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Antibiotic susceptibility and molecular analysis of invasive *Haemophilus influenzae* in Canada, 2007 to 2014

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Background: Previously we studied the antibiotic susceptibility of invasive *Haemophilus influenzae* collected in Canada from 1990 to 2006 and characterized isolates by serotype, MLST and *ftsI* gene sequencing for significant PBP3 mutations.

Objectives: To provide an update based on isolates collected from 2007 to 2014.

Methods: A total of 882 case isolates were characterized by serotype using slide agglutination and PCR. MLST was carried out to determine ST. Isolates were tested for β-lactamase production, presence of significant PBP3 mutations and antibiotic susceptibility by disc diffusion against 14 antibiotics. MIC values of three antibiotics were determined for 316 isolates using microbroth dilution.

Results: Non-typeable *H. influenzae* accounted for 54.6% of the isolates and 45.4% were serotypeable, predominantly type a (23.1%), type b (8.3%) and type f (10.8%). The overall rate of ampicillin resistance due to β-lactamase production was 16.4% and increased from 13.5% in 2007–10 to 19% in 2011–14. Significant PBP3 mutations were identified in 129 isolates (14.6%) with 23 (2.6%) also producing β-lactamase. MLST identified related STs (ST-136, ST-14 and ST-367) associated exclusively with genetically β-lactamase-negative, ampicillin-resistant isolates and confirmed previously reported associations between significant PBP3 mutations and ST.

Conclusions: A significant increase in β-lactamase-producing isolates was observed from 2007 to 2014; the rate of significant PBP3 mutations has increased since previously reported and 52.5% of non-typeable *H. influenzae* now show resistance markers. Resistance to trimethoprim/sulfamethoxazole was common and no resistance to fluoroquinolones or third-generation cephalosporins was found.

Introduction

Haemophilus influenzae is an important bacterial pathogen causing a wide variety of both invasive and respiratory infections. *H. influenzae* is divided into six serotypes based on polysaccharide capsule antigens that are denoted serotypes a–f, while strains that lack a polysaccharide capsule are designated non-typeable (NT-Hi). Prior to vaccination, serotype b (Hib) was a common cause of serious invasive disease, especially in children ≤5 years old.[1]

Since the introduction of the Hib conjugate vaccine, the rate of Hib disease has decreased dramatically and many reports now point to non-Hib and NT-Hi as responsible for the majority of invasive *H. influenzae* infections.[2–5]

Ampicillin resistance in *H. influenzae*, first described in the 1970s,[6] is now a well-documented global phenomenon.[7,8] Ampicillin resistance is divided into two main categories based on the production of β-lactamase enzymes. Isolates producing

β-lactamase are termed β-lactamase positive, ampicillin resistant (BLPAR) and constitute the most common type of ampicillin resistance observed in *H. influenzae*, with most isolates producing either the TEM-1- or ROB-1-type β-lactamase.[9] Isolates that do not produce β-lactamase, but contain alterations in PBP3, resulting in decreased affinity for β-lactams, are termed β-lactamase negative, ampicillin resistant (BLNAR).[10] In addition to BLPAR and BLNAR, isolates that possess both β-lactamase as well as have significant PBP3 mutations are termed β-lactamase positive, amoxicillin/clavulanic acid resistant (BLPACR).[10]

Frequently, isolates that have significant PBP3 mutations have only low-level resistance and may not show the phenotypes of ampicillin or amoxicillin/clavulanic acid resistance when tested by disc diffusion or microbroth dilution methods. Such isolates are classified as genetically BLNAR/BLPACR (gBLNAR/gBLPACR), respectively.[11]

A previous study of 236 invasive *H. influenzae* collected from 1990 to 2006 in Canada revealed an overall β-lactamase-positive, ampicillin-resistant rate of 17.8%, with no significant difference between serotypeable versus NT-Hi strains.[12] However, significantly more NT-Hi (12.9%) than serotypeable strains (2.3%) demonstrated resistance to trimethoprim/sulfamethoxazole.[12] From the same collection of isolates, the rate of gBLNAR was 8.1% and the rate of gBLPACR was 0.9%.[13] The present study also examines invasive isolates collected in the same manner as before and covers the period 2007–14 with the aim of providing an update on the current susceptibility of invasive *H. influenzae* in Canada.

Materials and methods

A total of 882 *H. influenzae* isolates from individual invasive disease cases in Canada from 1 January 2007 to 30 June 2014 were included in this study, and were collected in the same manner as described in our previous study.[12] Isolates were submitted from Provincial and Territorial Public Health Laboratories and hospitals to the National Microbiology Laboratory (NML) as part of our laboratory surveillance activities. Since practice varies from province to province, the specimens received at the NML do not necessarily represent a systematic collection of all invasive case isolates across Canada. Most isolates were submitted for identification and serotyping and very few isolates were submitted solely for antibiotic susceptibility testing.

Serotyping of *H. influenzae* isolates was performed by slide agglutination using commercial antisera (Difco, Oakville, Ontario, Canada) and by PCR detection of serotype-specific genes and the capsule transport gene, *bexA*, as described by Falla *et al.*[14] All non-typeable isolates were confirmed to be *H. influenzae* by partial 16S rRNA gene sequencing.[15] MLST was carried out using methods reported by Meats *et al.*,[16] and allele number and ST assignment was done through the *H. influenzae* MLST website, http://pubmlst.org/hinfluenzae/.

β-Lactamase production was detected using DrySlide nitrocefin (BBL, Becton Dickinson, Oakville, Ontario, Canada) and typed as TEM-1 or ROB-1 using primers described by Molina *et al.*[17] Disc diffusion was carried out according to CLSI guidelines[18] and the following antibiotic discs were tested: ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), cefaclor (30 μg), azithromycin (15 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clarithromycin (15 μg), imipenem (10 μg), levofloxacin (5 μg), meropenem (10 μg), moxifloxacin (5 μg), tetracycline (30 μg) and trimethoprim/sulfamethoxazole (25 μg). Ampicillin, cefixime and ceftriaxone MICs were determined by microbroth dilution using Sensititre plates (Oxoid, Nepean, Ontario, Canada) according to manufacturer's instructions. Control strain ATCC 49247 was used in each experiment for both disc diffusion and MIC determination.
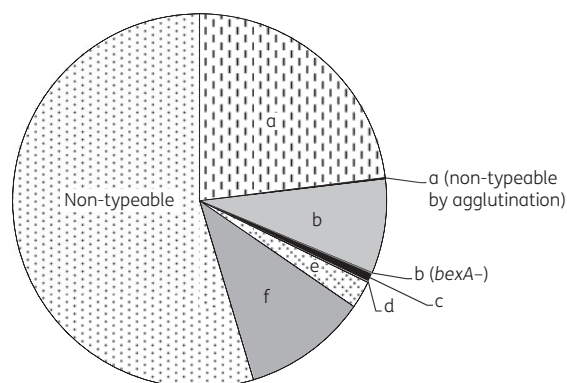
Figure 1. Serotype distribution of 882 invasive *H. influenzae* case isolates in Canada from 1 January 2007 to 30 June 2014.

Detection of gBLNAR and gBLPACR strains was done by PCR amplification and partial gene sequencing of the *ftsI* gene, which encodes PBP3.[10] Amino acid substitutions in PBP3 associated with reduced susceptibility to β-lactams were determined through alignment with the β-lactamase-negative, ampicillin-susceptible strain Rd KW20 (GenBank accession number L42023) and grouped accordingly.[19,20]

Statistical analysis and *P* value determination were performed using the online *z* score calculator for two population proportions (www.socialstatistics.com).

Results

Production of *β*-lactamases and susceptibility of invasive H. influenzae to ampicillin and ceftriaxone

Figure 1 shows the serotype distribution for the 882 invasive *H. influenzae* isolates collected from 1 January 2004 to 30 June 2014.

Over twice as many NT-Hi isolates (103/482 or 21.4%) produced β-lactamase compared with serotypeable isolates (42/400 or 10.5%) and all were found to be resistant to ampicillin when tested by the disc diffusion method. TEM-1 type β-lactamase was found in 93.8% (*n* = 136), ROB-1 was found in 4.1% (*n* = 6) and 2.1% (*n* = 3) were negative for both TEM-1 and ROB-1. Among the serotypeable isolates, β-lactamase was most commonly found in Hib (25/73, 34.2%), followed by Hic (1/6, 16.7%), Hif (10/95, 10.5%), Hie (2/21, 9.5%) and Hia (4/204, 2.0%) (Table 1).

Ampicillin, cefixime and ceftriaxone MICs were determined for 316 of the 882 isolates. Originally, 317 isolates were chosen for MIC determination, but 1 gBLNAR did not grow on the MIC plate. The 316 isolates included all 23 gBLPACR, 105 gBLNAR as well as randomly selected BLPAR (*n* = 24) and BLNAS (*n* = 164) isolates and were comprised of 155 serotypeable and 161 NT-Hi (see below and Table 2). All β-lactamase-producing isolates had an ampicillin MIC >4 mg/L, while the corresponding values for the β-lactamase-negative isolates ranged from ≤0.125 mg/L to 4 mg/L. There was only one NT-Hi isolate that was β-lactamase negative, but ampicillin resistant, according to CLSI guidelines with an MIC = 4 mg/L and had significant PBP3 mutations Asp-350-Asn, Ser-357-Asn, Met-377-Ile, Ser-385-Ile, Leu-398-Phe and Asn-526-Lys (group III+) and was typed by MLST as ST-586.

Table 1. Antibiotic resistance profiles of 882 invasive *H. influenzae* case isolates in Canada

Serotype	Number of isolates	β-Lactamase	Antibiotic resistance profile[a]
a	198	−	susceptible to all antibiotics tested
	1	−	CLR R
	1	−	TET R
	3	+	AMP R
	1	+	AMP R, CHL R, SXT R, TET R
b	44	−	susceptible to all antibiotics tested
	3	−	SXT R
	1	−	CHL R, SXT R, TET R
	19	+	AMP R
	4	+	AMP R, CHL R, SXT R
	1	+	AMP R, CHL R, TET R
	1	+	AMP R, CHL R, CEC R, SXT R
c	5	−	susceptible to all antibiotics tested
	1	+	AMP R
d	1	−	susceptible to all antibiotics tested
e	18	−	susceptible to all antibiotics tested
	1	−	SXT R
	2	+	AMP R
f	83	−	susceptible to all antibiotics tested
	1	−	SXT R
	1	−	CLR R
	10	+	AMP R
Non-typeable	283	−	susceptible to all antibiotics tested
	76	−	SXT R
	10	−	CEC R
	1	−	TET R
	1	−	AMP R[b]
	7	−	SXT R, CEC R
	1	−	SXT R, CLR R
	85	+	AMP R
	14	+	AMP R, SXT R
	2	+	AMP R, CEC R
	1	+	AMP R, SXT R, TET R
	1	+	AMP R, CHL R, SXT R, TET R

R, resistant.
[a]Antibiotics tested: AMP = ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg); CEC = cefaclor (30 μg); azithromycin (15 μg), ceftriaxone (30 μg); CHL = chloramphenicol (30 μg), ciprofloxacin (5 μg); CLR = clarithromycin (15 μg), imipenem (10 μg), levofloxacin (5 μg), meropenem (10 μg), moxifloxacin (5 μg); TET = tetracycline (30 μg); SXT = trimethoprim/sulfamethoxazole (25 μg).
[b]BLNAR isolate.

Table 2. Rate of gBLNAR and gBLPACR *H. influenzae* in Canada among serotypeable and non-typeable isolates

	β-Lactamase production	Total	Significant PBP3 mutations	No significant PBP3 mutations	gBLNAR rate (%)	gBLPACR rate (%)
Serotypeable	+	42	4	38		4/42 (9.5)
	−	358	1	357	1/358 (0.3)	
Non-typeable	+	103	19	84		19/103 (18.4)
	−	379	105	274	105/379 (27.7)	

Regardless of β-lactamase status, all 882 isolates were susceptible to ceftriaxone by disc diffusion and the 316 isolates tested for MICs were susceptible to both cefixime and ceftriaxone, according to CLSI guidelines.

Analysis of ftsI genes of invasive H. influenzae and the correlation to their ampicillin, cefixime and ceftriaxone MICs

Sequencing of the *ftsI* gene to determine significant PBP3 mutations associated with reduced susceptibility to β-lactams was carried out on all 882 isolates. Significant PBP3 mutations were found in five encapsulated isolates (1.3% of 400 isolates), including three Hib (all β-lactamase positive) and two Hif (one β-lactamase positive and one β-lactamase negative). In contrast, of the 482 NT-Hi isolates tested, 124 (25.7%) were found to contain significant PBP3 mutations (19 were β-lactamase positive) (Table 2). Of the 129 isolates with significant PBP3 mutations, 119 (92.2%) belonged to group II and were further classified into groups IIa ($n = 22$), IIb ($n = 61$), IIc ($n = 9$) and IId ($n = 27$) based on amino acid substitutions in addition to the Asn-526-Lys mutation (Table S1, available as Supplementary data at *JAC* Online). There were seven isolates belonging to group I (characterized by Arg-517-His), two group III+ (characterized by Ser-385-Thr, Leu-389-Phe and Asn-526-Lys) and one group III-like (characterized by Ser-385-Thr and Arg-517-His) (Table S1).

When the ampicillin MIC values of 269 β-lactamase-negative isolates (105 gBLNAR plus 164 BLNAS) were analysed, those with significant PBP3 mutations showed MIC values eight times higher than those without significant PBP3 mutations (Figure 2). The MIC_{50}/MIC_{90} values for isolates with and without significant PBP3 mutations were 1/2 mg/L and ≤0.125/0.25 mg/L, respectively.

Cefixime MICs for the 316 isolates ranged from ≤0.125 to 1 mg/L. The six isolates that showed the highest cefixime MIC values (either 0.5 or 1 mg/L) all had significant PBP3 mutations with three isolates belonging to group I, two group III+ and one group III-like. Ceftriaxone MIC values ranged from ≤0.03 to 0.25 mg/L. There were only two isolates with ceftriaxone MIC = 0.25 mg/L and both had significant PBP3 mutations and belonged to group III+.

Susceptibility of invasive H. influenzae to other antibiotics

Resistance to trimethoprim/sulfamethoxazole was significantly more common in NT-Hi ($n = 100$ or 20.7%) than in serotypeable isolates ($n = 12$ or 3.0%) ($P < 0.05$). Multi-drug resistance was also more common among NT-Hi: 26 (5.4%) versus 8 (2.0%) for serotypeable isolates ($P < 0.05$). Resistance to chloramphenicol, cefaclor, tetracycline and clarithromycin was also detected in small numbers (Table 1). No resistance to fluoroquinolones or to third-generation cephalosporins was observed.

Clonal analysis of invasive H. influenzae showing PBP3 mutations

Of the five serotypeable isolates showing significant PBP3 mutations, three were β-lactamase-positive Hib and belonged to three different, but related, STs (ST-95, ST-92 and ST-54): ST-92 is a
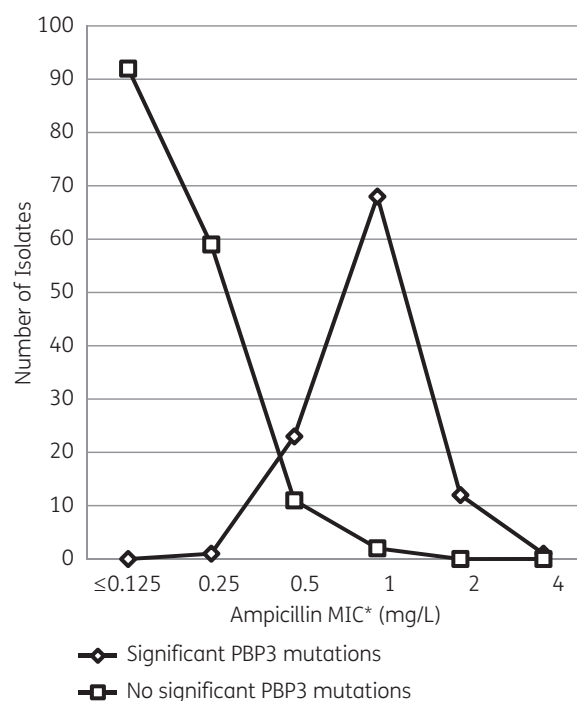
Figure 2. Ampicillin MIC values for 269 β-lactamase-negative invasive *H. influenzae* case isolates with ($n = 105$) and without ($n = 164$) significant PBP3 mutations. *According to CLSI guidelines: ≤1 mg/L = susceptible, 2 mg/L = intermediate and ≥4 mg/L = resistant.[18]

double-locus variant (DLV) of ST-95, and ST-54 is a triple-locus variant (TLV) of ST-95 and a DLV of ST-92. There were two Hif isolates with significant PBP3 mutations that belonged to ST-124 (gBLNAR) and its single-locus variant (SLV) ST-698 (gBLPACR) (Table S2).

Of the 124 NT-Hi isolates that showed significant PBP3 mutations, 19 were also β-lactamase positive (gBLPACR). Ten of the 19 NT-Hi gBLPACR isolates (52.6%) belonged to two unrelated STs, ST-165 and ST-396, while the remaining nine isolates belonged to eight other unrelated STs (Table S2).

Of the 105 gBLNAR NT-Hi isolates, 60 or 57.1% were found to belong to 8 STs (ST-14, ST-136, ST-156, ST-201, ST-245, ST-367, ST-396 and ST-556), while the remaining isolates were found to belong to 27 different STs. Five STs, namely ST-14 (10/10), ST-136 (4/4), ST-201 (10/10), ST-367 (10/10) and ST-396 (11/11), were exclusively found in isolates with significant PBP3 mutations. ST-14, ST-136 and ST-367 are either DLV or TLV of each other and are part of the same clonal complex. Other STs associated with isolates with β-lactamase production and/or significant PBP3 mutations are described in Table S2.

Discussion

In this study of 882 invasive *H. influenzae* isolates, the majority (83%) were β-lactamase negative and susceptible to ampicillin and only one true BLNAR isolate was found. However, it is interesting to note that over half of NT-Hi (253/482 or 52.5%) were resistant to one or more antibiotics when tested by disc diffusion and/or

contained significant PBP3 mutations, which is significantly higher than previously reported among Canadian invasive NT-Hi (56/147, 38.1%) ($P < 0.05$).[12,13] In contrast, only 13% (52/400) of serotype-able isolates from this study showed antibiotic resistance and/or significant PBP3 mutations, with the exception of Hib, where 29 isolates (39.7%) showed antibiotic resistance and/or significant PBP3 mutations.

The previous study found that the overall rate of β-lactamase-producing isolates was 17.8%, but decreased from 21.4% in 1990–99 to 16.4% in 2000–06.[12] However, in this study, even though the overall rate of β-lactamase production was lower (16.4%), the rate increased significantly throughout the study period, going from 13.5% in the years 2007–10 to 19% in 2011–14 ($P < 0.05$), with a high of 22.7% in 2014. Both studies found that more NT-Hi were β-lactamase positive compared with serotype-able isolates: 20.5% versus 13.5% in 1990–2006[12] and 21.4% versus 10.5% in this study.

In the previous study, 8.9% (21/236) of all invasive *H. influenzae* isolates tested were found to have significant PBP3 mutations;[13] however, the rate has increased significantly to 14.6% (129/882) in the present study ($P < 0.05$). The rates of significant PBP3 mutations observed in this study fluctuated between 8.8% in 2007 and 19.2% in 2014, but did not appear to increase consistently with each year.

The majority of gBLNAR isolates belonged to group II based on the first-stage substitution Asn-526-Lys[19] and displayed low-level resistance or reduced susceptibility to ampicillin, but remained susceptible to cefixime and ceftriaxone. When the MIC data were interpreted according to CLSI guidelines, only 1 out of 105 gBLNAR isolates tested could be classified as ampicillin resistant with MIC = 4 mg/L (group III+). However, using EUCAST breakpoints,[21] an additional 12 would be considered ampicillin resistant with MIC = 2 mg/L (1 group I and 11 group II). Despite this, it is important to note that the gBLNAR isolates had MIC values eight times higher than those of BLNAS strains (Figure 2). Group II isolates with low-level ampicillin resistance are also predominant in Spain, Sweden, Norway and Korea,[22–25] while the majority of Japanese isolates belong to group III and subgroups of group III.[26] The group III+/III-like isolates in this study were all from the province of British Columbia and contained the second-stage substitution Ser-385-Thr.[22] In addition, the group III-like isolate contained Arg-517-His and the group III+ isolates contained Leu-385-Phe and Asn-526-Lys. The Leu-389-Phe substitution is considered a third-stage mutation and is often associated with higher levels of resistance to β-lactams.[22,23] The two group III+ isolates in this study had ampicillin MICs of 1 and 4 mg/L, cefixime MIC = 0.5 mg/L and ceftriaxone MIC = 0.25 mg/L. The group III-like isolate had ampicillin MIC = 1 mg/L, cefixime MIC = 1 mg/L and ceftriaxone MIC = 0.06 mg/L. Using CLSI guidelines, all were susceptible to cefixime and ceftriaxone; however, the two group III+ isolates would have been resistant to ceftriaxone using EUCAST guidelines. These results are consistent with previous reports of group III+ isolates showing decreased susceptibility to third-generation cephalosporins.[24]

The finding of only two group III+ isolates 2 years apart may seem insignificant, but it is nevertheless an important observation due to the potential for rapid emergence of these strains. A Norwegian study noted the Leu-389-Phe substitution in only 13% of high-level resistant gBLNAR isolates from 2006–10 and 93% in 2011–13.[19] Japan has also reported a 2-fold increase in BLNAR isolates occurring between 2001 and 2004.[27] Even though these studies are mainly focused on respiratory isolates, it is possible to assume that invasive isolates may follow a similar path.

The link between significant PBP3 mutations and MLST has been previously reported and shows a relationship between specific PBP3 substitution patterns and certain STs.[25] Our data confirmed earlier findings that ST-14, ST-201, ST-367 and ST-396 were exclusively associated with isolates containing significant PBP3 mutations,[24,25] and identified four isolates belonging to ST-136 (part of the same clonal complex as ST-14 and ST-367), also associated with isolates containing significant PBP3 mutations. All ST-367 ($n = 10$) and 9 out of 10 ST-14 isolates had identical PBP3 mutation patterns between amino acid positions 350 and 526 (Asp-350-Asn, Met-377-Ile, Ala-502-Val, Asn-526-Lys) and matched previously reported patterns for these STs,[25] as did the four ST-136 isolates. All ST-396 isolates ($n = 11$) also had identical patterns (Ile-449-Val, Asn-526-Lys) as did ST-201 isolates ($n = 10$) (Asp-350-Asn, Gly-390-Glu, Asn-526-Lys) and again, both matched previously reported patterns for these STs.[25]

One limitation of this study is that not all invasive *H. influenzae* are captured due to our current passive laboratory surveillance system. As we have not changed our method of specimen collection since our last national study, isolates from this study can be compared with those from that previous study.[12] Also, isolates were not pre-selected for inclusion in this study and hence may be regarded as a representative of invasive strains in Canada. Compared with our last reports,[12,13] the key findings of this study are (i) the significant increase in β-lactamase-producing isolates (from 13.5% in 2007–10 to 19% in 2011–14), (ii) 14.6% of isolates with significant PBP3 mutations, (iii) >50% of NT-Hi are either resistant to one or more antibiotics and/or contained significant PBP3 mutations and (iv) confirmation of the relationship between ST and significant PBP3 mutations. Continued monitoring of antibiotic susceptibility and isolate characterization are important tools for detecting changes in disease-causing strains and providing guidance for empirical antibiotic treatment of patients.

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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