Clonal relationship of recent invasive *Haemophilus influenzae* serotype f isolates from Denmark and the United States

Brita Bruun, 1,2 Bente Gahrn-Hansen, 3 Henrik Westh 4 and Mogens Kilian 5

1 Department of Clinical Microbiology, Statens Serum Institut, Artillerivej 5, DK 2300 Copenhagen S, Denmark
2 Department of Clinical Microbiology, Hillerød Hospital, Helsevej 2, DK 3400 Hillerød, Denmark
3 Department of Clinical Microbiology, Odense University Hospital, J. B. Winslevsvej 21-2, DK 5000 Odense C, Denmark
4 Department of Clinical Microbiology, Hvidovre Hospital, Kettegaard Alle 30, DK 2650 Hvidovre, Denmark
5 Department of Medical Microbiology and Immunology, Aarhus University, Universitetsparken, DK 8000 Aarhus C, Denmark

Surveillance performed after the introduction of general *Haemophilus influenzae* serotype b (Hib) vaccination in Denmark identified 13 cases of invasive bacteraemic *H. influenzae* serotype f (Hif) disease in adults over a period of 7 years. Bacteraemic respiratory tract infections accounted for 61% of cases, but meningitis, epiglottitis and osteoarthritis were also seen. Recent Danish isolates were compared to recent American isolates, historical Hif strains and non-Hif invasive strains. Results of conventional serotyping were confirmed by PCR detection of the serotype-f-specific cap and bexA gene sequences. Multilocus enzyme electrophoresis typing revealed that recent Danish and American isolates belonged to a single Hif clone, which may be undergoing expansion. The need for accurate serotyping of *H. influenzae* to enable reliable monitoring for Hib replacement by other capsular types is emphasized.

INTRODUCTION

Concern about a possible rise in the incidence of invasive infection with non-serotype b capsulate *Haemophilus influenzae* after widespread vaccination against *H. influenzae* type b (Hib) has been expressed by a number of investigators around the world (Adderson et al., 2001; Campos, 2001; Cerquetti et al., 2003; Ogilvie et al., 2001; Omikunle et al., 2002; Ribeiro et al., 2003; Urwin et al., 1996). A general Hib vaccination programme was introduced in Denmark in 1993, with a subsequent substantial reduction in the incidence of Hib disease. Concomitantly, the number of non-type b capsulate *H. influenzae* strains, predominantly type f, from invasive infections sent to the Reference Laboratory, Statens Serum Institut (SSI), increased.

We report on the clinical and epidemiological characteristics of 13 recent cases of invasive *H. influenzae* type f (Hif) infection in Denmark. In addition, recent Danish Hif isolates were compared to recent non-capsulate invasive *H. influenzae* isolates, recent invasive American Hif isolates and historical Hif isolates by multilocus enzyme electrophoresis (MLEE) in order to determine the genetic relatedness of these bacteria.

METHODS

Clinical study. A case was defined as isolation of Hif from blood or cerebrospinal fluid (CSF). Cases of pneumonia, acute exacerbation of chronic obstructive pulmonary disease (COPD) and otitis media were only included if there was accompanying bacteraemia. Cases were identified from the records of the Reference Laboratory, which receives strains for identification and typing from the clinical microbiology laboratories in Denmark. The first registered invasive case since 1975 was seen in 1995. Collection of cases for the study was stopped in July 2002. Complete clinical records were collected from all patients except no. 1, where only a discharge letter was obtained.

Strains. Inclusion of the 13 Danish clinical isolates from systemic infections was based on positive agglutination in *H. influenzae* serotype f antiserum and in polyvalent *Bacto H. influenzae* antiserum a–f (Difco). These tests were initially performed in the Reference Laboratory at SSI. Only nine of the 13 invasive Hif isolates that initially were examined
were available for further study. For comparative purposes, an additional 23 strains were examined. They included three recent non-invasive Danish Hif isolates (HK 2026, HK 2028, HK 2036); four recent invasive American Hif strains received from Monica Farley, Centers for Disease Control and Prevention, Atlanta, GA, USA (Urwin et al., 1996); three Danish Hif strains from the 1970s (HK 172, HK 192, HK 247), two of which were isolated from CSF and one from otitis media; four invasive Hif strains from 1945 to 1951 from the collection of H. C. Engbæk, SSI (2547–2550); eight recent invasive non-capsulate Danish H. influenzae isolates (2529/01, 2117/01, 2118/01, 2116/01, 2069/01, 2119/01, 2429/02, 2444/02); and Hif strain NCTC 8473, an original strain of the now deceased Margaret Pittman isolated from CSF. The strains subjected to further examination are seen in Fig. 1.

Serotyping. Serotyping of each strain was repeated using alternative antisera. A suspension of bacteria collected from an 18 h chocolate agar culture was prepared in saline. A drop of the suspension was mixed on a microscope glass slide with antisera raised in burros (kindly provided by Rachel Schnersson, National Institutes of Health, Bethesda, MD, USA). Agglutination developing with one serum within 10 s of gentle rocking was considered a positive reaction in the absence of reactions in other antisera.

Capsular PCR typing. Capsular PCR typing based on detection of serotype-specific capsular polysaccharide biosynthesis gene sequences (a through f) and transport gene bexA was carried out according to the principles described by Falla et al. (1994) with Ready-To-Go PCR Beads (Amersham Pharmacia Biotech), added genomic DNA and the respective primer sets for each of the serotypes and bexA for a final concentration of 5 pmol l−1.

Biotyping. Biotyping based on reactions for indole, urease and ornithine decarboxylase production was performed according to methods described previously (Kilian, 1976).

MLEE. Analysis of the phylogenetic relationships of the bacterial strains was performed by MLEE. Bacterial lysates for MLEE were prepared by sonication, electrophoresed in starch gels, and selectively stained for activity of each of 13 metabolic enzymes as described by Selander et al. (1986). Electromorphs (allozymes) of each enzyme equated with alleles at the corresponding structural gene locus were scored and assigned a

---

**Fig. 1.** Phylogenetic relationship, biotype, serotype and origin of 32 Haemophilus influenzae isolates examined in the study.
number according to decreasing rate of anodal migration. A pairwise
distance matrix based on the MLEE data was produced in the program
ETMEGA of T. S. Whittam (www.foodsafe.msu.edu/whittam/#Pro-
grams) and the phylogenetic tree was constructed using the neigh-
bour-joining algorithm in the MEGA version 2.1 software package
(Kumar et al., 2001).

RESULTS

Clinical study

A summary of important clinical data from the 13 cases of
invasive Hif disease is presented in Table 1. All patients were
above the age of 55 years and the majority (77%) were
female. Underlying diseases or other factors predisposing to
infections were present in eight of the patients. The following
remarkable cases are described in more detail.

Meningitis

The patient (case 4) was hospitalized because of a blurred
sensorium and vomiting preceded by a common cold and
pain in the right first toe. There was a past history of recurrent
otitis media and cortical mastoidectomies in young adult-
hood. The patient had cared for two grandchildren suffering
from acute otitis media during the week before hospitaliza-
tion. The patient’s symptoms resolved rapidly on ceftriaxone
treatment. The pain, swelling and tenderness of the big toe
were judged to be a metastatic infection.

Osteoarthritis

The patient (case 6) was hospitalized after 4 weeks of
influenza-like symptoms with pain eventually concentrating
in the left arm and shoulder. During the following weeks of
hospitalization she underwent a number of examinations
and tests, including blood cultures, with negative results;
bone and leukocyte scans did, however, demonstrate in-
creased uptake of tracers in the left shoulder. As intermittent
fever continued, three blood cultures were obtained on three
consecutive days, and one of these yielded Hif. Magnetic
resonance imaging demonstrated arthritis and osteomyelitis
of the shoulder. The patient’s fever resolved readily on
ceftriaxone, and she was discharged with oral ciprofloxacin
for a planned duration of 3–6 months.

Supra-epiglottitis

The patient (case 10) was hospitalized for dysphagia and sore
throat. Physical examination disclosed audible respiration,
fever and a 10×5 cm swelling of the neck on the left side.
After blood culturing the patient was started on cefuroxime
and metronidazole plus hydrocortisone. When a CT scan
demonstrated pronounced swelling with compression of the
trachea, the patient was intubated. Hereafter the patient
improved rapidly.

Cholangitis and liver abscess

The patient (case 12) was hospitalized after intermittent
cholangitis symptoms for 2–3 months. As she became
increasingly hypotensive and anuric she was placed in
intensive care and started on cefuroxime and metronidazole.
Six days later a cholecystectomy was performed. The gall
bladder was found to be heavily inflamed and a 10×8 cm
liver abscess was drained. A stent was placed in the bile duct.
After this the course was uncomplicated.

Microbiological study

The results of biotyping and serotyping by serological
examination and by PCR detection of serotype-specific cap
gene sequences are summarized in Fig. 1 together with the
origin of the isolates. A total of 24 isolates were recorded as
serotype f based on serological analysis in two independent
laboratories and using two different sets of antisera. The

Table 1. Thirteen cases of invasive infections with Haemophilus influenzae serotype f in Denmark from 1995 to 2002

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>Year seen</th>
<th>Predisposing factors</th>
<th>Clinical presentation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69</td>
<td>F</td>
<td>1995</td>
<td>COPD, steroids</td>
<td>Pleuropneumonia</td>
<td>Blood</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>M</td>
<td>1996</td>
<td>Myelomatosis, COPD, steroids</td>
<td>Acute exacerbation in COPD</td>
<td>Blood</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>F</td>
<td>1996</td>
<td>None</td>
<td>Pneumonia</td>
<td>Blood</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>F</td>
<td>1998</td>
<td>Mastoidectomy antea</td>
<td>Meningitis, otitis media</td>
<td>CSF, blood</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>M</td>
<td>1998</td>
<td>Cirrhosis, encephalopathy hepatica</td>
<td>Pneumonia</td>
<td>Blood</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
<td>F</td>
<td>1999</td>
<td>None</td>
<td>Pneumonia</td>
<td>Blood</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>F</td>
<td>1999</td>
<td>COPD, mesothelioma</td>
<td>Pneumonia, pleural empyema</td>
<td>Blood, BAL</td>
</tr>
<tr>
<td>8</td>
<td>68</td>
<td>F</td>
<td>2001</td>
<td>None</td>
<td>Osteoarthritis</td>
<td>Blood</td>
</tr>
<tr>
<td>9</td>
<td>84</td>
<td>F</td>
<td>2001</td>
<td>Chronic lymphatic leukaemia</td>
<td>Pneumonia</td>
<td>Blood, tracheal suction</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>F</td>
<td>2001</td>
<td>None</td>
<td>Supra-epiglottitis</td>
<td>Blood</td>
</tr>
<tr>
<td>11</td>
<td>59</td>
<td>M</td>
<td>2002</td>
<td>Lung cancer, steroids, neutropenia</td>
<td>Bacteraemia without clinical focus</td>
<td>Blood</td>
</tr>
<tr>
<td>12</td>
<td>58</td>
<td>F</td>
<td>2002</td>
<td>None</td>
<td>Cholangitis, cholelithiasis, liver abscess</td>
<td>Blood</td>
</tr>
<tr>
<td>13</td>
<td>70</td>
<td>F</td>
<td>2002</td>
<td>COPD</td>
<td>Acute exacerbation in COPD</td>
<td>Blood</td>
</tr>
</tbody>
</table>
serotyping results were confirmed by PCR detection of serotype-f-specific cap gene sequences, apart from one isolate (2244/02), which gave a positive result in both serotype f antisera, but yielded no PCR product with the serotype-f- and bexA-specific primers. We concluded that the positive agglutination reaction obtained for this strain is falsely positive and due to cross-reacting somatic antigens. Likewise, none of seven recent blood isolates that agglutinated in several typing antisera, including the serotype f antiserum (‘polyagglutinable’), yielded amplification products with the serotype- and bexA-specific primers. This was also true for a single blood isolate (2190/01) that was recorded as non-capsulate on the basis of colony appearance and lack of reaction in antisera a–f.

All serotype f strains, including recent and historical Danish isolates, as well as historical and more recent invasive isolates from the USA (Adderson et al., 2001), were biotype I. Six of seven polyagglutinable isolates belonged to biotypes II or III.

The phylogenetic analysis revealed that all recent Danish type f isolates were closely related to the four invasive isolates from the USA and two Danish serotype f isolates from a case of meningitis in 1970 and a case of otitis media in 1973 (Fig. 1). These isolates were joined by four historic serotype f isolates from Denmark (1945–1951) and the USA (isolated before 1951) at a genetic distance of 0·15. Another Danish serotype f isolate from a case of meningitis in 1972 was more distantly related. One serotype f reference strain (NCTC 8473) isolated from meningitis in the USA in 1944 was phylogenetically distinct and more related to the non-capsulate isolates than to other serotype f strains.

**DISCUSSION**

The clinical syndromes in the 13 adults with invasive Hif disease seen in the present Danish study resemble the adult cases described from the USA (Slater et al., 1990; Urwin et al., 1996) and Spain (Campos et al., 2003). Lower respiratory infections, pneumonia and acute exacerbations in COPD predominated in all studies, but other syndromes known from classical Hib disease, such as meningitis, epiglottitis and osteoarthritis, were also seen. Underlying diseases or other conditions predisposing to infection were present in 78 % of patients in the study of Urwin et al. (1996) and in 61 % of patients in the present study. Overall mortality was 30 % among adults in the American study and 14 % in the Spanish study, while none died in the present study.

In contrast to the study of Urwin et al. (1996) where 26 % of invasive Hif cases occurred in children younger than 5 years, no strains from paediatric cases were found in the present study. This is most likely due to the low number of cases seen in the Danish series. Another explanation might be that some type f isolates from children have been misidentified as type b by local clinical microbiology laboratories using slide agglutination and not submitting isolates to reference laboratories for confirmation. Serotyping of H. influenzae by slide agglutination is difficult, especially since local laboratories perform the once-routine method less frequently after infection with Hib has become rare. In a recent study from the CDC, 68 % of 40 isolates identified as type b by slide agglutination by local laboratories did not contain the correlating serotype b capsule gene, as determined by PCR (LaClaire et al., 2003). This emphasizes the need for accurate serotyping preferably performed by reference laboratories in order to ensure reliable monitoring for Hib replacement by other serotypes.

The question of whether there has been an increase in invasive Hif infection in Denmark cannot be answered by the present study. Non-type b invasive strains have been sent to the Reference Laboratory regularly on a voluntary basis, and increased awareness and interest after introduction of Hib vaccination may account for the rise in case ascertainment. This may also explain the fact that three additional invasive Hif cases were identified within 6 months of termination of inclusion of cases into the present study. Urwin et al. (1996) found a modest increase of invasive Hif from 0·5 to 1·9 cases per 1 000 000 population from 1989 to 1994 in an American multistate study with 99 Hif cases. Perdue et al. (2000) reported that the incidence of invasive non-type b (including both non-typable and other H. influenzae serotypes) increased from 0·5 to 1·1 cases per 100 000 Alaskan inhabitants more than 10 years old from 1980–1990 to 1991–1995; four Hif cases occurred in 1980–1990 and five in 1991–1995, indicating a twofold increase. On the other hand, Campos et al. (2003) in their study of 49 Hif strains, of which 25 were isolated from blood or CSF, stated that their findings did not support the hypothesis of Hif replacement after introduction of Hib vaccination in the mid-1990s in Spain.

Whether an increase in Hif and other encapsulated non-type b disease is taking place worldwide needs further epidemiological studies involving longer post Hib vaccination observation periods in order to compensate for naturally occurring fluctuations in incidence. A decisive factor determining a possible increase may well be whether encapsulated non-type b strains acquire the mutated capsulation locus structure present in most invasive Hib strains, which facilitates amplification of capsule expression and increased virulence. Recently, the presence of the necessary bexA gene deletion has been demonstrated in the capsulation locus of a sizeable proportion of Hif and H. influenzae type a isolates from invasive infections in the Gambia, West Africa (Kroll et al., 1994; Ogilvie et al., 2001).

The limited genetic diversity of the recent Danish clinical isolates of Hif revealed by our phylogenetic analysis (Fig. 1) is in full agreement with previous observations from the USA (Anyanywu et al., 2003; Omikunle et al., 2002) and Spain (Campos et al., 2003). The close relationship of our isolates to strains representing cases from the USA suggests that these recent cases of Hif disease are caused by a single clone (according to the definition of Maynard Smith, 1995) in which genetic diversity is very limited. This is in contrast to the pre-vaccination situation with Hib infections, which
showed a distinct geographical distribution of different clones (Musser et al., 1990; Van Alphen et al., 1987). The current clone of Hif is furthermore closely related to isolates from Denmark and the USA dating from 1945 to 1951 (Fig. 1), demonstrating long-term stability of the situation. Comparison of genetic distances suggests that all these isolates belong to a single of the three phylogenetic lineages detected among 50 serotype f strains by Musser et al. (1988) (joining at a genetic distance of 0.47). The exclusive isolation of strains belonging to only one of these lineages suggests that one clonal lineage has recently undergone expansion. It will therefore be of interest to perform continued worldwide surveillance of encapsulated H. influenzae isolates in order to monitor possible Hib replacement by this specific Hif clone and other non-Hib clones.

REFERENCES


