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Invasive *Haemophilus influenzae* in Manitoba, Canada, in the Postvaccination Era

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Fifty-two *Haemophilus influenzae* isolates from patients with invasive disease in the province of Manitoba, Canada, were examined for serotype, biotype, genotype, and antibiotic susceptibility. Half of the 52 isolates were found to be serotype a, and 38.5% (20 isolates) were found to be nonserotypeable (NST). There were only three serotype b strains and one each for serotypes c, d, and f. All 26 serotype a isolates belonged to biotype II and demonstrated identical or highly similar DNA fingerprints by pulsed-field gel electrophoresis. An analysis of these isolates by multilocus sequence typing showed that they belong to the clonal complex ST-23. While 69% (18 of 26) of the serotype a cases were found in males, only 9 (45%) of the 20 patients with NST isolates were males. Twenty (77%) of the 26 serotype a isolates were from patients who were <24 months old. Twelve (63%) of the NST isolates were from adult or adolescent patients. In contrast to the clonal nature of serotype a isolates, the 20 NST isolates were found to belong to 18 different sequence types. Most of these 18 different sequence types were unrelated to each other, with the exception of 7 sequence types grouped into three clonal groups. Two (6.25%) out of 32 serotypeable isolates (1 serotype a and 1 serotype b) and 6 (30%) of 20 NST isolates were resistant to ampicillin due to β-lactamase production. These results suggest a change in the epidemiology of *H. influenzae* disease, with the majority of invasive *H. influenzae* isolates being associated with serotype a and NST strains.

Surveillance for invasive *Haemophilus influenzae* disease in Canada dates back to 1979 (http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/list_e.html), before the introduction of *H. influenzae* serotype b (Hib) vaccine. Prior to the introduction of Hib vaccines, i.e., from 1969 to 1985 (29), most cases of invasive *H. influenzae* disease were caused by Hib; these cases occurred primarily in children aged 6 to 24 months, when natural serum antibody levels to the capsular polysaccharide of polyribosylribitol phosphate are at the lowest levels. The introduction of the polyribosylribitol phosphate conjugate vaccine in Canada occurred in 1992. Since then, the incidence of Hib has decreased dramatically, reaching an all-time low in 2000, with only four cases noted by a network of 12 pediatric centers across Canada monitoring for vaccine-preventable diseases (26, 27). However, surveillance for invasive *H. influenzae* disease in Canada captures only cases due to Hib, and there is limited information on the prevalence or incidence of invasive *H. influenzae* disease due to non-type b *H. influenzae*. Currently, it is not known if Hib vaccination alters the epidemiology of invasive *H. influenzae* disease by inducing capsule replacement. Capsule replacement in *H. influenzae* disease has been reported from at least two countries (Brazil and Portugal) after extensive use of the Hib vaccine (2, 25). Also, questions have recently been raised in an editorial comment (4) postulating that non-type b *H. influenzae* isolates are becoming a more common cause of invasive *H. influenzae* disease. This raises the question of what the appropriate public health response is to invasive non-type b *H. influenzae* disease. In order to address this question, we studied invasive *H. influenzae* strains (defined by isolation from normally sterile body sites, such as blood and cerebrospinal fluid [CSF]) isolated at the University of Manitoba Health Sciences Centre (UM-HSC) and Children’s Hospital in Winnipeg, Manitoba, during the last five years (2000 to 2004, inclusive) and characterized them according to their serotype, biotype, and genotype using a number of standard procedures.

**MATERIALS AND METHODS**

**Bacterial isolates and biochemical testing.** Isolates from patients with invasive *H. influenzae* disease were identified from the electronic database of the Clinical Microbiology Laboratory of the University of Manitoba Health Sciences Centre in Winnipeg, Manitoba. We were able to retrieve the corresponding *H. influenzae* isolates from 52 of the 53 individual cases: 1 isolate did not grow from the frozen stock. Identities of all isolates were reconfirmed by standard biochemical tests (14) and their biotypes determined according to current nomenclature (13).

**Serotyping by antiserum and PCR analysis of capsular polysaccharide synthesis genes.** Serotyping was done by slide agglutination assay using antisera from two commercial sources (Difco, Oxsville, Ontario, Canada; Denka Seiken, Tokyo, Japan). PCR amplification of serotype-specific and capsule transport *bexA* genes was done using primers described by Falla et al. (10). The detection of deletion of the *bexA*-IS1016 capsular genetic structure was done by PCR according to the method described by Kroll et al. (16).

**Clonal analysis by molecular methods.** For pulsed-field gel electrophoresis (PFGE), cultures were suspended in Tris-EDTA (TE) buffer (100 mM) and adjusted to a turbidity of 0.5 using a Dade Behring MicroScan turbidity meter (Dade Behring, West Sacramento, California). plugs for PFGE were prepared by mixing the cell suspensions with 1.6% SeaKem Gold agarose (Mandel, Guelph, Ontario, Canada). The plugs were then treated with lysis buffer (100 mM TE buffer containing 0.5 mg/ml proteinase K and 1% Sarkosyl) at 50°C for 90 min to lyse the bacteria. The released DNA within the plugs was cleaned by a series of

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TABLE 1. Serotype distribution of 52 H. influenzae isolates from patients with invasive disease

<table>
<thead>
<tr>
<th>Serotype</th>
<th>No. of isolates by isolation yr</th>
<th>Total no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>b</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>c</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>f</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NST</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

**RESULTS**

Invasive H. influenzae disease identified from 2000 to 2004 at the UM-HSC. The HSC and Children’s Hospital comprise the largest tertiary health care center in the province of Manitoba, serving residents of Manitoba, northwestern Ontario, and Nunavut. According to the UM-HSC 2004–2005 annual report (http://www.hsc.mb.ca/annual_report/HSC_04-05_AnnualReport.pdf), approximately 19,748 adults and 5,452 children patients were admitted to the HSC and Children’s Hospital, respectively.

During the 5-year period of 1 January 2000 to 31 December 2004, 53 cases of invasive H. influenzae disease were identified by the Clinical Microbiology Laboratory of the UM-HSC. We were able to retrieve 52 isolates matched to these 53 cases, and the serotype distribution of these 52 cases by year is presented in Table 1. The serotype identity of the 52 isolates was confirmed by PCR detection of their serotype-specific capsular polysaccharide synthesis genes. The capsular transport *bea* gene was also detectable in all serotypeable strains. The most frequently identified serotype was a (26 cases), followed by nonserotypeable (NST) strains (20 cases). There were only three serotype b isolates and one isolate each of serotypes c, d, and f. There was no trend observed in the frequency of serotype a and NST isolates during the 5-year period. Although we did not have the vaccination history of the patients, it was of interest that the three cases of Hib infection were found in infants aged 5, 6, and 9 months. In Canada, the routine immunization schedule for the Hib vaccine is a primary dose given at 2 months of age, followed by boosters at 4, 6, and 18 months of age. Therefore, none of the three patients with Hib would have received all four doses of the vaccine. It was very possible that at least one or two of the patients had received only two doses of the vaccine.

The overall age distribution of the patients with invasive H. influenzae infection is presented in Fig. 1. Thirty-three cases occurred in patients under 2 years of age, while 9 cases occurred in patients aged 50 years or over. Serotype a was more likely to be isolated from children than NST H. influenzae (chi-square test, \( P < 0.01 \); odds ratio, 5.0; 95% confidence interval, 1.2 to 22.4). More males than females were infected with serotype a isolates than with NST isolates; however, this trend was not significant (\( P = 0.10 \)). Overall, most (41) H. influenzae isolates were from blood culture, while 9 isolates were from CSF. Three isolates were positive by both blood and CSF cultures.

**Microbiological characterization of invasive H. influenzae isolates.** All 26 serotype a organisms belonged to biotype II, and the three serotype b isolates were biotype I. Eleven of the 20 NST strains were typed as biotype II, 6 as biotype III, 1 as biotype IV, and 2 as biotype V. Eight of the 52 invasive H. influenzae isolates produced a \( \beta \)-lactamase and are thus ampicillin resistant. \( \beta \)-Lactamase production was found more often in the NST strains (6/20, or 30%) than in the serotypeable strains (2/32, or 6%). Of the two \( \beta \)-lactamase-positive serotypeable H. influenzae strains, one was identified as serotype a and the other as serotype b. All 52 isolates were found to be sensitive to both chloramphenicol and ceftriaxone; three NST isolates (15%) were found to be resistant to sulfamethoxazole-trimethoprim. No \( \beta \)-lactamase-negative ampicillin-resistant strains were found in this collection (Table 2).

**Clonal population of serotype a and NST H. influenzae isolates from cases of invasive disease.** When analyzed by PFGE, the 26 Hia isolates showed either identical or highly similar DNA fingerprint patterns with serotype a isolates than with NST isolates; however, this trend was not significant (\( P = 0.10 \)). Overall, most (41) H. influenzae isolates were from blood culture, while 9 isolates were from CSF. Three isolates were positive by both blood and CSF cultures.

**Microbiological characterization of invasive H. influenzae isolates.** All 26 serotype a organisms belonged to biotype II, and the three serotype b isolates were biotype I. Eleven of the 20 NST strains were typed as biotype II, 6 as biotype III, 1 as biotype IV, and 2 as biotype V. Eight of the 52 invasive H. influenzae isolates produced a \( \beta \)-lactamase and are thus ampicillin resistant. \( \beta \)-Lactamase production was found more often in the NST strains (6/20, or 30%) than in the serotypeable strains (2/32, or 6%). Of the two \( \beta \)-lactamase-positive serotypeable H. influenzae strains, one was identified as serotype a and the other as serotype b. All 52 isolates were found to be sensitive to both chloramphenicol and ceftriaxone; three NST isolates (15%) were found to be resistant to sulfamethoxazole-trimethoprim. No \( \beta \)-lactamase-negative ampicillin-resistant strains were found in this collection (Table 2).

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TABLE 2. Antibiogram, determined by disk diffusion test, of 52 $H. influenzae$ isolates from patients with invasive disease

<table>
<thead>
<tr>
<th>Serotype</th>
<th>β-lactamase production$^b$</th>
<th>AMP (2 µg)</th>
<th>AMP (10 µg)</th>
<th>CHL (30 µg)</th>
<th>CRO (30 µg)</th>
<th>SXT (25 µg)</th>
<th>No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>1</td>
</tr>
<tr>
<td>a</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>25</td>
</tr>
<tr>
<td>b</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>1</td>
</tr>
<tr>
<td>b</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>2</td>
</tr>
<tr>
<td>c</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>1</td>
</tr>
<tr>
<td>d</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<td>1</td>
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<tr>
<td>f</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>1</td>
</tr>
<tr>
<td>NST</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>5</td>
</tr>
<tr>
<td>NST</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>2</td>
</tr>
<tr>
<td>NST</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td>NST</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$ Antibiotics: AMP, ampicillin; CHL, chloramphenicol; CRO, ceftriaxone; SXT, sulfamethoxazole-trimethoprim (19:1). Susceptibility patterns: S, sensitive; R, resistant, based on NCCLS guidelines for disk diffusion susceptibility testing of $H. influenzae$ (21).

$^b$ β-lactamase production was determined by DrySlide nitrocefin.

prints, with only three patterns being recognized (Fig. 2A). This result suggests that the Hia strains that cause invasive $H. influenzae$ disease are clonal. The PFGE observation was confirmed by MLST, with all 26 Hia belonging to the ST-23 clonal complex. Twenty-five isolates were identified as ST-23, and the sequence type of the remaining isolate differed from ST-23 by only one allele among the seven housekeeping gene loci studied (Fig. 3).

In contrast to the homogeneity of the Canadian Hia population, the NST $H. influenzae$ strains appeared to display a more heterogeneous pattern of DNA fingerprints (Fig. 2B). The heterogeneity of the NST $H. influenzae$ strains was also confirmed by MLST analysis. There were, altogether, 18 different STs among the 20 NST isolates, with only 2 STs being represented by 2 isolates in each case. For the remaining 16 STs, each was represented by only a single isolate (Fig. 3).

One of the Hib isolates was identified as ST-44 (identical to the ST of the Eagan strain, which belongs to the clonal lineage of A1a), and the remaining two Hib isolates were identified as ST-95, which differed from ST-44 in three alleles. The Hia and Hib isolates had different STs among the 20 NST isolates, with only 2 STs being associated with virulence, our study did not confirm such an association (although only a limited number of Hia isolates were analyzed for this phenomenon).

The other striking feature of this study is the almost equally large number of cases due to NST strains (20 of the 52 cases, or 38.5%) as to serotypeable strains. The virulence properties of NST $H. influenzae$ have been disputed (22); however, our findings do indicate a clear link between NST $H. influenzae$ and invasive disease. Unlike the cases of Hia infection, there were more (63%, or 12 out of 19 cases; age information was not available for 1 NST case) cases of NST infection among adolescents or adults than infants or children (37% or 7 out of 19 cases were under the age of 13 months). Also unlike cases of Hia infection, there is no gender predilection in the cases of NST infection. Another virulence factor, β-lactamase production, was seen more frequently in NST isolates than in the Hia isolates. Microbiologically, the NST isolates were more heterogeneous, with no particular DNA fingerprint pattern predominating among the 20 isolates. This heterogeneity was also evident from the MLST data. Their sequence types were scattered over the minimum spanning tree diagram (Fig. 3), with no particular sequence or clonal type predominating. The ob-

DISCUSSION

Although several studies have reported invasive disease due to Hia (1, 11, 20, 25), most of them were based on reports of only a few cases. This is one of the few studies to date in which a large number of invasive Hia isolates were examined. Twenty-six (50%) of the 52 cases of invasive $H. influenzae$ infection in this study were caused by Hia. This pattern is in contrast to the three cases of Hib infection (6%) among all of the cases of invasive $H. influenzae$ disease in Manitoba in the last five years. Only 6% of the 52 cases of invasive $H. influenzae$ infection were caused by serotypes other than Hia and Hib. Before the introduction of Hib vaccines, Hib was responsible for the majority of serious infections, such as meningitis, epiglottitis, pneumonia, and septicemia, in early childhood, suggesting that it has a more virulent nature than other serotypes. With the declining incidence of Hib, it appears from this study, as well as from others, that Hia may be the second most clinically virulent type among the six serotypes of $H. influenzae$. Since capsular structure is related to virulence and serotyping of $H. influenzae$, it is of interest that the Hia capsule structure is more similar to that of Hib than to those of other $H. influenzae$ serotypes. Both the Hia and Hib capsules contain the five-carbon sugar ribitol. In Hia, the ribitol is linked to glucose to form the polymer of glucose-ribitol phosphate (3), while in Hib, the capsule is made up of a polymer of ribose-ribitol phosphate (8).

Similar to findings of other studies (1, 11, 20, 25), most cases of Hia infection were in children. Twenty-one (81%) of the 26 patients with Hia infection were children under the age of 4, and 14 of those were under the age of 12 months. In this study, there were also five cases in adults: one was 27 years of age, two were 37 years of age, and two were between 50 and 60 years of age. Unlike other studies, including one that examined 76 cases of Hia infection among Navajo and White Mountain Apache children in the United States (20), our Hia cases involved twice as many males as females (69% males and 31% females). This apparent higher proportion of males versus females was found in patients aged 2 years or under and in the adult patients (there was only 1 female out of the 5 adults; there were 7 females out of the 20 patients with Hia infection in those aged ≥36 months).

All 26 Hia isolates in this study belonged to biotype II, showed very similar DNA fingerprints, and were related to one another by belonging to the ST-23 clonal complex. This limited genetic diversity has also been reported for encapsulated $H. influenzae$ strains other than Hib (including Hia, Hie, and Hif) (23). Unlike cases of Hia infection reported from The Gambia, western Africa (17), or the United States (11, 20), where a deletion involving the becA IS1016 gene segment in the capsular polysaccharide synthesis operon has been suggested to be associated with virulence, our study did not confirm such an association (although only a limited number of Hia isolates were analyzed for this phenomenon).
FIG. 2. (A) Genetic relationship of 26 H. influenzae (Hi) serotype a isolates by pulsed-field gel electrophoresis analysis of SmaI-digested DNA. (B) Genetic relationship of 20 NST H. influenzae isolates by pulsed-field gel electrophoresis analysis of SmaI-digested DNA.
Observation of heterogeneity among NST \textit{H. influenzae} strains has already been found in a number of other studies (2, 5, 6, 9, 24). The finding of NST \textit{H. influenzae} causing invasive disease in the adult population may be related to the fact that NST \textit{H. influenzae} is an important respiratory pathogen in patients with chronic obstructive pulmonary disease (15). \textit{H. influenzae} strains isolated from patients with an acute exacerbation of chronic obstructive pulmonary disease have been reported to induce more inflammation than colonizers, which may explain their increased pathogenicity in these patients (7). However, it is not possible from our laboratory-based study to know the proportion of the cases of NST \textit{H. influenzae} infection with pulmonary involvement. Therefore, our second phase of characterizing the changing epidemiology of invasive \textit{H. influenzae} disease would involve chart reviews to determine host factors that may play a role in invasive NST \textit{H. influenzae} disease.

Although our data are based on findings from only one province in Canada, the observations have far-reaching implications regarding the changing epidemiology of invasive \textit{H. influenzae} disease in Canada. Therefore, we propose to enhance the national surveillance of invasive \textit{H. influenzae} disease by suggesting that the current national case definition of invasive \textit{H. influenzae} disease be modified to include cases with positive isolation of any of the six serotypes of \textit{H. influenzae}, as well as NST \textit{H. influenzae}, from normally sterile body sites. Such an initiative would potentially have an impact upon vaccine development for invasive \textit{H. influenzae} infection. Furthermore, we advocate that serotypes determined by bacterial agglutination testing should be confirmed by PCR genotyping in order to minimize potential errors in serotyping (18) and to ensure the accuracy of the serotype information for all cases of invasive \textit{H. influenzae} infection.

As a follow up to this study, we are conducting a chart review of the 52 patients with invasive \textit{H. influenzae} disease in order to describe disease severity and outcomes and to determine whether host risk factors can be identified in association with invasive Hia or invasive NST \textit{H. influenzae} disease. We are also planning further analyses of the NST \textit{H. influenzae} isolates in
order to determine if there are common virulence traits within this genetically diverse group of bacteria.

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REFERENCES


