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Antimicrobial susceptibility of *Haemophilus influenzae* strains isolated from invasive disease in Italy

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***Objectives*: *Haemophilus influenzae* invasive disease is potentially life threatening and requires prompt antibiotic therapy. The aim of this study was to assess the antimicrobial susceptibility of *H. influenzae* strains isolated from invasive disease in Italy and to investigate ampicillin-resistant isolates by molecular biology techniques.**

***Materials and methods*: One-hundred and seventy-six invasive *H. influenzae* isolates, collected during 1998–2003, were analysed for susceptibility to ampicillin, azithromycin, chloramphenicol and ciprofloxacin. Ampicillin-resistant isolates were further tested against cefotaxime and imipenem. MICs were determined by Etest and interpreted according to NCCLS criteria. The ampicillin resistance genes, bla_{TEM} and bla_{ROB}, were searched for by PCR. Genetic relatedness among ampicillin-resistant isolates was investigated by PFGE.**

***Results*: Overall, ampicillin resistance was 10.2% (all β-lactamase producer strains). The prevalence of ampicillin-resistant isolates increased from 6.9% in 1998/1999 to 19% in 2002/2003. Resistance to azithromycin and chloramphenicol was 6.8% and 1.7%, respectively. No strains were resistant to ciprofloxacin. Co-resistance between ampicillin and chloramphenicol and between ampicillin and azithromycin was observed in three and one isolates, respectively. All ampicillin-resistant isolates were susceptible to cefotaxime and imipenem and all harboured the bla_{TEM} gene. PFGE demonstrated that most of the ampicillin-resistant isolates showed little genetic homology.**

***Conclusions*: An upward trend in resistance to ampicillin due to β-lactamase production was demonstrated In Italy. According to PFGE results, clonal dissemination of ampicillin-resistant isolates does not occur. Imipenem may represent an appropriate alternative for treatment of *H. influenzae* invasive disease caused by ampicillin-resistant isolates when third-generation cephalosporins cannot be used.**

Keywords: ampicillin resistance, β-lactamases, molecular typing, meningitis

Introduction

Haemophilus influenzae causes a variety of infections in children and adults, ranging from respiratory tract infection to invasive disease (meningitis, bacteraemia, epiglottitis, cellulitis and septic arthritis).

Antibiotic treatment plays an essential role in managing *H. influenzae* invasive disease. For years ampicillin was the cornerstone of therapy. Resistance to ampicillin was first reported in the 1970s and during the following decades it steadily increased.[1] Plasmid-mediated production of TEM or ROB β-lactamase is the most common mechanism of resistance.[2] Therapeutic options in the treatment of *H. influenzae* meningitis included chloramphenicol in combination with ampicillin and, more recently, third-generation cephalosporins or carbapenems. The last two have also been successfully used in the treatment of other invasive infections. Resistance to chloramphenicol among *H. influenzae* isolates has been reported recently.[1] Until now, resistance to third-generation cephalosporins and carbapenems has been virtually absent in *H. influenzae*. Since

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H. influenzae invasive disease is potentially life threatening, antimicrobial susceptibility surveillance studies are required in order to identify resistance trends and to detect emerging resistance.

In this study, 176 invasive *H. influenzae* isolates, collected during 1998–2003 were genotyped and tested for susceptibility to ampicillin and chloramphenicol. Ampicillin-resistant isolates were further studied in order to: (i) assess susceptibility to cefotaxime and imipenem; (ii) identify the presence of structural genes for TEM or ROB β-lactamase; and (iii) investigate genetic relatedness by cluster analysis of the PFGE patterns. Moreover, since several elderly patients with *H. influenzae* invasive disease suffered from previous severe respiratory tract infections, susceptibility to azithromycin and ciprofloxacin was also examined.

Material and methods

Bacterial strains and patients

One hundred and seventy-six *H. influenzae* strains isolated from invasive disease in Italy during January 1998–December 2003 were analysed. Strains had been isolated from various clinical specimens: CSF (97 isolates), blood (73 isolates), pleural fluid (three isolates), peritoneal fluid (two isolates) and synovial fluid (one isolate). Age was known for 169 patients: 87 were ≤5 years and 82 were aged 6–98 years. One Hib isolate (strain 40F) belonging to the clone endemically present in Italy several years ago was included in PFGE analysis.[3]

PCR capsular genotyping

The capsular genotype of each isolate was identified by PCR, following procedures previously reported.[4] Primers were supplied by M-Medical, Cornaredo, Milano, Italy. This method allowed us to identify unequivocally the capsular type and to differentiate between non-encapsulated (NC) and b− strains. The latter are spontaneous capsule-deficient type b mutants in which a single copy of the *capB* genes is present although the capsule is not expressed following the loss of a gene (*bexA*) necessary for capsule export.

Antimicrobial susceptibility testing

For each isolate, MICs of ampicillin, ciprofloxacin, chloramphenicol and azithromycin were determined by Etest (AB Biodisk, Solna, Sweden) using *Haemophilus* test medium according to NCCLS guidelines.[5] Strains resistant to ampicillin were further tested against cefotaxime and imipenem by the same method. *H. influenzae* ATCC 49247 and *H. influenzae* ATCC 10211 were used as controls. The interpretative breakpoints were based on NCCLS criteria.

Each isolate was tested for β-lactamase activity by the cefinase disc test (BBL, Beckton Dickinson, MD, USA).

Detection of bla_{TEM} and bla_{ROB} genes by PCR

The presence of the bla_{TEM} gene was investigated in the ampicillin-resistant isolates by PCR as previously described.[6] The bla_{ROB} gene was amplified as previously reported.[2] *H. influenzae* ATCC 49247 was used as a negative control for both PCRs.

PFGE

All the ampicillin-resistant isolates were examined by PFGE after digestion of their genomic DNAs with *Sma*I (30 U) restriction enzyme (New England BioLabs, Hitchin, Herts, UK), following procedures previously described.[4]

Dendrogram

Similarity analysis was performed with Dice's coefficient and clustering was carried out by means of the unweighted pair group mean association (UPGMA) method, with the Diversity Database Fingerprinting Software, version 2 (Bio-Rad Laboratories).

Results

By PCR capsular genotyping, 107 *H. influenzae* isolates (60.8%) were identified as type b, 59 (33.5%) as NC, five (2.8%) as type e, four (2.2%) as type f and one (0.6%) as b−.

Table 1 shows susceptibility results of the 176 *H. influenzae* isolates to the four antimicrobial agents tested. Ampicillin resistance was the most frequently detected resistance. Eighteen ampicillin-resistant isolates (10.2%) were identified with MICs in the range 8–>256; all of them were β-lactamase producers. The prevalence of ampicillin-resistant isolates has progressively increased over time, ranging from 8/116 (6.9%) in 1998/1999, to 6/39 (15.4%) in 2000/2001, to 4/21 (19%) in 2002/2003. Ampicillin-resistant isolates were found among Hib (13/107, 12.1%) and NC (5/59, 8.4%) isolates but not among type e, f and b− isolates. Isolates from CSF had a higher rate of resistance to ampicillin (14/97) compared with isolates from other sterile sites (4/79) ($P = 0.04$). β-Lactamase production did not affect susceptibility to cefotaxime among our isolates, since all β-lactamase-positive isolates were susceptible (MIC_{50} and $MIC_{90} = 0.016$ mg/L). Similarly, all were inhibited by imipenem ($MIC_{50} = 1.5$ mg/L; $MIC_{90} = 3.0$

Table 1. Antimicrobial susceptibility of 176 invasive *H. influenzae* strains isolated in Italy during 1998–2003

	MIC (mg/L)			Susceptibility category		
Antibiotic	MIC_{50}	MIC_{90}	range	S (%)	I (%)	R (%)
Ampicillin	0.250	8.0	≤0.012–≥256	89.8	0.0	10.2[a]
Azithromycin[b]	2.0	4.0	≤0.25–12.0	93.2	–	6.8
Chloramphenicol	0.50	0.75	≤0.012–12.0	97.2	1.1	1.7
Ciprofloxacin[b]	0.012	0.016	≤0.004–0.080	100	–	0.0

[a]All the ampicillin-resistant isolates were β-lactamase producers.
[b]No intermediate breakpoint.

mg/L). Ciprofloxacin inhibited 100% of our isolates with an MIC_{90} of 0.016 mg/L. Azithromycin could not be considered a very active drug since the MIC_{90} (4.0 mg/L) of the isolates corresponded to the breakpoint. Overall, 12 azithromycin-resistant isolates (6.8%) were detected with MICs in the range 6–12 mg/L. The frequency of azithromycin-resistant isolates slightly decreased during the study period, from 9/116 (7.8%) in 1998/1999 to 2/39 (5.1%) in 2000/2001 to 1/21 (4.8%) in 2002/2003. Azithromycin resistance was more frequent among NC (11/59) than among Hib isolates (1/107) ($P = 0.00002$) and was significantly associated with strains from blood of adult patients (blood 9/72 versus other sterile sites 3/104, $P = 0.012$; and adults 11/82 versus children 1/87, $P = 0.019$). Chloramphenicol inhibited more than 97% of the isolates, only three resistant strains were detected (two Hib and one NC isolate) with MICs in the range 8–12 mg/L and two intermediate strains (both Hib). Co-resistance between ampicillin and chloramphenicol and between

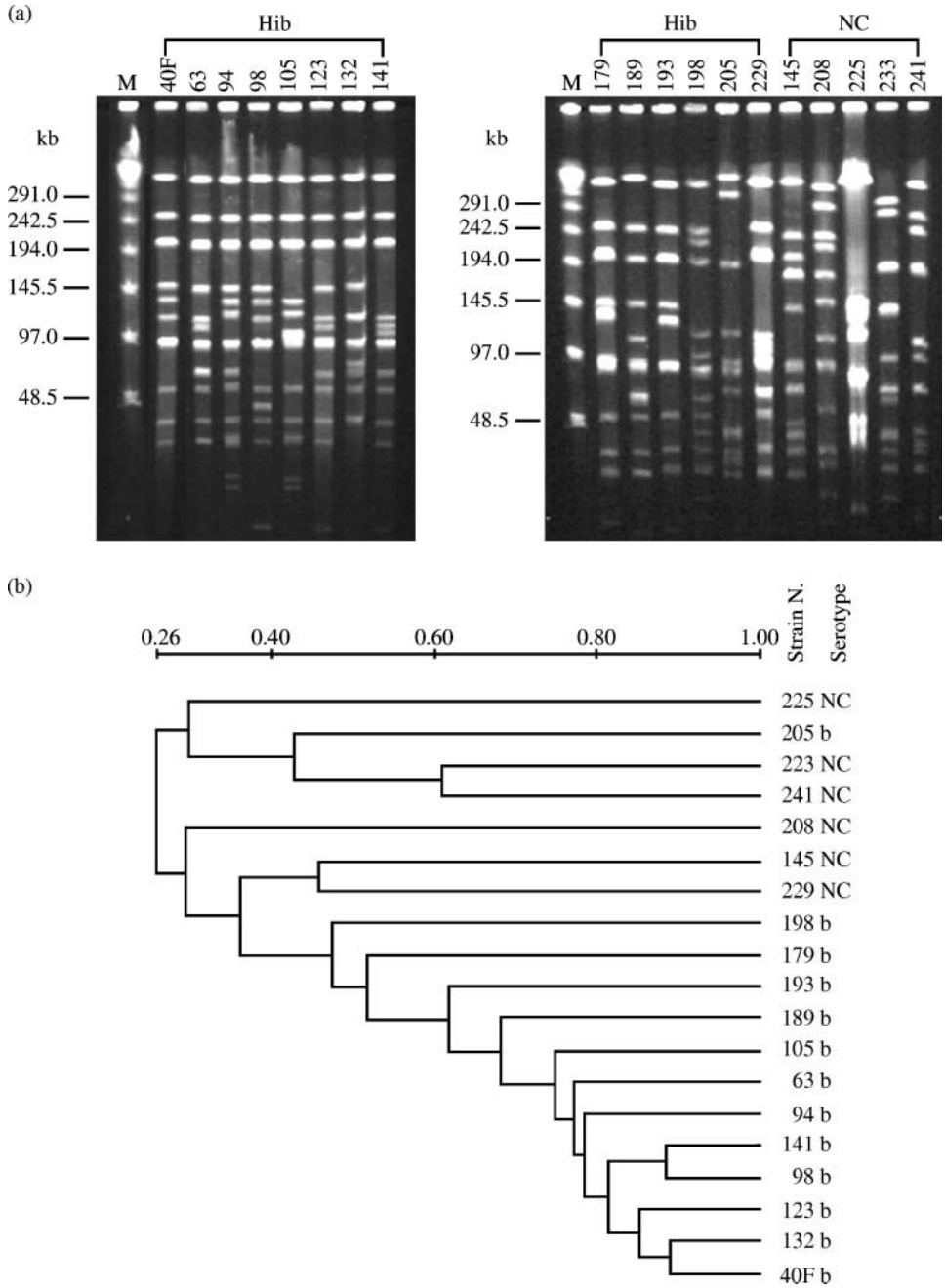

Figure 1. Genetic relatedness of 18 ampicillin-resistant *H. influenzae* isolates by cluster analysis of the PFGE patterns. (a) PFGE patterns of *Sma*I-digested chromosomal DNAs of the *H. influenzae* isolates. Strain code numbers and serotype designations are indicated above lanes. M, Lambda ladder PFGE marker (New England BioLabs). (b) Dendrogram based on PFGE *Sma*I restriction pattern analysis. On the right, the strain code number and the serotype are reported. Similarity analysis was performed with Dice's coefficient and clustering by the UPGMA method. Isolates with a coefficient of similarity value of ≥0.8 were considered to belong to the same clonal group.

ampicillin and azithromycin was observed in three isolates (one Hib and two NC) and one NC isolate, respectively.

TEM β-lactamase sequences were identified by PCR in all 18 β-lactamase-producing isolates. No amplification products were obtained for the bla_{ROB} gene.

When analysed by PFGE the 18 ampicillin-resistant isolates yielded 18 distinctive profiles (Figure 1a). The computer-generated dendrogram (Figure 1b) showed that most isolates had little genetic homology. Among Hib isolates, five (strains 132, 123, 98, 141 and 94) belonged to the same clonal group as the 40F strain (~80% similarity), whereas the others showed greater genetic distance. The NC isolates did not cluster among themselves, the greatest similarity being about 60% between strains 233 and 241.

Discussion

The main mechanism of resistance observed among the invasive *H. influenzae* isolates analysed was β-lactamase production. Notably, an upward trend in the rate of β-lactamase producers was detected, increasing from 6.9% in 1998/1999 to 19% in 2002/2003. Compared with a previous study on invasive Hib isolates that were collected during 1994–1996 in Italy, which reported an ampicillin-resistance rate of ~1.5%,[3] a steady increase in the prevalence of β-lactamase producers among invasive isolates has occurred.

Worldwide, a considerable variation in the extent of prevalence of β-lactamase production among *H. influenzae* isolates has been reported.[1,7] However, the majority of data referred to strains isolated from community-acquired respiratory tract infections, with little information on isolates from invasive disease. A Spanish study on invasive isolates collected in 1999–2000 recorded a prevalence of ampicillin resistance by β-lactamase production of 24.2%.[8] A similar rate was reported in another study.[9]

Two β-lactamases, TEM and ROB, have been identified in *H. influenzae*, with TEM being more prevalent.[2] In this study, all β-lactamase-positive isolates harboured a bla_{TEM} gene, suggesting that the TEM β-lactamase was widely prevalent also among invasive isolates. The finding, that all 18 β-lactamase-producing strains were susceptible to cefotaxime, confirms previous data indicating that evolution of TEM β-lactamase toward expanded-spectrum activity has not occurred so far in *H. influenzae*. Analysis of the genetic relatedness of the β-lactamase-producing isolates showed they exhibited a considerable genetic heterogeneity. On the basis of this finding, the hypothesis of clonal dissemination of β-lactamase-positive isolates is not supported. Instead, our data suggest that the bla_{TEM} gene, probably carried by a plasmid, had been horizontally acquired by strains belonging to distinct clones.

Concern about the optimal efficacy of macrolides in the therapy of respiratory tract infections has been raised recently. In Italy, a rapid increase in the prevalence of macrolide-resistant bacteria has been observed, probably relating to high consumption. This study confirms the presence of azithromycin resistance also among *H. influenzae* invasive isolates, although no increase was detected over the study period. The rate of resistance to chloramphenicol is similar to that reported in another Italian study on non-invasive isolates.[10] Neither ciprofloxacin-resistant strains nor strains with reduced susceptibility to ciprofloxacin were found, confirming that resistance to this class of antibiotic remains rare. Finally, co-resistance between some drug classes is present in *H. influenzae* isolates, although it is not a major issue compared with other Gram-negative bacteria.

In conclusion, the data collected demonstrate the presence of an upward trend in resistance to ampicillin due to TEM β-lactamase production, whose frequency nearly tripled over the study period. Clonal dissemination of the ampicillin-resistant isolates did not occur, suggesting the bla_{TEM} gene is horizontally acquired by the isolates. Cefotaxime and imipenem maintain high activity against β-lactamase-producer isolates and imipenem may represent an appropriate alternative for treatment of *H. influenzae* invasive disease caused by ampicillin-resistant isolates when third-generation cephalosporins cannot be used. Azithromycin exhibits low activity against the isolates tested whereas ciprofloxacin has been shown to be remarkably active.

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