Antimicrobial resistance patterns of *Haemophilus influenzae* isolated from patients with meningitis in São Paulo, Brazil

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Abstract

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From 1989 to 1995, a total of 391 *Haemophilus influenzae* isolates were recovered from the cerebrospinal fluid (CSF) of hospitalized patients in São Paulo, Brazil. The majority of strains were isolated from infants aged less than 5 years. Strains belonging to biotype I (64.7%), biotype II (34.5%) and biotype IV (0.76%) were detected. Ninety-nine percent of these strains were serotype b. Minimal inhibitory concentration (MIC) was determined for ampicillin, chloramphenicol and ceftriaxone. The ß-lactamase assay was performed for all strains. The rate of ß-lactamase producer strains ranged from 10 to 21.4% during a period of 7 years, with an overall rate of 13.8%. Of the 391 strains analyzed, none was ß-lactamase negative ampicillin resistant (BLNAR). A total of 9.7% of strains showed resistance to both ampicillin and chloramphenicol; however, 4% of them were resistant to ampicillin only and 2% to chloramphenicol. All strains were susceptible to ceftriaxone and the MIC_{90} was 0.007 µg/ml, suggesting that ceftriaxone could be an option for the treatment of bacterial meningitis in pediatric patients who have not been screened for drug sensitivity.

Introduction

Meningitis, especially in infants, young children and in the elderly, is the most serious clinical manifestation of invasive disease caused by *Haemophilus influenzae* (1,2). Substantial declines in invasive *Haemophilus influenzae* type b diseases have been reported in many countries where routine vaccination against *H. influenzae* type b has been implemented (3-6). Although very successful in developed countries, *H. influenzae* type b vaccination is still very costly and unfeasible in the majority of developing countries. In São Paulo State, Brazil, *H. influenzae* is the second most common cause of meningitis, mainly among infants aged less than 5 years. The average annual rate of incidence of meningitis caused by *H. influenzae* was 11.4 cases per 100,000 inhabitants from 1989 to 1995 (Respiratory Diseases Division, Epidemiological Surveillance Center, São Paulo, SP, Brazil).

Resistance to ampicillin due to a plasmid-mediated extracellular TEM-1 type ß-lactamase was recognized in *H. influenzae*

for the first time in 1974 (7-9). Subsequently, strains producing a second ß-lactamase named ROB-1 enzyme were reported (10). More recently, *H. influenzae* strains referred to as ß-lactamase negative ampicillin resistant (BLNAR) have also been described, although such strains seem to be relatively uncommon as shown by recent national and multinational surveillance studies (2,11-14). The prevalence of ampicillin resistance in *H. influenzae* strains has been the subject of multinational surveillance studies in several countries and the overall rate reported was between 10 and 60% (8,9,11-14). The emergence of resistant *H. influenzae* strains, particularly to ampicillin, all over the world has limited the routine use of ampicillin and as a result third-generation cephalosporins have become a therapeutic option (15,16).

Few data are available in Brazil on ampicillin resistance and ß-lactamase production in *H. influenzae* isolated from young children. In the present study we report the antimicrobial resistance pattern of *H. influenzae* isolated chiefly from young children in São Paulo, Brazil, from 1989 through 1995.

Material and Methods

Bacterial strains

A total of 391 *H. influenzae* strains isolated between 1989 and 1995 were studied. All strains were isolated from cerebrospinal fluid (CSF) samples, 76% of them from infants aged less than 5 years and hospitalized in São Paulo municipality. These strains and CSF were sent by hospital laboratories to the National Center for Meningitis, Adolfo Lutz Institute, Central Public Health Laboratory, São Paulo, Brazil, for routine microbiological analysis.

Strain identification

Isolates were identified by colony and cell morphology and by the demonstration of growth requirements for V and X factors. The V requirement was tested by observing the satellite phenomenon and the X factor dependence was determined by testing the ability to convert δ-aminolevulinic acid to porphyrins. Strains were further characterized by biochemical reactions using the method described by Kilian and Biberstein (17). Capsulated strains were serotyped by the method of Pitman (18) using slide agglutination with type-specific antisera a through f.

Susceptibility testing

All strains were subcultured twice onto a chocolate agar plate (Difco Laboratories, Detroit, MI, USA) and incubated at 37°C in 5% CO_2 for 18-24 h before testing. The minimal inhibitory concentrations (MIC) of different antimicrobial agents were determined by the broth microdilution method using *Haemophilus* test medium (HTM) (Cation-supplemented Mueller-Hinton Broth, BBL Microbiology Systems, Cockeysville, MD, USA), supplemented with 15 µg/ml of bovine hematin, 15 µg/ml of NAD and 5 µg/ml of yeast extract (Difco Laboratories), containing one of the following antimicrobial agents at 2-fold dilution: 0.03-32 µg/ml ampicillin, 0.015-16 µg/ml chloramphenicol, 0.001-2 µg/ml ceftriaxone (19) (Sigma Chemical Co., St. Louis, MO, USA). Antibiotic panels were prepared by dispensing media containing two-fold concentration increments of antimicrobial agents in a 50-µl volume into plastic 96-well trays (Difco). Growth from 18- to 24-h cultures was suspended in distilled water and diluted to match the turbidity, this being equivalent to 0.5 McFarland standard read using a spectrophotometer at 625 nm. The suspension was further diluted 1:100 and added to the dilution trays to achieve a final inoculum of 5 x 10^5 CFU/ml.

Colony counts were performed to determine the desired final inoculum (19). Immediately following inoculation, the microdilution panel was incubated at 37°C in ambient

air for 20 to 24 h. After incubation the MIC was defined as the lowest concentration of an antimicrobial agent required for an organism to show no evidence of growth. According to the NCCLS criteria (19), the organisms were considered resistant/susceptible if the MIC was ≥/≤ (µg/ml) 4/1 for ampicillin and 8/2 for chloramphenicol, and susceptible if the MIC was ≤2 µg/ml for ceftriaxone. *Haemophilus influenzae* ATCC 49247 was used as control.

ß-Lactamase assay

Production of ß-lactamase was determined by the chromogenic cephalosporin method (7) using reconstituted lyophylized nitrocefin (Glaxo 87/312 Glaxo Research, Unipath Ltd., Hampshire, England). The test was considered positive if the color changed from yellow to purple for cephalosporin. *Haemophilus influenzae* ATCC 49247 was used as negative control and *Staphylococcus aureus* ATCC 29213 as positive control.

Results

Biotypes and serotypes

Strains belonging to biotype I 253 (64.7%), biotype II 135 (34.5%) and biotype IV 3 (0.76%) were detected. Type b was the serotype accounting for 99% of strains.

Antimicrobial resistance patterns

The annual percentage of resistant strains is shown in Table 1. The overall rate of ampicillin-resistant strains that produced ß-lactamase was 13.8%. The percentage of ß-lactamase producer strains isolated was 14.0% for children aged less than 5 years and 1.6% for children older than 6 years. This difference was not statistically significant, as demonstrated by the chi-square test (P = 0.3). Resistance to both ampicillin and chloramphenicol was observed in 9.7% (38/391) of strains. Strains isolated in 1993 showed the highest rate of ß-lactamase-producing strains (21.4%) and also of strains resistant to both chloramphenicol and ampicillin (14.2%). The overall rate of strains resistant only to chloramphenicol was 2.0%, and the overall rate of strains resistant only to ampicillin was 4.0%. Among the 391 strains analyzed, none was BLNAR. Data concerning the MIC_{50} and MIC_{90} of ß-lactamase-positive and -negative strains are summarized in Table 2. All *H. influenzae* strains were extremely susceptible to ceftriaxone,

Table 1 - Antimicrobial resistance (number and percentage) and the production of ß-lactamase in H. influenzae isolated from CSF in the municipality of São Paulo between 1989 and 1995.

[1]The values considered for the calculation of resistance are in accordance with the National Committee for Clinical Laboratory Standards (19): ampicillin, ≥4.0 µg/ml; chloramphenicol, ≥8.0 µg/ml; ceftriaxone, >2.0 µg/ml.

Year	No. of strains	No. (%) of ß-lactamase-positive	No. (%) of resistant strains[1] to:		
			ampicillin	chloramphenicol	chloramphenicol and ampicillin
1989	82	9 (10.9)	3 (3.6)	–	6 (7.3)
1990	72	9 (12.5)	1 (1.4)	3 (4.2)	8 (11.1)
1991	85	10 (11.7)	3 (3.5)	1 (1.2)	7 (8.2)
1992	63	10 (15.9)	3 (4.8)	1 (1.5)	7 (11.1)
1993	42	9 (21.4)	3 (7.1)	1 (2.4)	6 (14.2)
1994	27	4 (15.0)	1 (3.7)	1 (4.0)	3 (11.1)
1995	20	3 (15.0)	2 (10.0)	1 (5.0)	1 (5.0)
Total	391	54 (13.8)	16 (4.0)	8 (2.0)	38 (9.7)

exhibiting MIC_{90} values of 0.007 µg/ml (Table 2).

Discussion

Since the late 1970's, antibiotic resistance has increased among strains of *H. influenzae*, mainly in terms of ß-lactamase-mediated ampicillin resistance, representing a serious clinical concern all over the world. The present study shows that the overall rate of ß-lactamase producers in *H. influenzae* was 13.8%. Similar results have also been obtained in a collaborative study conducted in the US during the 80's and in São Paulo during the 90's (20,21). In Finland and in South Africa the overall rate of ß-lactamase-mediated ampicillin resistance in *H. influenzae* was 10% (11,22,23).

The ß-lactamase-producing strains presented a MIC_{90} of 32 µg/ml for ampicillin and 16 µg/ml for chloramphenicol, with most strains resistant to ampicillin being also resistant to chloramphenicol (Table 1). The resistance to ampicillin is coded by a plasmid which harbors the transposon TnA, and the transposition mechanism is important for the evolution to plasmids simultaneously carrying multiple resistance (24,25). Significant changes in MIC_{90} values over a period of time could be the best indicators of increasing resistance to the ß-lactam antibiotics in *H. influenzae* (24).

The overall rates of ß-lactamase production were highest in the age groups of less than 5 years and among isolates from systemic sources such as CSF. These observations could be explained by the relatively higher percentage of *H. influenzae* strains isolated from young children. Non-ß-lactamase-mediated ampicillin resistance was not detected among the *H. influenzae* strains studied. This is in agreement with numerous previous surveillance studies, which have indicated that such observations remain uncommon (11,13,14). It has been postulated that BLNAR is associated with altered penicillin-binding proteins (PBPs) and might be due to a lower virulence of strains with abnormal PBPs (13,16,26).

Although in the 80's it was reported that 57% of *H. influenzae* strains isolated in Spain were ampicillin and chloramphenicol resistant, resistance to chloramphenicol only is still rare (8,9,14,21). Among our strains, 11.7% were resistant to chloramphenicol, including the associated resistance to ampicillin (9.7%), and 2% were resistant only to chloramphenicol. In addition, MIC_{90} to chloramphenicol showed the same range (8-16 µg/ml), in agreement with data reported in other countries (8,27). Our data indicate that 9.7% of all strains were resistant both to ampicillin and chloramphenicol, in agreement with reported results (21).

The rapid detection of resistance and early initiation of alternative therapy are important factors in the treatment of meningitis caused by multiple resistance strains. It is of fundamental importance to perform the ß-lactamase test and susceptibility tests in order to detect resistance to ampicillin and

Table 2 - MIC_{50} and MIC_{90} of ß-lactamase-positive and -negative H. influenzae strains isolated from CSF in São Paulo, Brazil, between 1989 and 1995.

	ß-Lactamase-positive		Range	ß-Lactamase-negative		Range
	MIC_{50}	MIC_{90}		MIC_{50}	MIC_{90}	
	(µg/ml)			(µg/ml)		
Ampicillin	16	32	2-32	0.125	0.25	0.06-0.25
Chloramphenicol	8.0	16	8-16	0.5	1.0	0.03-1.0
Ceftriaxone	0.003	0.007	0.001-0.007	0.003	0.007	0.001-0.007

chloramphenicol. No relationship between antimicrobial resistance patterns and biotypes was seen in our study, as also reported elsewhere (23,28). We observed a higher frequency of biotype I among strains isolated from invasive disease mainly affecting children, as also reported previously (29).

All strains were susceptible to ceftriaxone with a MIC_{90} of 0.007 µg/ml for ß-lactamase-positive and -negative strains. In developed countries, the alternative treatment of choice for meningitis caused by multiple resistant *H. influenzae* strains is the use of third-generation cephalosporins (11-13). In several countries resistance to ampicillin and chloramphenicol was found to be sufficiently high to warrant their replacement by a combination of ampicillin with a ß-lactamase inhibitor, e.g., sulbactam or a third-generation cephalosporin as first-line therapy, depending on the severity of infection (8,15,22-24).

In Brazil there is a need for further evaluation of the resistance profile in *H. influenzae* to ensure a better therapy option, especially where the combination of ampicillin and chloramphenicol is extensively applied because of its low cost and effectiveness (23,30,31).

The emergence of drug resistance is a serious challenge for the management of invasive *H. influenzae* disease, which em- phasizes the fundamental role of laboratory-based surveillance for antimicrobial resistance. The continued usefulness of antimicrobial agents will probably depend on the rational use of antimicrobial agents in the community.

We showed an increasing antimicrobial resistance to ampicillin and chloramphenicol among *Haemophilus influenzae* strains isolated from children. Since all strains were susceptible to ceftriaxone, we conclude that this antimicrobial agent could be an option for the treatment of bacterial meningitis in pediatric patients who have not been screened for drug sensitivity.

Acknowledgments

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