76.8)	15 (15.8)	6 (6.3)	0	1 (1.1)	0

Abbreviations: PCR, polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; rMLST, ribosomal multilocus sequence typing; BLAST, basic local alignment search tool.

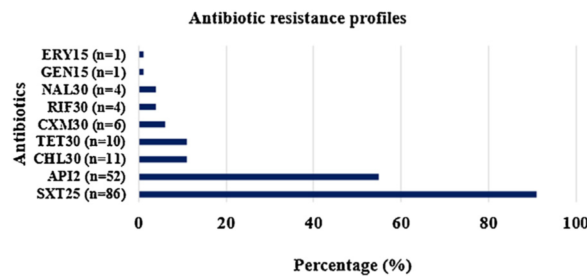

Figure 1. Antibiotic resistance profile of *Haemophilus* isolates.
N, number of resistant isolates; API2, ampicillin 2 μg; CHL30, chloramphenicol 30 μg; NAL30, nalidixic acid 30 μg; CXM30, cefuroxime 30 μg; SXT25, co-trimoxazole 25 μg; GEN15, gentamicin 15 μg; TET30, tetracycline 30 μg; RIF30, rifampicin 30 μg; ERY15, erythromycin 15 μg

decreased susceptibility. Of the 14 *Haemophilus* isolates producing β-lactamase, four (28.6%) also exhibited mutations in the *ftsI* gene encoding PBP3. The genetic platform bearing TEM-1 was detected in all β-lactamase-producing isolates. Table 3 summarises the amino acid changes that were observed. Among the 31 resistant *H. influenzae* isolates, 20 isolates showed mutations in *ftsI*, of which 10 were of group II and 10 belonged to group III (Table 3), according to Deghmane et al. in 2019. In addition to the common E398D and I488 V substitutions, five of the six *H. parainfluenzae* ampicillin-non-susceptible isolates displayed mutations, as shown in Table 3. Of the 15 *H. haemolyticus,* 14 were ampicillin-resistant and all showed mutations in the *ftsI* gene.

Table 3
Mutations in part of PBP3 among 40 ampicillin-resistant *Haemophilus* isolates.

***Haemophilus* species**	**Number of isolates**	***fstI***	***blaTEM-1***	***fstI* group**	**Amino acid substitutions**
Haemophilus influenzae	2	2	0	III	D350N; M377I; A502V; N526K
	3	6, 55, 127	0	II	D350N
	4	43	0	III	D350N; G490E; N526K
	2	97	0	II	A502V; R517H
	1	119	0	III	D350N; M377I; G490E; A502V; N526K
	2	120	0	III	D350N; G490E; A502V; N526K
	1	121	0	II	G490E; N526K
	1	122	0	III	I449 V; N526K
	1	123	0	II	A437S
	3	126	3	II	A502S
Haemophilus haemolyticus	8	/	1	/	F332L; K344R; I348V; D350N; T352G; K355T; L356 V; M377I; S406G; P408S; V418A; A437S; V461I; I519L
	1	/	0	/	F332L; K344R; I348V; D350N; T352G; S353A; K355T; L356 V; M377I; P392A; S406G; P408S; V418A; A437S; V461I; I519L
	4	/	0	/	K344R; D350N; T352G; K355T; L356V; M377I
	1	/	0	/	K344R; D350N; T352G; K355T; L356 V; A368V; M377I
	1	/	0	/	K344R; D350 N; T352G; K355T; L356V; M377I; K486Q; G490E
	2	/	0	/	V342A; K344R; I348V; D350N; T352G; K355T; L356V; A368P; M377I; S406G; P408D; D410E; V418R; I420V; A444S; V461I; K477Q; I488V; I491M
Haemophilus parainfluenzae	2	/	0	/	V342A; K344R; I348V; D350N; T352G; K355T; L356V; A368P; M377I; E398D; S406G; P408D; D410E; V418R; I420V; A444S; V461I; K477Q; I491M
	1	/	0	/	V342A; K344R; I348V; D350N; T352G; K355T; L356V; A368P; M377I; S406G; P408D; D410E; V418R; I420V; A444S; V461I; K477Q; I491M

Abbreviations: /, not applicable; A, alanine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; *blaTEM-1*: *β-lactamase TEM-1.*

Table 4
Inhibitory diameters, MICs of fluoroquinolones and the amino acid mutations of the quinolone resistance-determining regions (QRDRs) of *gyrA*and *parC* in the *Haemophilus*isolates.

Isolates	*Haemophilus* species	Disk diffusion inhibitory zone diameter (mm)		MIC (μg/mL)	Mutation(s) in QRDR	
		NAL	CIP	CIP	*gyrA*	*parC*
108−13Cr	*H. parainfluenzae*	10	14	4	S84L, D88Y	S84F, D88Y
173-AN*	*H. haemolyticus*	31	34	0.19	S84L	None
178-AN	*H. influenzae*	6	14	2	S84L, D88N	S84I
283-CN*	*H. influenzae*	33	35	0.012	S84L	None
326-CN	*H. influenzae*	15	25	1	S84L	S84R

Abbreviations: D, aspartate; F, phenylalanine; I, isoleucine; L, leucine; N, asparagine; R, arginine; S, serine; Y, tyrosine.
* Susceptible *Haemophilus* isolates with amino acid substitutions in *gyrA* gene.

Chloramphenicol acetyltransferase production

Of the 10 *Haemophilus* isolates resistant to chloramphenicol, there were one *H. parainfluenzae,* two *H. haemolyticus* and seven *H. influenzae.* The molecular mechanism underlying this resistance was the production of chloramphenicol acetyltransferase enzyme in these isolates, except for two *H. influenzae* isolates; these two resistant isolates did not display 50S subunit ribosomal mutations.

Mutations in the QRDRs

All QRDRs of gyrA (DNA gyrase subunit A), *parC* (DNA topoisomerase IV subunit A) and *parE* (DNA topoisomerase IV subunit B) sequences of susceptible and non-susceptible *Haemophilus* isolates were compared with those of *H. influenzae* loci HEAM01394, HAEM01649 and HAEM01650, respectively, through the MLST website (http://pubmlst.org/h*influenzae*/). Five isolates showed mutations in the QRDRs of gyrA, of which three were resistant to ciprofloxacin (on the basis of the diameter of the inhibition zone) and two susceptible isolates that exhibited only an S84L mutation, as shown in Table 4. The resistant isolates presented changes at position 84 in *gyrA* and mutations in *parC*. Among resistant isolates with MIC > 2 μg/mL, mutations involved two substitutions at the 84 and 88 positions in QRDRs of gyrA. No mutation was obtained in *gyrB* or *parE*.

Mutations in the rpoB *gene*

None of the isolates that were identified as *H. influenzae* was resistance to rifampicin. However, four non-*H. influenzae* isolates (4.3% of all tested isolates) were rifampicin-resistant, including one *H. haemolyticus* and three *H. parainfluenzae* isolates. Several mutations were detected in the *rpoB* genes encoding the beta subunit of the RNA polymerase in the three *H. parainfluenzae*. Mutation within the cluster I region (507–533) of *rpoB* gene (D516 N and N518D) were observed in the two *H. parainfluenzae* isolates with MIC of 32 μg/mL. The *H. haemolyticus* isolate showed no mutation and showed MIC of rifampicin of 1.5 μg/mL (Table 5). None of the rifampicin-susceptible isolates revealed mutations within the rifampicin-resistance determining region of the *rpoB* gene, which are described to be associated with resistance.

Genetic relationships among the isolates

The genetic relatedness among *Haemophilus* species was displayed from the alignment of protein sequences of *ftsI, gyrA* and *rpoB* (Figure 2). The *ftsI* phylogenetic tree allowed separation of the three species (*H. influenzae*, *H. haemolyticus* and *H. parainfluenzae*). This was also the case for the *rpoB*-based phylogenic tree. The *gyrA* (Figure 2B) gave the less discriminant profile, while the phylogenetic tree from *ftsI* (Figure 2A) gave a better profile, with the different species well separated.

The MLST-based genetic relatedness of *H. influenzae* isolates was then analysed. Of the 73 *H. influenzae* isolates, 46 had a complete MLST profile with all the seven housekeeping genes (*adk, atpG, fucK, frdb, mdh, pgi,* and *recA*) that showed 40 distinct STs. A single isolate represented 34 unique STs, while 12 other isolates represented six other unique STs (two isolates per ST) (Figure 3). The diversity among the 46 *H. influenzae* isolates was also reflected by the high Simpson's Index of diversity, which was 1 with 95% CI [1.0, 1.0]. The isolates were highly diverse and several STs corresponded with new STs that were included in the pubMLST database. It is noteworthy that the unique serotype b isolate belonged to ST-222, which is quite different from the ST-6 to which the majority of invasive serotype b isolates belong (Deghmane et al., 2019). The GrapeTree analysis also showed a highly diverse structure of the tree and few isolates were linked by fewer than three different alleles of the seven MLST genes (Figure 3). The metadata of these 46 isolates were also very diverse (polysaccharide capsule, age, sex, sample type, study site). The remaining 27 isolates lacked one or more housekeeping genes of the seven loci of the MLST scheme. Therefore, the whole set of the 73 isolates was compared using WGS analysis with “Gene Comparator” of the BIGSdb against the loci of the reference strain Rd KW20. The neighbour network is presented in Figure 4, which also shows the highly diverse structure of the bacterial isolates in this study.

The eBURST algorithm generated from the 40 different STs, a single clonal complex and 46 singletons revealed a high level of genetic diversity in this population structure of *H. influenzae*.

Discussion

All *Haemophilus* species were correctly identified by *rpoB* analysis and MALDI-TOF on the basis of WGS-based identification.

Table 5
Inhibitory diameters, MICs of rifampicin and the amino acid mutations of the *rpoB* gene in *Haemophilus* isolates.

Isolates	***Haemophilus* species**	**RIF30 inhibition zone (mm)**	**RIF, MIC (μg/mL)**	***rpoB* mutation**
080−6Cr	*H. parainfluenzae*	17	32	F506S, N518D, T724I, L979V
117−9Cr	*H. parainfluenzae*	17	2	V634I, L979V
157-Acr	*H. parainfluenzae*	11	32	D516N, T724I, L979V
340-AN	*H. haemolyticus*	16	1.5	No mutation

Abbreviations: RIF, rifampicin; MIC, minimal inhibitory concentration; D, aspartate; F, phenylalanine; I, isoleucine; N, asparagine; S, serine; T, threonine; V, valine.

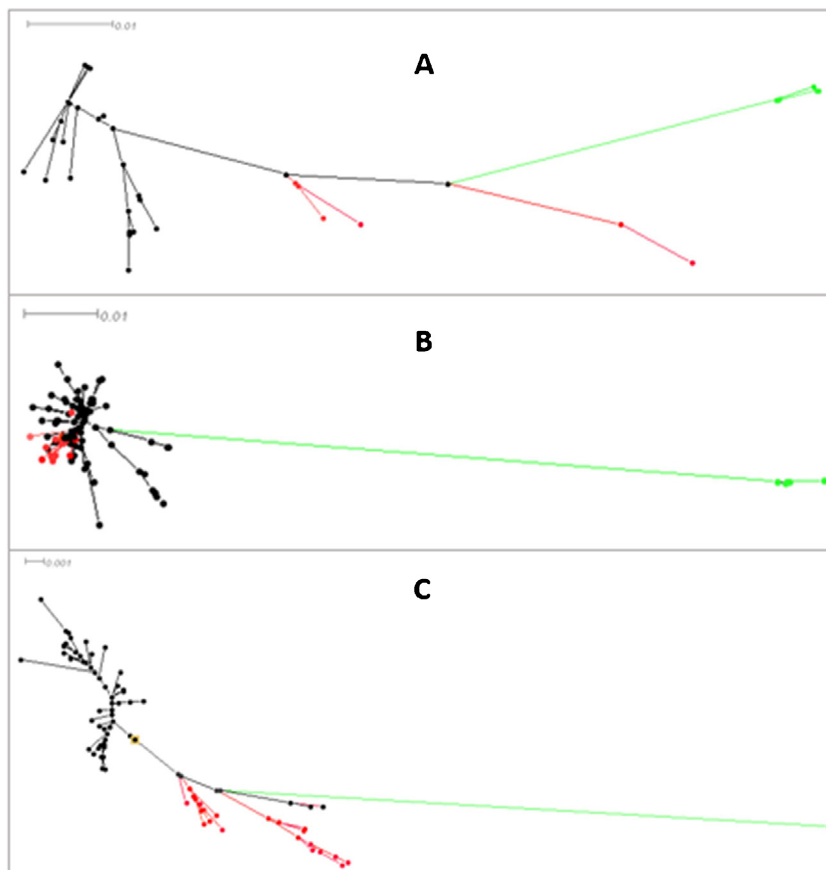

Figure 2. Neighbour-Net SplitsTree graphs generated using SplitsTree4 to visualise trees of *Haemophilus* species isolated from patients with respiratory infections. They show the genetic relatedness of *Haemophilus* species based on three genes: *ftsI* (A), *gyrA* (B) and *rpoB*(C).
Black indicates *Haemophilus influenzae*; green indicates *Haemophilus parainfluenzae*; red indicates *Haemophilus haemolyticus*.

The rMLST failed to identify *H. parainfluenzae* isolates because they are most closely related to *Aggregatibacter segnis* (Murphy et al., 2015). NTHi represented 95.9% of *H. influenzae* isolates (70/73). This result is similar to other studies (Chang et al., 2011; Setchanova et al., 2013). One of 73 (1.4%) *H. influenzae* was type b isolated in a non-vaccinated participant in the current study, but was genetically distinct from invasive Hib isolates.

Resistance towards the folate pathway inhibitors was frequent and encountered in 85% of the tested isolates. This result is similar to those in Ethiopia, Thailand and Turkey (Kuvat et al., 2015; Lulitanond et al., 2012; Mulu et al., 2018). As in the current study, isolates from these studies were mainly form patients with RTIs. Further studies may need to distinguish between invasive and non-invasive isolates as antibiotic resistance frequencies may differ between these isolates (Deghmane et al., 2019). Moreover, carriage isolates from asymptomatic carriers should be considered.

Resistance to β-lactams was of great importance since they are first-line drugs for many bacterial infections. A total of 55.3% of isolates were resistant to β-lactams. It was reported that 14.7% of isolates were β-lactamase-positive, which was similar to 13.3% reported in North African countries (Algeria, Morocco and Tunisia) (Benouda et al., 2009) but lower than the observed frequencies (>20%) in several sub-Saharan African countries (Senegal, Democratic Republic of Congo and Central African Republic) (Bercion et al., 2007; Kacou-Ndouba et al., 2016; Ndiaye et al., 2009). All β-lactamase producing *Haemophilus* isolates harboured the *TEM-1* gene (Tristram et al., 2007).

Among the 25% of *Haemophilus* isolates that had a mutation in their PBP3, the N526 K mutation was the most recurrent in *H. influenzae* followed by the D350 N mutation. This result is similar to many other studies in Spain (Puig et al., 2013) and Portugal (Barbosa et al., 2011). The mutations associated with resistance in *H. haemolyticus* were similar to those published in many studies using the sequence of the Rd KW20 strain as reference (Maddi et al., 2017; Marti et al., 2016; Witherden and Tristram, 2013). The analysis of mutations conferring ampicillin resistance in *H. haemolyticus* took *H. influenzae* as a reference strain (Marti et al., 2016). Twelve resistant isolates did not exhibit any mutations. The antibiotic resistance mechanism of these remaining β-lactam antibiotic-resistant isolates could be due to altered antibiotic permeability and efflux, as demonstrated in Gram-negative bacteria (Wilke et al., 2005).

Resistance to fluoroquinolones that was previously considered extremely rare in *Haemophilus* species (Pérez-Trallero et al., 2010; Puig et al., 2015) is emerging worldwide, with 4.2% recorded in the present study. It is associated with mutations in the genes encoding the DNA gyrase (*gyrA*) and topoisomerase IV (*parC* and *parE*) in *Haemophilus* species. The amino acid substitutions are S84 L, D88 N/Y in GyrA and S84 F/I/R, D88 N/Y in ParC, as reported in previous studies (Abotsi et al., 2017; Puig et al., 2015; Rodriguez-Martinez et al., 2011). Only *Haemophilus* isolates with MICs >2 μg/mL of ciprofloxacin exhibited mutations 84 and 88 in both *gyrA* and *parC*. This result is different from many other studies where isolates with 2 > MICs (μg/mL)>0.5 presented at least two mutations (Faccone et al., 2016; Shoji et al., 2014). However, two isolates had mutations at position 84 of GyrA but showed susceptible phenotypes. Similarly, in Japan, three susceptible *H. influenzae* isolates had a single mutation (two Ser84-Leu mutations

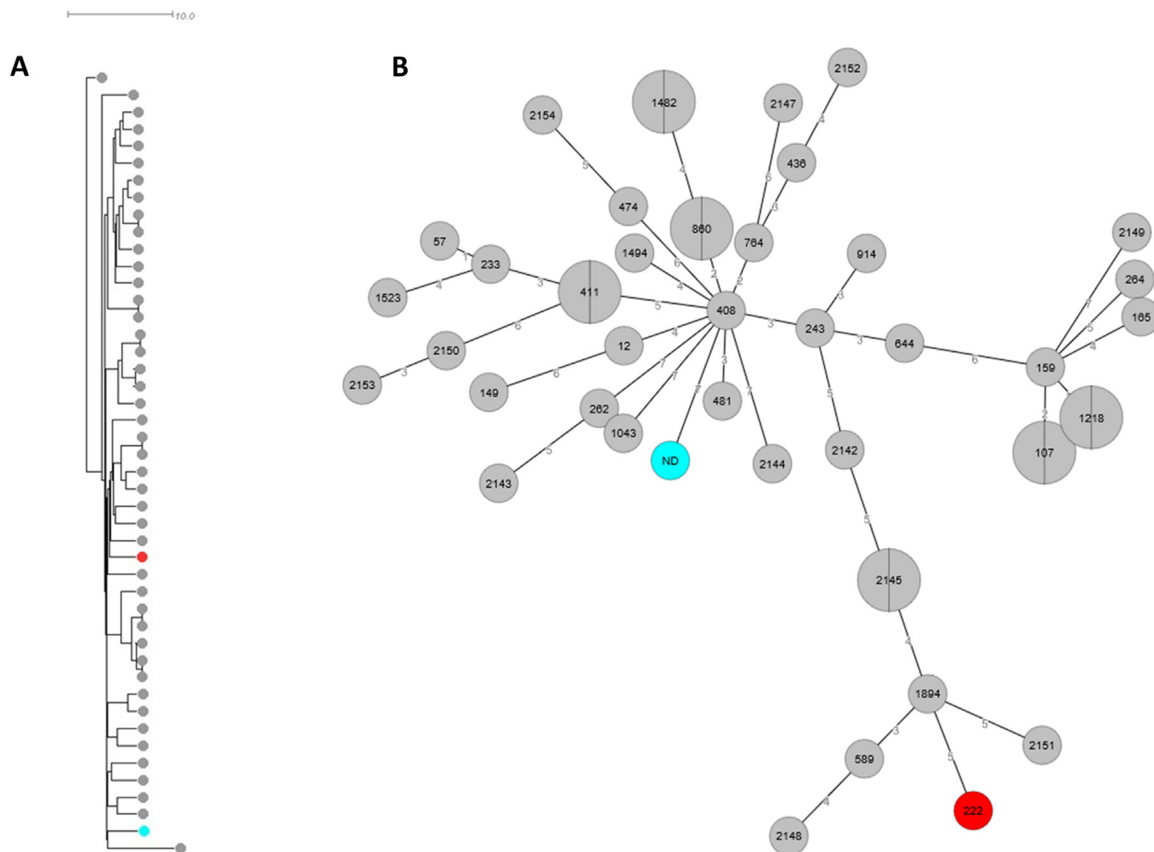

Figure 3. (A) UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree of 46 *H. influenzae* sequences constructed from the seven MLST loci. The linkage distance shows the number of nucleotide substitutions. (B) A GrapeTree based on the seven MLST loci from the 46 genome of isolates with complete MLST data. The nodes were drawn to scale according to the number of isolates (indicated by the pie chart) of each node. The branches between the nodes were drawn to scale and the number of different alleles between the two connected nodes is indicated on the branch. The grey node corresponded to non-typeable isolates (HiNT). The two typeable isolates were indicated in red (serotype b) and cyan (serotype f).

in the *gyrA* gene and one mutation at Gly82-Arg in *par*C) (Shoji et al., 2014). The current findings are different from most studies, which show that the mutation at position 84 was associated to fluoroquinolone resistance. In South Africa, it has been found that the only mutation at position 84 in GyrA was associated with fluoroquinolone resistance (Elliott et al., 2003). The S84 L mutation was found in both susceptible and resistant isolates, suggesting that it is an initial but not a sufficient step in the development of fluoroquinolone resistance (Seyama et al., 2017; Shoji et al., 2014).

After analysing the sequence of the rifampicin-resistance determining region of the *rpoB* gene, two resistant isolates of four showed two substitutions in the conserved cluster I region (507–533) of *rpoB* gene (D516 N and N518D), considered as markers of rifampicin resistance (Abadi et al., 1996; Cruchaga et al., 2003; Goldstein, 2014). Other amino acid changes outside clusters (amino acids 507–533; amino acids 563–572 and amino acid 687) – including F506S, V634I, T724I, and L979V – were detected in resistant isolates of *H. parainfluenzae* but not in susceptible isolates, suggesting that they could play a role in rifampicin resistance. The resistance mechanism in *H. haemolyticus* with MIC of 1.5 μg/mL without any mutation in the rifampicin-resistance determining region of the *rpoB* gene highlighted the fact that amino acid substitution in the *rpoB* gene is not the only resistance mechanism in *Haemophilus* species (Abadi et al., 1996; Cruchaga et al., 2003; Goldstein, 2014).

Among the 94 *Haemophilus* isolates, 10.6 % were resistant to chloramphenicol, which is lower than the 21.7 % reported in Cameroon in 2001 (Fonkoua et al., 2001). The difference in resistance profiles can be linked to the fact that most *Haemophilus* isolates were not invasive. Additionally, chloramphenicol is no longer routinely used due to the side effects. Production of chloramphenicol acetyltransferase enzyme was recorded in 81.8 % of resistant isolates. Indeed, enzymatic inactivation by acetylation of the drug via different types of chloramphenicol acetyltransferases is the first and still most frequently encountered mechanism of bacterial resistance to chloramphenicol (Tristram et al., 2007). However, two resistant isolates of *Haemophilus* species (18.2 %) remained with unknown resistance mechanisms and it was hypothesised that this could be due to other mechanisms such as efflux systems, inactivation by phosphotransferases, mutations of the target site, and permeability barriers (Schwarz et al., 2004).

High genetic diversity was observed but no association was found between the ST and clinical and demographic parameters. Similar results have already been reported in the USA, Spain and Italy (Giufre et al., 2018; Puig et al., 2013; Schumacher et al., 2012). In summary, *Haemophilus* respiratory infections are dominated by highly diverse NTHi in Cameroon, showing high levels of antibiotic resistance. Vaccines for NTHi would be of great interest with regards to its detection rate.

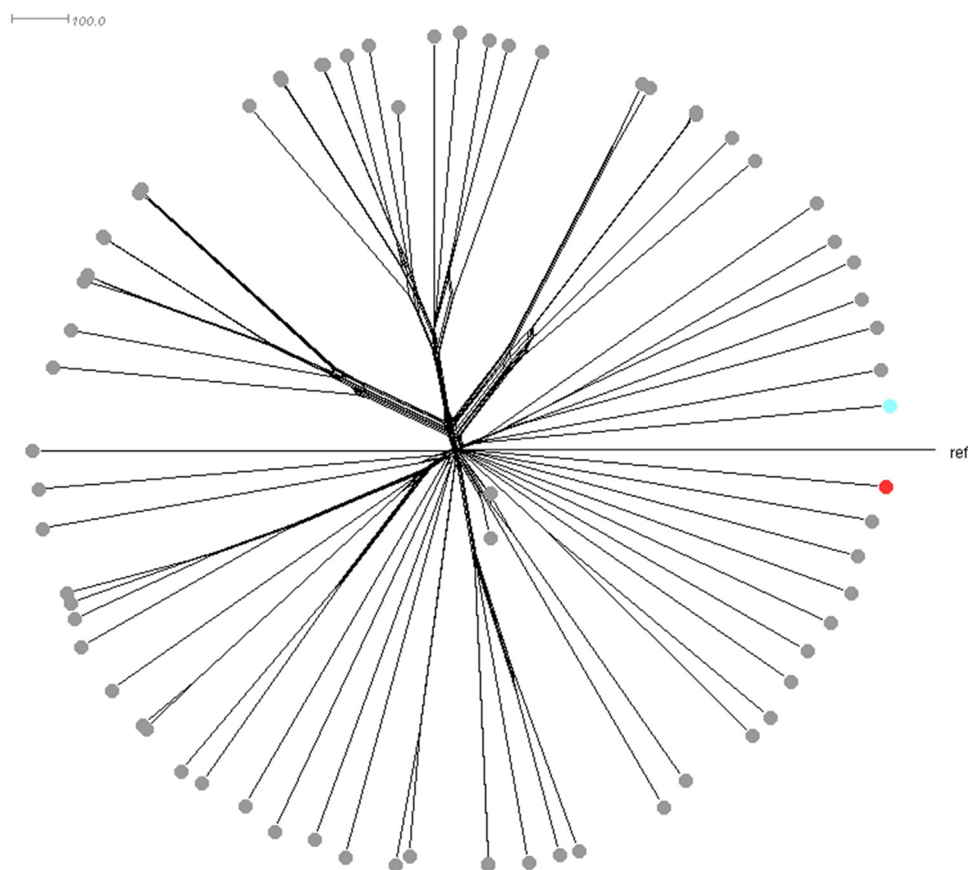

Figure 4. A neighbour-network based on allelic profiles all the 73 isolates compared with the annotated loci of the reference strain Rd KW20 (ref). Individual isolates are represented by circles and the colour of the circle indicates the serotype of the corresponding isolate. The non-typable isolates (HiNT) are represented by grey circles. The two typeable isolates were indicated in a red circle (serotype b) and cyan circle (serotype f).

Conflict of interest

No conflict of interest to declare.

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Ethical approval

Ethical approval for this study was granted from the National Research Ethics Committee of Cameroon N°2017/03/876/CE/CNERSH/SP. Written informed consent was obtained from all participants. For minors to enter the study, parents or guardians gave written informed consent.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.08.040.

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