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Persistent Hib Invasive Disease and Rising Dominance of Highly Resistant NTHi in Chinese Children: A Retrospective Single-Centre Study, 2013–2023

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Abstract:

Background: *Haemophilus influenzae* (*H. influenzae*) is a major cause of both invasive and non-invasive infections in children. Following the introduction of serotype b (Hib) vaccination, non-typeable *H. influenzae* (NTHi) has become the predominant in many countries, accompanied by increasing antimicrobial resistance. In China, Hib vaccine has been available on a self-pay basis since the late 1990s, but comprehensive data integrating molecular typing, resistance mechanisms, and clinical infection characteristics remain scarce for Chinese children. This study investigated the serotype distribution, antimicrobial resistance, and molecular epidemiology of *H. influenzae* isolates from children with pneumonia, suppurative otitis media, and invasive infections in China.

Methods: A total of 111 clinically significant isolates were included, comprising 25 isolates from sterile sites 28 from middle ear fluid collected between 2013 and 2023, and all 58 from bronchoalveolar lavage fluid collected in 2023. Serotyping was performed by slide agglutination and further confirmed by PCR targeting *bexA* and *bexB*. Antimicrobial susceptibility was determined using the E-tests. β -Lactamase production was detected by the nitrocefin test, and the presence of *bla*_{TEM-1} and *bla*_{ROB-1} genes was identified by PCR. *ftsI* sequencing was used to characterize PBP3 amino acid substitutions. Multilocus sequence typing (MLST) was performed to assign sequence types (STs) and clonal complexes (CCs).

Results: Only two encapsulated types were identified: Hib (13 isolates, 11.7%) and Hif (3 isolates, 2.7%), both predominantly found in the invasive isolates (11/13 and 2/3, respectively). NTHi accounted for 85.6% (95/111) of all isolates, comprising 48.0% (12/25) of invasive isolates and 96.5% (83/86) of non-invasive isolates. Patients with invasive disease were significantly younger than those with non-invasive infections (median age 12 months vs. 49 months). NTHi showed higher ampicillin (75.8%) and azithromycin resistance rates (73.7%) than encapsulated strains (31.3%, 12.5%, respectively). Among all 111 isolates, β -Lactamase positivity was 65.8% (all *bla*_{TEM-1}; two also *bla*_{ROB-1}), and *ftsI* sequencing revealed 17 substitutions, with Group III+ (31.5%) and III-like+ (29.7%) as the dominant groups. MLST identified 52 STs and 23 CCs. CC107 was the most prevalent clonal complex (27.9%), exclusively comprising NTHi isolates that were uniformly β -lactamase positive and predominantly carried Group III+ or III-like+ mutations. All Hib isolates belonged to CC6, and all Hif isolates belonged to CC124. The four novel STs identified were all confined to NTHi isolates.

Conclusions: Although the Hib vaccine has been available for over two decades, Hib remains a major cause of invasive *H. influenzae* infections in Chinese children, supporting its inclusion in the national immunization programme. Meanwhile, NTHi has become the predominant type in both invasive and non-invasive settings and carries a significantly higher resistance burden, underscoring the need for NTHi-targeting vaccines.

Keywords : *Haemophilus influenzae*; Antimicrobial Resistance; Non-typeable *Haemophilus influenzae*; Multilocus Sequence Typing (MLST); *ftsI* Mutation

Introduction

Haemophilus influenzae (*H. influenzae*) is a common commensal of the human nasopharynx and a significant pathogen responsible for a broad spectrum of childhood infections, ranging from mild upper respiratory tract illnesses to severe, life-threatening invasive conditions such as pneumonia, meningitis, bacteraemia, and epiglottitis. Based on the expression of a polysaccharide capsule, clinical isolates are categorized into six encapsulated serotypes (a–f) and non-typeable *H. influenzae* (NTHi)[1]. Prior to the widespread implementation of conjugate vaccines, serotype b (Hib) was the leading cause of invasive paediatric disease, accounting for approximately 95% of such cases globally. The introduction of Hib immunization programmes has significantly decreased the burden of Hib-associated invasive infections in numerous countries. However, this public health achievement has been accompanied by a rise in diseases attributed to NTHi and non-b serotypes—a phenomenon commonly referred to as serotype replacement[2]. In China, although the Hib vaccine has been available since 1996, it remains a non-National Immunization Program vaccine requiring self-payment and voluntary uptake. A systematic review and meta-analysis of 33 studies conducted between 2007 and 2016 across 12 provinces in mainland China revealed significant regional variation in Hib conjugate vaccine coverage, with rates ranging from 30.0% to 88.5% and an overall pooled coverage rate of 54.9% (95% CI 52.9% – 57.0%). The pooled coverage was higher in eastern China (59.7%) than in the central and western regions (48.5%). Moreover, only 14.3% of children received the full four-dose series[3]. This low and geographically uneven coverage has likely contributed to the persistent circulation of Hib and the emergence of serotype replacement in China.

The issue of antimicrobial resistance in *H. influenzae* has emerged as an increasing global concern. Data from the global SENTRY surveillance programme (2013–2022), encompassing 13,869 *H. influenzae* isolates from over 150 medical centres across 51 countries, indicated an overall β -lactamase positivity rate of 24.1%. Notably, the Asia and West Pacific region exhibited the lowest susceptibility rates to ampicillin (59.5%) and amoxicillin-clavulanate (82.1%) globally. This region also demonstrated a prevalence of 9.4% for β -lactamase-negative ampicillin-resistant (BLNAR) isolates and 10.9% for β -lactamase-positive amoxicillin-clavulanate-resistant (BLPACR) isolates, which carry both β -lactamase and *ftsI* mutations, representing the highest rates worldwide[4]. In contrast, the 2024 CHINET surveillance data from China reported an ampicillin resistance rate of 78.9% among paediatric *H. influenzae* isolates[5], substantially higher than the global and Asian averages from the SENTRY study, highlighting a particularly severe resistance burden in Chinese children.

Given that serotyping and genetic analysis are not part of routine clinical practice, coupled with the absence of a national surveillance programme for *H. influenzae* in China, reports on the serotype distribution and genetic characteristics of this pathogen in Chinese children have been scarce, with the majority of published studies focusing on antimicrobial resistance profiles. Moreover, previous studies have rarely considered the clinical significance of the isolates, and a substantial proportion were recovered from upper respiratory tract specimens such as sputum, making it difficult to distinguish pathogens from colonizers. This methodological limitation has frequently cast doubt on the validity of their findings. To address these issues, the present study focused on isolates from normally sterile body fluids, purulent otitis media secretions, and bronchoalveolar lavage fluid. The aim was to characterize the aetiological features of *H. influenzae* infections in Chinese children after more than two decades of Hib vaccine availability, and to provide evidence to guide clinical antimicrobial therapy and vaccine immunization strategies.

Material and methods

1. Bacterial Isolates and Patient Data

A total of 111 non-duplicate clinical isolates of *H. influenzae* were recovered from 111 paediatric patients with confirmed infections. Of these, 25 isolates were obtained from blood (13 isolates), cerebrospinal fluid (10), or pleural fluid (2) between 2013 and 2023, and 28 from middle ear fluid during the same period. An additional 58 isolates from bronchoalveolar lavage fluid were collected in 2023. Clinical data including age, sex, diagnosis, and specimen type were extracted from medical records. Infections were classified as invasive (e.g., bacteraemia, meningitis, empyema) or non-invasive (e.g., pneumonia, suppurative otitis media) according to established clinical criteria.

2. Bacterial Identification and Serotyping

All isolates were initially identified as *H. influenzae* using standard microbiological methods, including colony morphology, Gram staining, and growth requirements for X and V factors (Oxoid, Basingstoke, UK). Serotyping was performed by slide agglutination (SAT) using the ImmunLex™ *H. influenzae* antisera (Statens Serum Institut, Copenhagen, Denmark) specific for serotypes a through f. All isolates were subjected to polymerase chain reaction (PCR) for the detection of capsule-specific genes *bexA* and *bexB*, using previously described primer pairs [6]. Isolates negative for both *bexA* and *bexB* by PCR were designated as non-typeable *H. influenzae* (NTHi). For *bexB*-positive isolates, serotype was further confirmed by PCR using type-specific primers for serotypes a through f [7]. In this study, an isolate was considered typed if it was identified by either serotyping or PCR.

3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined using the E-test (Liofilchem s.r.l., Roseto degli Abruzzi, Italy) for the following agents: ampicillin, amoxicillin, amoxicillin–clavulanic acid, ceftriaxone and azithromycin. Susceptibility results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints for *H. influenzae* (M100, 2026 edition). *H. influenzae* ATCC10211, ATCC 49247 and ATCC 49766 were used as quality control strains. Based on β -lactamase production and susceptibility to ampicillin and amoxicillin-clavulanate, the isolates were phenotypically classified as BLNAS (β -lactamase-negative,

ampicillin-susceptible), BLNAI (β -lactamase-negative, ampicillin intermediate), BLNAR, BLPAR (β -lactamase-positive, ampicillin-resistant), BLPACI (β -lactamase-positive, amoxicillin-clavulanate intermediate), and BLPACR.

4. β -Lactamase and PBP3 Mutation Detection

β -lactamase production was detected using the chromogenic nitrocefin disk test (Oxoid, Basingstoke, UK). All β -Lactamase positive isolates were screened for the presence of β -lactamase genes, including *bla_{TEM-1}* and *bla_{ROB-1}*, by PCR using specific primers[8]. PCR products were visualized on agarose gels, and amplicons from representative isolates were sequenced to confirm the gene identity. The gene *ftsI* was sequenced and compared with that of Rd KW20 strain (GenBank accession no. NC_000907) to identify PBP3 amino acid substitutions. The deduced amino acid sequences were analyzed for known mutations associated with β -lactam resistance, including those corresponding to stages I, II, and III. Isolates were classified into mutation groups (e.g., Group I, II, III, III-like, etc.) based on the combination of amino acid substitutions, following the classification scheme described by previous studies[9]. Isolates with no amino acid substitutions were recorded as wild-type.

5. Multilocus Sequence Typing (MLST)

MLST was performed according to the *H. influenzae* MLST scheme available at the PubMLST database (<https://pubmlst.org/hinfluenzae/>). Internal fragments of seven housekeeping genes (*adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA*) were amplified by PCR and sequenced. Allelic profiles and sequence types (STs) were assigned using the PubMLST database. Novel STs were submitted to the database for new allele and ST number assignment. Clonal complexes (CCs) were defined as groups of STs sharing at least five identical alleles with the founder ST, using the eBURST algorithm.

6. Statistical Analysis

Statistical analyses were performed using SPSS software (version 26.0, IBM Corp., Armonk, NY, USA). Continuous variables were compared using the Mann-Whitney U test, and categorical variables were compared using the chi-square

test or Fisher's exact test, as appropriate. A P value of <0.05 was considered statistically significant.

7. Ethical Statement

This study was approved by the Ethics Committee of Beijing Children's Hospital. Informed consent was waived because of the retrospective nature of the study and the use of anonymized clinical data and isolates.

Results

1. Clinical Features of Infection

The clinical characteristics of the 111 patients with *H. influenzae* infections are summarized in Table 1. Patients with invasive infections were significantly younger than those with non-invasive infection, with a median age of 12 months (IQR 6.5–24) compared with 49 months (IQR 18.75–76.25) for the non-invasive group ($p < 0.001$). The majority of invasive cases (52.0%) occurred in children under 1 year of age, particularly those aged 6 to 12 months (36.0%). In contrast, non-invasive infections predominated in children older than 3 years.

Table 1. Clinical characteristics of 111 patients with *H. influenzae* infection

Characteristic	Invasive (n=25)	Non-invasive (n=86)	p-value
Gender, male/female (ratio)	18: 7 (2.6: 1)	47: 39 (1.2: 1)	0.167 ^a
Age range	4 months – 12 years	4 months – 16 years	
Age (months), median (IQR)	12 (6.5-24)	49 (18.75-76.25)	$<0.001^b$
Age group, n (%)			
4-6m	4(16.0)	3(3.5)	
>6-12 months	9(36.0)	7(8.1)	
>12-18 months	3(12.0)	8(9.3)	
>18-36 months	4(16.0)	16(18.6)	
>36-72 months	1(4.0)	27(31.4)	
>72 months	4(16.0)	25(29.1)	

a: Chi-square test; b: Mann-Whitney U test. BALF: bronchoalveolar lavage fluid; IQR: interquartile range.

2. Serotype distribution

Among the 111 *H. influenzae* isolates examined by SAT, only 2 (1.8%) were capsular positive, both identified as serotype f. PCR analysis revealed that none of the isolate was positive for *bexA*, whereas a *bexB* band of the expected size was detected in 16 isolates, the remaining 95 were classified as NTHi. Among the 16 *bexB*-positive isolates, 13 were serotype b and 3 were serotype f. Of these, 13 isolates (11 serotype b and 2 serotype f) were recovered from invasive infection cases. Encapsulated isolates accounted for 52.0% of invasive isolates but only 3.5% of non-invasive isolates.

3. Analysis of antimicrobial susceptibility phenotypes and genotypes

3.1 β -lactamase production

Of the 111 *H. influenzae* isolates, 73 (65.8%) were β -lactamase-positive, all of which produced TEM-1, with two also producing ROB-1. The β -lactamase positivity rate was lower among invasive isolates (48.0%) than among non-invasive isolates (70.9%), although the difference did not reach statistical significance (Table 2).

3.2 Antimicrobial susceptibility test

The antimicrobial susceptibility results are summarized in Table 2. The resistance rates to ampicillin, amoxicillin, and amoxicillin-clavulanic acid were 70.3% (78/111), 55.0% (61/111), and 9.0% (10/111), respectively. The non-susceptibility rate to azithromycin was 64.9% (71/111). All isolates remained susceptible to ceftriaxone. BLNAR accounted for 4.5% (5/111) and BLNAI for 8.1% (9/111) of all isolates. BLPACR comprised 5.4% (6/111), and BLPACI accounted for 12.6% (14/111).

When stratified by infection type, invasive isolates exhibited lower resistance rates to ampicillin (52.0% vs. 75.6%) and lower non-susceptibility to azithromycin (36.0% vs. 73.3%) compared with non-invasive isolates (Table 2).

Compared with the 16 encapsulated isolates, the 95 NTHi isolates displayed significantly higher β -lactamase-positive rate (72.6% vs. 25%), resistance rates to ampicillin (75.8% vs. 31.3%), amoxicillin (58.9% vs. 31.3%), and azithromycin (73.7% vs. 12.5%) (Figure 1A).

Table 2 β -lactamase production and antimicrobial susceptibility of 111 *H. influenzae* isolates and comparison between invasive and non-invasive groups

β -lactamase/Agent	Total (N=111)	Invasive isolates (N=25)	Non-invasive isolates(N=86)	χ^2 / Fisher exact test	P-value
β -lactamase positive (n(%))	73 (65.8%)	12 (48%)	61 (70.9%)	$\chi^2=4.523$	0.054
TEM-1 (n(%))	73 (100%)	12 (100%)	61 (100%)	-	-
ROB-1 (n(%))	2 (1.8%)	0	2 (2.3%)	-	-
Ampicillin					
Susceptible (n(%))	25 (22.5%)	8 (32%)	17 (19.8%)	-	-
Intermediate (n(%))	8 (7.2%)	4 (16%)	4 (4.7%)	-	-
Resistant (n(%))	78 (70.3%)	13 (52%)	65 (75.6%)	$\chi^2=4.089$	0.043
MIC ₅₀ (mg/L)	12	6	12	-	-
MIC ₉₀ (mg/L)	≥ 256	≥ 256	≥ 256	-	-
MIC range (mg/L)	0.094- $\geq$ 256	0.25- ≥ 256	0.094- ≥ 256	-	-
Amoxicillin					
Susceptible (n(%))	31 (27.9%)	8 (32%)	23 (26.7%)	-	-
Intermediate (n(%))	19 (17.1%)	7 (28%)	12 (14%)	-	-
Resistant (n(%))	61 (55%)	10 (40%)	51 (59.3%)	$\chi^2=2.915$	0.088
MIC ₅₀ (mg/L)	12	6	12	-	-
MIC ₉₀ (mg/L)	≥ 256	≥ 256	≥ 256	-	-
MIC range (mg/L)	0.094- $\geq$ 256	0.25- ≥ 256	0.094- ≥ 256	-	-
Amoxicillin/Clavulanic Acid					
Susceptible (n(%))	77 (69.3%)	17 (68%)	60 (69.8%)	-	-
Intermediate (n(%))	24 (21.6%)	7 (28%)	17 (19.8%)	-	-
Resistant (n(%))	10 (9%)	1 (4%)	9 (10.5%)	Fisher exact test	0.451
MIC ₅₀ (mg/L)	1	0.75	1	-	-
MIC ₉₀ (mg/L)	6	4	8	-	-

MIC range (mg/L)	0.094- $\geq$ 256	0.094-12	0.094- $\geq$ 256	-	-
Ceftriaxone					
Susceptible (n(%))	111 (100%)	25 (100%)	86 (100%)	-	-
Resistant (n(%))	0	0	0	-	-
MIC ₅₀ (mg/L)	0.125	0.023	0.19	-	-
MIC ₉₀ (mg/L)	0.38	0.25	0.5	-	-
MIC range (mg/L)	0.016-1.5	0.016-0.5	0.016-1.5	-	-
Azithromycin					
Susceptible (n(%))	39 (35.1%)	16 (64%)	23 (26.7%)	-	-
Non-susceptible rate (n(%))	72 (64.9%)	9 (36%)	63 (73.3%)	$\chi^2=11.797$	<0.001
MIC ₅₀ (mg/L)	≥ 256	4	≥ 256	-	-
MIC ₉₀ (mg/L)	≥ 256	≥ 256	≥ 256	-	-
MIC range (mg/L)	2- ≥ 256	2- ≥ 256	2- ≥ 256	-	-

χ^2 , Chi-square test statistic

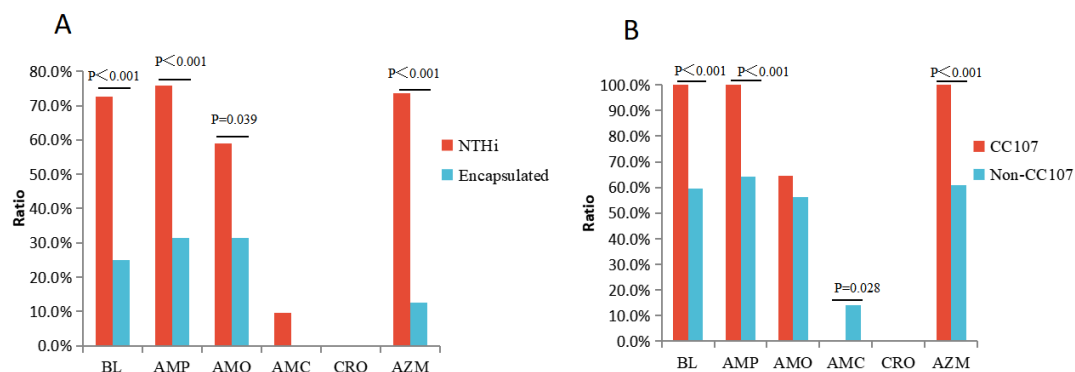


Figure 1 Comparison of β -lactamase positive rates and resistance rates to five antimicrobial agents among different groups of *H. influenzae*. AZM data represent non-susceptibility. (A) Comparison of β -lactamase positive rate (BL) and resistance rates to ampicillin (AMP), amoxicillin (AMO), amoxicillin-clavulanate (AMC), ceftriaxone (CRO) and non-susceptible rates to azithromycin (AZM) between NTHi and encapsulated isolates; (B) Comparison of β -lactamase positive rate and resistance rates to five antimicrobial agents between CC107 clonal complex and

non-CC107 clonal complex within NTHi isolates. The y-axis represents the positive/resistance ratio (%). P values for intergroup differences are shown above the corresponding groups, and $P < 0.05$ indicates statistically significant differences.

3.3 *ftsI* mutation

Sequencing of the *ftsI* gene across the 111 isolates identified 17 distinct amino acid substitutions, including Q339H, D350N, S357N, M377I, S385T, L389F, R451L, G490E, A502V, R517H, N526K, A530S, T532S, V547I, Y557H, V562L, and N569S. At least one substitution was detected in 98 isolates (88.3%), and these substitutions formed 31 different combination patterns (**Supplementary Table 1**). The two most prevalent patterns were Group III+ (35/111, 31.5%) and Group III-like+ (33/111, 29.7%). Both groups shared the stage-II core mutation S385T, the stage-III potentiating mutation L389F, and a stage-I mutation, either N526K or R517H. Additional accompanying substitutions, such as D350N, S357N, M377I, V547I, and N569S, were frequently observed in these groups. In contrast, 13 isolates (11.7%) carried no non-synonymous *ftsI* mutations, and 6 isolates (5.4%) exhibited incomplete or atypical mutation patterns that could not be assigned to any of the defined groups.

3.3 *ftsI* mutation types stratified by β -lactam resistance phenotype

In the β -lactamase-producing group ($n = 73$), the III+ subgroup was the most frequently observed (39.7 %, 29/73), followed by the III-like+ subgroup (37.0%, 27/73). The wild-type subgroup was rarely observed (4.1%, 3/73). Among the 6 BLPACR isolates, the III+ subgroup accounted for 50.0 % (3/6) and group II accounted for 33.3% (2/6). In the non- β -lactamase-producing group, the wild-type subgroup was the most frequently observed (26.3 %, 10/38), followed by group II (21.1 %, 8/38) and the III-like+ subgroup (21.1 %, 8/38). Within this group, the BLNAR subgroup was predominantly composed of the III-like+ subgroup (80.0 %, 4/5), whereas the BLNAS subgroup was predominantly wild-type (40.0 %, 10/25). (detailed in table 3)

Table 3 Distribution of *ftsI* mutation groups among BLPAR, BLPACR, BLNAR, BLNIR, and BLNAS isolates

β -Lactamase production	β -Lactamase production (-)
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PBP3 Groups	(+) BLNAR BLNAIR		BLNAR (n=5)	BLNAIR (n=8)	BLNAS (n=25)	Total (n=38)
	BLPAR (n=73)	BLPACR* (n=6)				
I	2.7% (2)	0	0	0	4% (1)	2.6% (1)
II	8.2% (6)	33.3% (2)	0	50% (4)	16% (4)	21.1% (8)
III+	39.7% (29)	50% (3)	20% (1)	37.5% (3)	8% (2)	15.8% (6)
III-like+	37.0 (27)	16.7% (1)	80% (4)	12.5% (1)	12% (3)	21.1% (8)
III like	6.8 (5)	0	0	0	0	0
other	1.4% (1)	0	0	0	20% (5)	13.2% (5)
wild-type	4.1% (3)	0	0	0	40% (10)	26.3% (10)

BLPAR: β -lactamase-positive ampicillin-resistant ; BLPACR: β -lactamase-positive amoxicillin-clavulanate-resistant; BLNAR: β -lactamase-negative ampicillin-resistant; BLNAIR: β -lactamase-negative ampicillin-intermediate-resistant; BLNAS: β -lactamase-negative ampicillin susceptible. *:Among the 73 β -lactamase-producing isolates, 6 isolates additionally fulfilled the criteria for the BLPACR phenotype.

4. MLST

MLST analysis identified 52 distinct STs among the 111 isolates. ST107 was the most frequent (24 isolates, 21.6%), followed by ST95 (9 isolates, 8.1%), ST155 (8 isolates, 7.2%), ST1002 (6 isolates, 5.4%), and ST57 (6 isolates, 5.4%). The remaining STs were each found in one to four isolates. Four novel STs were identified, all of which were recovered from non-invasive isolates.. The eBURST algorithm

grouped the isolates into 23 clonal complexes. CC107 was the most prevalent (31 isolates, 27.9%), followed by CC6 (13 isolates, 11.7%), CC3 (9 isolates, 8.1%), CC155 (8 isolates, 7.2%), and CC57 (6 isolates, 5.4%). Detailed results are summarised in Supplementary Table 2.

All 13 Hib isolates belonged to CC6, and the 3 Hif isolates belonged to CC124. Neither of these two CCs was detected among NTHi isolates. Among NTHi isolates, CC107 was the most frequent clonal complex (31/95), comprising 5 invasive and 26 non-invasive isolates. Other commonly identified CCs in NTHi included CC3 (9 isolates), CC155 (8 isolates), CC57 (5 isolates), and CC395 (4 isolates), most of which were found exclusively in non-invasive isolates. Among the CCs associated with NTHi, only CC107 and CC57 were shared by invasive and non-invasive isolates.

All 31 CC107 isolates were NTHi. Compared to other 64 NTHi isolates, the CC107 isolates showed significantly higher β -lactamase-positive rate (100% vs. 59.4%, $P<0.001$), resistance rates to ampicillin (100% vs. 64.1%, $P<0.001$), and azithromycin non-susceptibility (100% vs. 60.9%, $P<0.001$) (Figure 1B). In addition, all CC107 isolates carried *ftsI* amino acid substitutions, with the majority harbouring Group III+ (16/31, 51.6%) or Group III-like+ (14/31, 45.2%) mutations. Only one isolate (3.2%) carried a Group I mutation.

Discussion

During the period from 2013 to 2023, only 25 invasive *H. influenzae* isolates were identified, of which 13 (52.0%) were encapsulated, including 11 Hib (44.0%) and 2 Hif (8.0%). Although invasive *H. influenzae* infections are relatively uncommon, Hib remains the predominant identifiable serotype among culture-positive cases. This finding is consistent with a Hebei-based study of 13 invasive paediatric isolates, in which Hib accounted for 61.5% of cases[10]. Given that Hib disease is vaccine-preventable and predominantly affects infants and young children, similar to the age distribution observed in our invasive cases, there is a clear

rationale for incorporating the Hib vaccine into China's routine childhood immunization programme.

A 2021 health economic modelling study projected that universal Hib vaccination would avert 93% of Hib cases and 92% of Hib-related deaths annually in children under 5 years of age, leading to a substantial reduction in both disease burden and healthcare expenditure[11].

All Hib isolates in this study all belonged to CC6, and the three Hif isolates to CC124. These indicate that encapsulated isolates display a high degree of clonality, which is a favorable factor for vaccine immunization prevention. The strong association between Hib and CC6 has been consistently documented worldwide. In Portugal, a pre-vaccination period study (1989–2001) reported that 89.5% (34/38) of invasive Hib isolates belonged to CC6, and this clonal predominance persisted in the post-vaccine era (2002–2010)[12]. A nationwide survey of Hib vaccine failures in Portugal further corroborated this clonal dominance, demonstrating that all 25 vaccine failure isolates belonged exclusively to CC6[13]. Notably, serotype replacement following Hib vaccine introduction has been documented in several countries, with Hif increasingly recognised as an emerging pathogen in the post-vaccine era[14]. This trend warrants close monitoring of Hif and other non-b serotypes in mainland China.

It is necessary to mention that SAT identified only two encapsulated isolates (both Hif), whereas PCR detected 13 Hib and 3 Hif isolates. Similar discrepancies between serotyping and genotyping results have been reported previously, with false-negative SAT rates for encapsulated strains reaching up to 40%[15]. This discrepancy is largely attributable to capsule loss during freezing preservation and subculture, which results in a non-typeable phenotype despite intact capsule genes. To minimise such misclassification, SAT should be performed promptly after bacterial isolation in the clinical setting.

The present study revealed that NTHi comprised 48.0% of invasive cases and accounted for 96.5% of non-invasive isolates, reinforcing its dominance in paediatric *H. influenzae* infections in China. This finding aligns with the global shift

observed in the post-Hib vaccine era, during which NTHi has supplanted encapsulated serotypes as the leading cause of both invasive and non-invasive disease. Similar trends have been reported elsewhere: in a US paediatric tertiary hospital, NTHi constituted 52.4% of invasive infections between 2011 and 2023, exceeding all encapsulated types combined[16]; In the EU/EEA, NTHi accounted for 81.0% (2,552/3,149) of invasive cases with known serotype in 2023[17]; Studies from Bulgaria on paediatric otitis media and from Guangzhou on respiratory infections both reported NTHi as the predominant type, with proportions of 90.1% and over 50%, respectively [18, 19]. Collectively, these observations indicate that widespread Hib vaccination has driven the emergence of NTHi as the major contributor to both invasive and non-invasive *H. influenzae* disease. Although the overall Hib vaccination rate among Chinese children remains relatively modest, regional coverage has exceeded 80% in some areas, and with the vaccine having been available for more than two decades, this selective pressure may have contributed to the rising dominance of NTHi.

The higher resistance burden observed in NTHi compared with encapsulated strains likely contributes to its epidemiological dominance. In this study, NTHi isolates exhibited significantly higher resistance rate to ampicillin (75.8% vs. 31.3%) and higher non-susceptibility to azithromycin (73.7% vs. 12.5%) than encapsulated isolates. Similar patterns have been reported elsewhere. A Brazilian study found that NTHi accounted for the majority of ampicillin-resistant invasive isolates (60.4%)[20], and a Taiwanese study reported an ampicillin resistance rate of 68.6% among invasive NTHi isolates, with identification of novel transposons carrying multiple resistance genes[21]. This resistance advantage is likely attributable to two factors. First, NTHi, as a long-term colonizer of the upper respiratory tract, is repeatedly exposed to antibiotics, promoting the acquisition of β -lactamase genes and *ftsI* mutations[22, 23]. Second, the high genetic diversity of NTHi enables rapid adaptation through horizontal gene transfer and clonal expansion of resistant lineages[21].

In the present study, BLPAR constituted the majority of resistant isolates, and resistance occurred regardless of *ftsI* status, indicating that β -lactamase production is the primary resistance mechanism. In contrast, BLPACR isolates predominantly carried Group III⁺ or III-like⁺ mutations, suggesting that combined β -lactamase production and *ftsI* mutations are required for resistance to amoxicillin-clavulanate. Notably, 15 of 25 BLNAS isolates carried various *ftsI* mutations yet remained susceptible to ampicillin, indicating that the presence of *ftsI* mutations does not invariably lead to phenotypic resistance. These findings highlight the complexity of genotype-phenotype correlations and the need for functional characterization of PBP3 variants[24].

Although a major clone with higher antimicrobial resistance, CC107 (32.3%, 31/96), was identified among NTHi isolates, more than 20 other CCs and numerous singleton STs were also detected, reflecting the high genetic heterogeneity of NTHi. This finding is consistent with previous observations. A study investigating nasopharyngeal carriage of NTHi in 190 children from Beijing and Youyang, Chongqing, reported 108 distinct STs [25]. Similarly, a study from Shanghai focusing on adult patients identified 36 STs among 51 NTHi isolates[26], indicating that the genetic diversity of NTHi is not confined to the paediatric population.

Several limitations should be acknowledged in this study. First, this was a single-centre study, and the relatively small number of invasive isolates, particularly encapsulated strains, limits the statistical power for subgroup comparisons. This is attributable to the fact that most children admitted to our hospital had already received antibacterial therapy before clinical specimen collection. Second, although we focused on clinically significant specimens, the retrospective design meant that isolate collection was not standardized across the entire study period, which may have introduced selection bias. Third, the absence of vaccination records for individual patients prevented us from directly assessing the impact of Hib immunization status on serotype distribution and disease severity. Despite these limitations, our findings remain robust. The consistent association between serotype and clinical presentation,

the strong clonal structure of Hib isolates, and the significantly higher resistance burden in NTHi, particularly the dominant CC107 clone, all point to important epidemiological trends. These results are also supported by comparable data from other regions. The study provides valuable evidence that Hib remains a major cause of invasive disease and that NTHi poses a growing challenge due to its resistance advantage, reinforcing the need for enhanced vaccination strategies and ongoing surveillance.

Conclusions

In summary, this study provides evidence that despite more than two decades of Hib vaccine availability in China, Hib remains a major cause of invasive *H. influenzae* infections in children and exhibits significant clonal transmission, highlighting the need to incorporate the Hib vaccine into the national immunization programme. At the same time, NTHi has emerged as the predominant type across both invasive and non-invasive infections and is associated with a considerably higher antimicrobial resistance burden. The emergence of the dominant CC107 clone, which is exclusively NTHi and uniformly carries both β -lactamase and Group III+ or III-like+ *ftsI* mutations, further underscores the declining utility of ampicillin and amoxicillin as empirical therapies. These findings reinforce the critical need for strengthened surveillance, antimicrobial stewardship, and accelerated development of vaccines targeting NTHi.

List of Abbreviations

Abbreviation	Full Form
BALF	Bronchoalveolar lavage fluid
BLNAI	β -Lactamase-negative, ampicillin-intermediate
BLNAR	β -Lactamase-negative, ampicillin-resistant

Abbreviation	Full Form
BLNAS	β -Lactamase-negative, ampicillin-susceptible
BLPACI	β -Lactamase-positive, amoxicillin–clavulanate-intermediate
BLPACR	β -Lactamase-positive, amoxicillin–clavulanate-resistant
BLPAR	β -Lactamase-positive, ampicillin-resistant
CC	Clonal complex
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
Hib	<i>Haemophilus influenzae</i> serotype b
Hif	<i>Haemophilus influenzae</i> serotype f
IQR	Interquartile range
ISPED	Chinese Pediatric Bacterial Resistance Surveillance Group
MEF	Middle ear fluid
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MSM	Men who have sex with men
NTHi	Non-typeable <i>Haemophilus influenzae</i>

Abbreviation	Full Form
OR	Odds ratio
PBP3	Penicillin-binding protein 3
PCR	Polymerase chain reaction
PWH	People with HIV
SAT	Slide agglutination test
ST	Sequence type
WGS	Whole-genome sequencing

Ethics approval and consent to participate

This study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of Beijing Children's Hospital. Due to the retrospective nature of the study and the use of anonymised clinical data and isolates, the Ethics Committee waived the requirement for informed consent from individual patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.-

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Authors' contributions

KY designed the study. HC and JZ collected the specimens from patients. XY, WG, and FD identified the bacteria and performed the antimicrobial susceptibility tests. JS and WS performed the molecular studies. JS and KY collected the data, analyzed them, interpreted the results, and drafted the manuscript. All authors reviewed and revised the manuscript and approved the final version.

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