Antibiotic resistance and clinical significance of

_Haemophilus influenzae_ type f

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Objectives: Little is known about the antibiotic susceptibility and clinical significance of non-type b capsulated _Haemophilus influenzae_. We studied the antibiotic resistance patterns, plasmid carriage and clinical features of _H. influenzae_ type f infections in Spain during 1996–2002.

Patients and methods: Forty-nine _H. influenzae_ type f recovered from Spanish hospitals were analysed at a central laboratory where full microbiological and molecular epidemiological studies were carried out. Antimicrobial susceptibility testing was performed in accordance with NCCLS guidelines.

Results: Twelve strains (24.5%) were resistant to ampicillin and 22 (44.9%) to co-trimoxazole. Decreased susceptibility to clarithromycin, tetracycline, chloramphenicol and rifampicin was found in 16.3%, 12.2%, 14.3% and 2% of strains, respectively. Multidrug resistance was present in nine (18.4%) of the 49 isolates. The most prevalent resistance phenotype was ampicillin/tetracycline/co-trimoxazole/chloramphenicol, which was detected in five isolates. All six strains that were simultaneously resistant to ampicillin, tetracycline and chloramphenicol had conjugative plasmids. The main clinical diagnoses were pneumonia (32.6%), sepsis (18.4%) and meningitis (16.3%). Thirty-two patients (65.3%) had previous underlying predisposing conditions, principally respiratory diseases (20.4%). Twenty-one patients (42.8%) had impaired immunity. Thirty-seven (75.5%) patients were >14 years old, 12 (24.5%) were ≤14 years, and seven were ≤5 years. Most isolates were clonally related.

Conclusions: A high prevalence of antibiotic resistance, including multiresistance, was detected in Spanish _H. influenzae_ type f isolates. Carriage of large conjugative plasmids was strongly associated with antibiotic resistance. _H. influenzae_ type f is mainly an opportunistic pathogen, although it may cause primary severe infections, such as meningitis in children.

Keywords: _H. influenzae_, plasmid carriage, opportunistic infections

Introduction

_Haemophilus influenzae_ is a frequent inhabitant of the human upper respiratory tract and a strictly human pathogen. It has been recognized as an important cause of a variety of severe clinical conditions such as pneumonia, bacteraemia, meningitis, epiglottitis, septic arthritis and cellulitis.1

_H. influenzae_ can be classified into six capsular types (a–f) and non-typeable strains according to its capsular antigen composition. _H. influenzae_ type b is the most invasive capsular type and was once one of the most prevalent bacterial pathogens, causing meningo-encephalitis and other invasive infections in children below the age of 5 years. Since the early 1990s, the widespread use of _H. influenzae_ type b conjugate vaccines in western countries has had a dramatic effect on preventing invasive infections due to this organism.2,3 However, such widespread vaccination has raised concerns about the possibility of serotype replacement, as other serotypes and/or non-typeable strains may fill the ecological niche left open by _H. influenzae_ type b.4 It has been suggested that _H. influenzae_ type f could be a new emerging pathogen.5 Serotype information is essential to evaluate vaccine efficacy and eventual changes in the epidemiology of _H. influenzae_ infections.5 Recently, the CDC have recognized

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important serotype discrepancies between standard slide agglutination typing and PCR-based capsule typing. Some authors have noted a trend towards an increased incidence of *H. influenzae* types e and f after widespread vaccination against *H. influenzae* type b.

Little information is available concerning the clinical, microbiological and epidemiological significance of *H. influenzae* type f. Most published reports are of individual clinical cases of meningitis, septic arthritis, osteomyelitis, pneumonia and sepsis.

During 1996–2002, we studied the antibiotic resistance patterns, plasmid carriage and clinical features of *H. influenzae* type f infections in Spain. Additionally, we investigated whether the IS1016-bex A deletion (a virulence factor found in invasive *H. influenzae* type b) had been acquired by *H. influenzae* type f.

**Patients and methods**

In 1996, vaccination against *H. influenzae* type b with conjugate vaccine was introduced into the regular vaccination programme, first in a few areas of Spain and then nationally. As part of a Europe-wide study, the Spanish national *Haemophilus* reference laboratory set up an active programme to characterize the phenotype and genotype of *H. influenzae* type b and other capsular types. Clinical microbiology laboratories, representing all geographical areas, aimed to send recovered *H. influenzae* to the reference laboratory. *H. influenzae* type f strains were analysed at our central laboratory, where full microbiological identification, susceptibility testing and molecular epidemiological studies were carried out. When an isolate was identified as *H. influenzae* type f, the clinical records of the patient were examined retrospectively, and a further clinical record was completed, which included the patient’s identification data, diagnosis, underlying conditions and outcome.

Identification was confirmed according to standard laboratory methods. These included Gram stain, X and V factors, ornithine, urea, indole and porphyrin tests. Two methods were used to carry out capsular typing in all isolates: the classic slide agglutination test, using type-specific antisera (Difco Laboratories, Detroit, MI, USA), and the PCR method of Falla et al., which was considered the reference test.

Antimicrobial susceptibility testing was performed in accordance with the guidelines of the NCCLS, using a *Haemophilus* test medium with a semi-automated commercialized microdilution method (Wider; Fco. Soria Melguizo S.A., Madrid, Spain). The antimicrobial agents tested were ampicillin, co-amoxiclav, cefuroxime, cefotaxime, ceftizoxime, cefpime, meropenem, clarithromycin, tetracycline, ciprofloxacin, levofloxacin, co-trimoxazole, chloramphenicol and rifampicin. *H. influenzae* ATCC 49247 and *H. influenzae* ATCC 49766 were used as quality control strains.

β-Lactamase production was determined by the chromogenic cephalosporin test with nitrocefin as substrate. Chloramphenicol acetyl transferase was measured as described previously.

To determine whether a putative virulence mutation genotype, described in *H. influenzae* types b and a, had been acquired by *H. influenzae* type f, the IS1016-bex A deletion was amplified by PCR, as previously described.

The Eagan strain of *H. influenzae* type b was used as a positive control.

All 49 clinical strains of *H. influenzae* type f and the *H. influenzae* type f reference strain ATCC 9833 were examined by PFGE. This examination was conducted following digestion of bacterial DNA with *Salmonella* (MBI Fermentas, Vilnius, Lithuania) and separation of the fragments in 1% agarose gel prepared in 0.5 X TBE buffer with Bio-Rad CHEF Mapper apparatus (Bio-Rad, Madrid, Spain). Gels were stained with ethidium bromide and photographed under ultraviolet light. A genetic similarity dendrogram was designed and calculated from the Dice correlation coefficient, represented by UPGMA with Molecular Analysis Software (Bio-Rad) and with a tolerance level of 2%. Well-resolved bands, corresponding to fragments exceeding 48.5 kbp, were included in the computer analysis.

Screening of large conjugative plasmid carriage relating to antimicrobial resistance was carried out by PCR, as described by Leaves et al.

The EPI-Info for Windows program, Release 6.04, was used for statistical analysis. Categorical variables were compared with Fisher’s exact test, two-tailed. The null hypothesis was rejected for values of *P* < 0.05.

**Results**

From January 1996–December 2002, a total of 6073 *H. influenzae* isolated from clinical samples were submitted to the national reference laboratory from all Spanish autonomous communities. Forty-nine (0.81%) of these strains were *H. influenzae* type f, both by molecular typing and agglutination. All belonged to biotype I, except two that were biotype II. The 49 isolates came from individual, unrelated patients from 18 institutions in seven geographical areas.

The MICs for the two ATCC control strains were always within recommended limits. Of the 49 isolates tested, 24.5% were resistant to ampicillin (all β-lactamase producers) and 44.9% to co-trimoxazole (Table 1). No resistance to co-amoxiclav, cefuroxime, cefotaxime, cefixime, cefepime, meropenem, ciprofloxacin or levofloxacin was detected.

A trend was found indicating that *H. influenzae* type f infections in children were more resistant than in adults to ampicillin (33.3% versus 21.6%) and co-trimoxazole (83.4% versus 59.4%), although the small number of cases prevents such differences being statistically significant.

Multidrug resistance (non-susceptibility to three or more antibiotics) was present in nine (18.4%) of the 49 isolates, and seven were isolated from patients who had at least one predisposing risk factor. The most prevalent resistance phenotype was ampicillin/tetracycline/co-trimoxazole/chloramphenicol, which was detected in five isolates, representing 55.5% of the multiresistant strains and 10.2% of strains overall. Of seven patients infected by isolates with simultaneous resistance to ampicillin and chloramphenicol, six were also resistant to tetracycline.

Overall carriage of large conjugative plasmids was detected by PCR in seven (14.3%) of the 49 *H. influenzae* type f strains. Its presence was strongly associated with antibiotic resistance, in particular with resistance to ampicillin, tetracycline and chloramphenicol. Plasmid was detected by PCR in all six strains that were simultaneously resistant to ampicillin, tetracycline and chloramphenicol. Seven of 12 ampicillin-resistant isolates (58.3%) had conjugative plasmids, whereas the 15 fully antibiotic susceptible strains did not (*P* < 0.001). Also, 100% of seven and six *H. influenzae* type f isolates resistant to chloramphenicol and tetracycline, respectively, carried conjugative plasmids (*P* < 0.001). In contrast, in the 21 isolates with resistance to co-trimoxazole and/or clarithromycin none carried them.

Twenty-four patients were male (49%) and 25 female (51%). Thirty-seven strains (75.5%) were isolated from patients aged >14 years, 12 (24.5%) from children ≤14 years, seven of whom were 5 years old or younger (the age of five patients was unknown). The mean age was 43.3 years (range: 5 months–95 years; median: 42 years).

Twenty-five isolates (51%) were recovered from blood (18) or cerebrospinal fluid (7), 19 (38.8%) from respiratory tract samples (14 from sputum and five from bronchoalveolar aspirates), two (4.1%) from abscesses, and in one (2%) each from conjunctiva, abdominal fluid and ear. The most frequent clinical diagnoses were...
pneumonia in 16 cases, sepsis in nine and meningitis in eight. Four of the 14 patients with *H. influenzae* type f in sputum were diagnosed with pneumonia, whereas the remaining 10 were diagnosed mainly with exacerbations of cystic fibrosis or chronic obstructive pulmonary disease. In some cases there was no clear evidence of any clinical implication and eventual colonization could not be excluded.

Seven (14.3%) patients died, all of whom were adults with severe underlying diseases. The antibiotic treatments most frequently used were third-generation cephalosporins and co-amoxiclav. Thirty-two patients (65.3%) had previous underlying predisposing conditions, and more than one risk factor was present in 13 of them. The most prevalent underlying diseases were respiratory diseases, in 10 cases; HIV infection in eight cases; tumoral pathologies in six cases; and liver pathology and diabetes in five cases each. Twenty-one (42.8%) patients had impaired immunity (HIV infection, Hodgkin lymphoma, multiple myeloma, IgG gammopathy, heart transplantation, leukaemia, corticosteroid treatments and diabetes).

Sixteen *H. influenzae* type f strains (32.6%) were isolated from a large hospital in Madrid with 1800 beds, covering a population of 640 000 inhabitants. In the community of Madrid (population more than 5 million), widespread vaccination with *H. influenzae* type b conjugate vaccines started in 1999. Over the study period, 2542 clinical isolates of *H. influenzae* were isolated in this institution, all of which were submitted to the reference laboratory, where 16 (0.6%) were identified as *H. influenzae* type f. In the area covered by this hospital, the overall incidence of *H. influenzae* infection during this period was 56.7 cases/100 000 inhabitants per year. In comparison, the incidence of *H. influenzae* type f infections was 0.40 cases/100 000 inhabitants per year. In the 7 full years included in the study, the incidence of *H. influenzae* type f infection was 0.31 cases/100 000 inhabitants in 1996, 0.62/100 000 in 1997, 0.16/100 000 in 1998, 0.47/100 000 in 1999, 0.31 cases/100 000 in 2000, 0.16/100 000 in 2001 and 0.47/100 000 in 2002. In the remaining hospitals with *H. influenzae* type f infections, the number of cases was too low to allow meaningful calculations.

As expected, the positive *H. influenzae* type b control (Eagan strain) presented the IS1016-bex A deletion when PCR-amplified. This was in contrast with the 49 *H. influenzae* type f strains, which did not present the deletion.

The genetic relatedness of 48 *H. influenzae* type f and the *H. influenzae* type f reference strain, estimated from the cluster analysis of the PFGE patterns obtained by DNA fingerprinting, is shown in Figure 1. The DNA of one isolate was not digested by *SmaI*. Overall, the Spanish *H. influenzae* type f population showed little genetic variability. Forty-four (91.6%) of the isolates had a genetic distance ≤12% and could be considered to belong to the same clone according to Tenover criteria, irrespective of their geographical origin, antibiotic resistance patterns or clinical significance.

### Discussion

Following the widespread use of effective conjugate vaccines, infections due to *H. influenzae* type b and the prevalence of *H. influenzae* type b carriers have decreased substantially. In Spain, widespread vaccination against *H. influenzae* type b has been in place since the mid-1990s. Vaccination campaigns create a novel epidemiological environment; it has been suggested that the decline in the rate of *H. influenzae* type b infections could have given rise to the emergence of diseases caused by other *H. influenzae* serotypes. An increased incidence of *H. influenzae* types e and particularly f has been noted in the USA.

In Europe, little information is available about the antibiotic susceptibility patterns and clinical significance of *H. influenzae* type f. In general, information on *H. influenzae* type f infections is limited as only sporadic clinical cases of meningitis, septic arthritis, osteomyelitis, pneumonia and sepsis have been described in the literature. We have shown that *H. influenzae* type f may be the aetiological agent of a wide range of illnesses including mild infections (otitis, conjunctivitis) and severe infections (meningitis, pneumonia, sepsis).

Until now, very little information about antimicrobial susceptibility, other than to ampicillin, has been reported for *H. influenzae* type f. β-Lactamase activity was detected in 21% of *H. influenzae* type f isolates studied by Urwin et al. In our study, 24.5% of 49 isolates produced β-lactamase and resistance to other antibiotics like...
co-trimoxazole, chloramphenicol and tetracycline and multiple antibiotic resistance was also remarkable. Carriage of large conjugative plasmids was strongly correlated with the expression of multiple antibiotic resistance. The ampicillin resistance was similar to that described previously in studies on *H. influenzae*, but clearly lower than the 50% and 61.5% found previously in Spain in *H. influenzae* types b and e, respectively.

Carriage of high-molecular-weight conjugative plasmids in *H. influenzae* type b constitutes the genetic bases of resistance to ampicillin, chloramphenicol and tetracycline. In seven of our antibiotic-resistant *H. influenzae* type f isolates, conjugative plasmids were detected by PCR, and all six strains with cross-resistance to ampicillin, tetracycline and chloramphenicol had a conjugative plasmid, as revealed by PCR. Our data suggest that resistance to ampicillin, chloramphenicol and tetracycline, and multiple resistance, in *H. influenzae* type f is plasmid mediated, in contrast to co-trimoxazole and clarithromycin resistance.

In this study, we have shown that the epidemiology and clinical significance of *H. influenzae* type f infections is very different from the classic invasive infections caused by *H. influenzae* type b. Only a small proportion (14.3%) of cases occurred in children ≤5 years, the majority being in patients of >14 years of age, often with predisposing factors and severe underlying conditions. In the series studied by Urwin et al., 26% of cases occurred in children younger than 5 years of age. We have found that the most frequent clinical presentations were infections of the respiratory tract (59.2%), these also being the commonest underlying diseases in infected patients (20.4%). However, meningitis was diagnosed mainly in adult patients (75%; 6/8), and in patients with predisposing factors (62.5%; 5/8).

We studied *H. influenzae* strains isolated in Spanish hospitals and, particularly, in one of the largest hospitals in the country. We found no increase in the incidence of *H. influenzae* type f in spite of there having been thorough vaccination against *H. influenzae* type b in Spain. Therefore, our data would not support the hypothesis of *H. influenzae* type f replacement after *H. influenzae* type b vaccination. Our results suggest that *H. influenzae* type f is an opportunistic pathogen. It causes infections in patients with serious underlying respiratory and liver pathologies or with impaired immunity. It is not a primary pathogen, as *H. influenzae* type b was in the pre-vaccination era. However, as in three of our clinical cases, occasional primary severe infections, such as meningitis or septicaemia, may appear in healthy children.

It has been suggested that invasive serotype a *H. influenzae* infections in children may be emerging as a consequence of molecular acquisition of a virulence genotype from *H. influenzae* type b. We found no such mechanism in our *H. influenzae* type f isolates since none—whether from invasive or non-invasive infections, isolated from children or adults—possessed the IS1016-bexA deletion.
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Omiakunle et al. 2003 studied 20 H. influenzae type f isolates by PFGE and found all but one to belong to a single clone. Our strains were also closely related since 91.6% of the strains had a genetic distance ≤12%.

In summary, we studied the antibiotic susceptibility and clinical and epidemiological characteristics of 49 consecutive clinical isolates of H. influenzae type f. A high proportion of isolates were resistant to single and multiple antibiotics. Most strains were isolated from adults with severe underlying predisposing conditions, although cases of sepsis and meningitis may appear in children. The Spanish H. influenzae type f bacterial population is clonally related.

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