

NASOPHARYNGEAL COLONIZATION BY *HAEMOPHILUS INFLUENZAE* IN CHILDREN ATTENDING DAY-CARE CENTERS, IN RIBEIRÃO PRETO, STATE OF SÃO PAULO, BRAZIL

Maria E.N. Bonifácio da Silva¹; Paulo da Silva²; Marta I.C. Medeiros²; Suzel N. Neme²; Claudia Macedo³; José Moacir Marin^{4*}

¹Laboratório de Análises Clínicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil; ²Secção de Bacteriologia, Laboratório de Ribeirão Preto, Instituto Adolfo Lutz, Secretaria da Saúde de São Paulo, Ribeirão Preto, SP, Brasil; ³Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil; ⁴Departamento de Morfologia, Estomatologia e Fisiologia, Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil.

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ABSTRACT

Nasopharyngeal carriage of *Haemophilus influenzae* (*Hi*) was studied in 114 healthy children < 3 years old, attending day-care centers (DCCs) in Ribeirão Preto, State of São Paulo, Brazil. Biotype, serotype (by specific antisera and PCR) and antibiotic susceptibility to 14 antibiotics of each isolate were determined. Carriage rates of *Hi* were 72.0%. Isolates belonged to biotype II (36.5%), I (21.5%), V (18.2%) and III (16.1%). The prevalence of encapsulated *Hi* carriers was 3.2% for type f, 1.0% for type b, 1.0% for type d and 1.0% for type e. Resistances to trimethoprim-sulphamethoxazole and ampicillin were 46.2% and 10.7% respectively. Multidrug resistance was found in 14 (15.0%) of the isolates tested. Among the isolates, 13.9% were β -lactamase producers; there were no β -lactamase negative ampicillin resistant isolates. DCCs are niches with a high potential for the spread of microorganisms and should be continuously monitored to detect elimination or replacement of *H. influenzae* type b colonization.

Key words: *Haemophilus influenzae*, day-care center, *Hib* colonization, *Hib* vaccination

INTRODUCTION

Haemophilus influenzae (*Hi*) is one of the major bacterial species causing infection in children. Strains of *Hi* are classified according to their polysaccharide capsule. There are six structurally and antigenically distinct capsular serotypes, designated from a to f (34). Encapsulated strains are responsible for a variety of invasive diseases, the most frequent being meningitis, but epiglottitis, arthritis, pneumonia and cellulites also occur (39).

Widespread use of *H. influenzae* capsular type b conjugate vaccine over the world has resulted in the virtual elimination of invasive *Hib* disease in countries that have included the vaccine in their infant immunization programs (21,32). In Brazil, the program

of immunization was started in the second semester in 1999, with apparently the same rate of success (36). With the decline in *Hib* disease, non type b *Hi* has become a more important cause of *Hi* disease. Serotype f (*Hif*) is reported to be the most common cause of invasive encapsulated non-b *Hi* disease in children (41). Serotype a is a rare cause of pneumonia and meningitis (37).

Nonencapsulated, i.e. nontypable *Hi* (*NTHi*), strains are well recognized causes of sinusitis and otitis media in children (25). Some data suggest that *NTHi* are a significant cause of invasive diseases, such as pneumonia in children of developing countries (17). Although this pathogen is associated with a variety of infections, it is also frequently isolated from the upper respiratory tract of healthy children, with reported carrier rates of up to 60.0% (12,17,26).

*Corresponding Author. Mailing address: Departamento de Morfologia, Estomatologia e Fisiologia, Faculdade de Odontologia de Ribeirão Preto, Av. do Café S/N - Campus USP. 14040-904, Ribeirão Preto, SP, Brasil. Tel.: (+5516) 602-4035, Fax: (+5516) 633-0999, E-mail: jmmarin@rge.fmrp.usp.br

Day care center (DCC) attendance has been reported as a major risk factor for increased rates of carriage of these respiratory bacterial pathogens (1), and for increased incidence of upper respiratory tract infection (4). Children attending DCCs carried potentially pathogenic bacteria such as *Hi* in the nasopharynx more often, and also had more frequently symptoms of upper respiratory tract infection (20).

Resistance to β -lactam antibiotics among *Hi* isolates has been increasing over the last few decades (11,22). The main mechanism of resistance detected in wild type *Hi* strains, is the production of β -lactamase with an overall prevalence of 13.4% in Europe (15), and up to 41.6% in the United States (23). In Japan, the prevalence of β -lactamase production is low (13.9%). In most cases, the mechanism for ampicillin resistance is the production of plasmid-mediated TEM or ROB β -lactamases, which are detected in >30.0% of isolates in some countries (11,22). It has been reported that children at DCC can be reservoirs for antibiotic-resistant bacteria (19).

In Japan, the prevalence of β -lactamase negative ampicillin-resistant (BLNAR) strains is 28.8% (40), while in the United States and Europe this phenotype is rare (<1.0%) (15,23). In Latin America (10) and in Brazil (6,8), the prevalence of β -lactamase production is almost the same as that found in Europe, around 9.0-14.5%, and BLNAR strains have not been detected.

The present study was designed to monitor nasopharyngeal carriage of *Hi* in healthy children attending DCCs in Ribeirão Preto, São Paulo State, Brazil and the resistance patterns of the isolates.

MATERIALS AND METHODS

Study population

From November 2002 to November 2003, boys and girls aged from 18-36 months attending municipal DCC in Ribeirão Preto, São Paulo State were recruited, and specimens collected. A total of 114 children attending three DCCs were enrolled in the study. DCCs were selected so as to represent the spectrum of social strata and different geographical areas in the city. Children were excluded from the study if they had taken antibiotics up to 1 week prior to the study and were considered adequately immunized against *Hib* if, prior to the study, they had received 3 doses of vaccine (at 2, 4 and 6 months of age). Only one swab for culture was obtained from each child on a single occasion.

The research protocol was approved by the Ethics Committee of the School of Dentistry of São Paulo University at Ribeirão Preto, and the study was initiated with the agreement of the Municipal Service of Health and Education of Ribeirão Preto and the approval of the DCC managers. Signed informed consent was obtained from a parent or guardian of each child and a questionnaire was completed with information regarding the

subject's age, sex, family size, history of recent respiratory infection or antibiotic therapy.

Sampling and cultures

Nasopharyngeal samples were obtained from all children with a twisted applicator rayon tipped swab (Copan, Brescia, Italy) in Amies media without charcoal and plated on the same day on a selective medium of 10% sheep blood-chocolate brain heart infusion agar plates, supplemented with 300 mg/L bacitracin (Difco Laboratories, Detroit, USA). After overnight incubation under a CO₂-enriched atmosphere at 36°C, plates were inspected for growth of *Hi*. Isolates were identified by colony and cell morphology and by the demonstration of growth requirements for V and X factors. Strain biotype was further characterized by biochemical reactions (24). Capsular serotyping was performed by transferring 10 μ L of a milky suspension of the bacterial cells made in 0.85% formalinized saline to 10 μ L of the antiserum; all specific antisera (a to f antisera Difco, Detroit, MI) were run in parallel, and the slide was rocked for 1 min. All strains were kept frozen at -80°C in brain heart infusion broth (Difco, Detroit, MI) with glycerol (40%) until further analysis.

Antimicrobial susceptibility testing

One colony from each primary culture was selected for further investigation. Susceptibility to amoxicillin-clavulanic acid (AMC-20/10 μ g), ampicillin (AMP-10 μ g), aztreonam (ATM-30 μ g), azithromycin (AZI-15 μ g), cefoxitin (CFO-30 μ g), ceftriaxone (CRO-30 μ g), ciprofloxacin (CIP-5 μ g), chloramphenicol (CLO-30 μ g), imipenem (IMI-10 μ g), levofloxacin (LVX-5 μ g), rifampin (RIF-5 μ g), trimethoprim-sulphamethoxazole (TMP/SMX-25 μ g), tetracycline (TET-30 μ g) and ticarcillin-clavulanic acid (TIC 75/10 μ g) (CEFAR, São Paulo, Brasil) was determined by the disk diffusion method of Bauer and Kirby, using Mueller Hinton base (Difco, Detroit, MI), supplemented with 15 μ g/mL of bovine hematin, 15 μ g/mL of NAD (Sigma, St. Louis, USA) and 5 mg/mL of yeast extract (Difco, Detroit, MI), following the recommendations of the National Committee for Clinical Laboratory Standards (30). *Hi* ATCC 49247 and 49766 were included as quality control standards in each assay. The production of β -lactamase was determined by the chromogenic cephalosporin method (38) using reconstituted lyophilized nitrocefin (Glaxo 87/312, Glaxo Research, Unipath Ltd., Hantsphire, England). *Hi* ATCC 49247 was used as a negative control and *Staphylococcus aureus* ATCC 29213 was used as a positive control.

DNA preparation

Isolates were recovered from frozen storage by being subcultured on chocolate agar plates and incubated under 5% CO₂ at 36°C for 18 h. High-molecular-weight chromosomal DNA was extracted from *Hi* isolates (33). A rapid DNA extraction method was also performed (27).

PCR

Primers specific for the *bexA* gene (primers H1 and H2), required for capsular exports, were used to differentiate *NTHi* isolates from encapsulated isolates, in the 93 strains tested. Primers and PCR conditions used were as previously described (13,27). Capsule typing was considered positive when DNA amplification of the *bexA* gene and one of the specific capsule genes occurred. Standard strains for all serotypes (ATCC 9006, ATCC 35533, ATCC 9007, ATCC 9332, ATCC 8142 and ATCC 9833) and a non-encapsulated strain (ATCC 49247) were used as positive controls for slide agglutination tests and PCR.

RESULTS

Between November 2002 to November 2003, 114 children < 3 years of age, with the complete cycle (3 doses) of anti *Hib* immunization, attending three DCCs in Ribeirão Preto, were enrolled in this study.

Hi was isolated from a nasopharyngeal culture from 82 (72.0%) of the 114 children and from a total of 93 of 114 samples (81.6%). *H. parainfluenzae* were isolated from 2 (1.7%) children and *H. parahaemolyticus* from 1 (0.8%) child.

A total of 93 isolates were serotyped and biotyped. *NTHi* isolates were identified into seven biotypes, biotype II being the most common (36.5%), followed by biotype I (21.5%) (Table 1). Thirteen isolates (13.9%) were β-lactamase-producing strains, and none were BLNAR (Table 1). Six isolates (6.4%) were encapsulated, three belonged to serotype f (3.2%), one belonged to serotype b (1.0%), one to serotype d (1.0%) and one to serotype e (1.0%).

Antibiotic susceptibility analysis showed that 43 (46.2%) of the isolates were resistant to trimethoprim-sulphamethoxazole (TMP-SMX), 10 (10.7%) to ampicillin, and 4 (4.3%) to ceftriaxone (Table 2). Resistance to both ampicillin and chloramphenicol was not found in any isolate. Forty five isolates (48.3%) were susceptible to all the antibiotics tested. Multidrug resistance,

Table 1. Distribution of biotypes and β-lactamase positive strains of *Haemophilus influenzae* isolated from the nasopharynx of healthy children in day care centers in Ribeirão Preto, State of São Paulo, Brazil.

Biotype	Nº of isolates (%)	β-lactamase positive (%)
I	20 (21.50)	4 (4.30)
II	34 (36.55)	6 (6.45)
III	15 (16.12)	0
IV	4 (4.30)	0
V	17 (18.27)	2 (2.15)
VII	2 (2.15)	1 (1.07)
VIII	1 (1.07)	0

Table 2. Antibiotic resistance rate of *Haemophilus influenzae* colonizing children attending day care centers in Ribeirão Preto, State of São Paulo, Brazil.

Antibiotic	Nº of isolates (%)
Amoxicillin/clavulanic acid	0
Ampicillin	10 (10.75)
Azithromycin	2 (2.15)
Aztreonam	2 (2.15)
Cefoxitin	3 (3.22)
Ceftriaxone	4 (4.30)
Ciprofloxacin	0
Chloramphenicol	3 (3.22)
Imipenem	0
Levofloxacin	0
Rifampin	0
Trimethoprim/Sulphamethoxazole	43 (46.23)
Tetracycline	0
Ticarcillin/clavulanic acid	0

defined as being resistant to two or more classes of antibiotics, was found in 14 (15.0%) of the isolates tested. The most frequently identified pattern was resistance to ampicillin and TMP-SMX (9 isolates, 9.6%).

DISCUSSION

Colonization of the human nasopharynx is the first step in the sequence of events resulting in *Hi* infections and disease. Previous studies have documented *Hi* point prevalence colonization rates among various populations to range from 11 to 88% (3,5,9,16,20,31) and most likely depending on differences in the children’s hygiene practices, behavior, or antibiotic use. In this study of nasopharyngeal cultures from children < 3 years old attending DCC in Ribeirão Preto- SP, the colonization rates are consistent with the variable rates reported. The increased carriage rates are most probably due to increased exposure resulting from crowding in the DCCs, this effect has been previously suggested to explain the higher carriage rates in children with more siblings (20,28).

Hi isolates were distributed among seven biotypes with the exception of biotype VI (Table 1). The most common colonizing biotypes were type II, I, V and III agreeing exactly with the result of a previous study (5). Recently, in a few studies in DCC in which *Hi* biotypes were reported, one in Brazil (5), one in France (35) where the most common biotypes for *Hi* were type II (62.2%), V (18.0%) and I (16.2%), and one in the USA (14), where the most common types were type II (37.0%), III (21.0%), I (13.0%), V (12.0%) matched the results of the present study.

The studies in Brazil showed to involve *Hib* diseases, particularly meningitis, and in these cases the more commonly isolated biotypes were of types I and II (7).

After the introduction of *Hib* immunization in Brazil, type a has been proven to replace type b as causative agent of meningitis (36). Surprisingly, in this study type a strains were not recovered from the children in day-care centers.

In DCCs studies, a small carriage rate of different serotypes has been found after *Hib* immunization; a French study with 1,683 healthy children reported 0.6% of type e and 0.4% of type f (9); an American study with 198 children and 404 isolates, reported the isolation of only one serotype f strain (14). In the present study three isolates (3.2%) of serotype f, one isolate of type d and one isolate of type e were found, a result not agreeing with the reported replacement (36); we have also found one isolate type b agreeing with a Dutch study (31) in which four isolates type b were found. Despite the success of the *Hib* vaccine, *Hib* isolates have not been fully eliminated. It is expected that *Hib* carriage will be maintained until the herd immunity levels become sufficient to avoid transmission; still, the risk of outbreaks of *Hib* disease even among fully vaccinated children in a DCC will continue to exist (29).

Young children attending DCCs have increased risk of more episodes of respiratory tract infections, including acute otitis media than children not attending DCC (2), this is reflected by the intensive use of antibiotics leading to increased resistance. Studies at DCCs in France, reported a high rate of β -lactamase positive strains (44.5-60.0%) (9,35) compared with those of the general population (15). In the present study, the rate of β -lactamase positive strains detected at DCCs (13.9%) was not different from those reported for the population (6,8).

In a recent report on worldwide prevalence of antimicrobial resistance, *Hi* resistance to TMP-SMX showed considerable regional variability (14.0-31.0% of isolates), highest rates being observed in Latin America (18), confirmed by the reported rates in Brazil 40.1- 45.2% (8), in this work we reported 46.2% of TMP-SMX resistance matching other rates reported in Brazil (8). Resistance to ampicillin reported in the present study was 10.7%. In Brazil the combination of ampicillin and chloramphenicol is extensively applied because of its low cost and effectiveness (6); both antibiotics have been checked in two Brazilian studies on *Hi* antibiotic resistance, showing rates for ampicillin ranging from 10.0 to 13.8% (7,8), again agreeing with those presently reported.

Bacteria carried in the nasopharynx of normal children reflect the strains currently circulating in the community, causing respiratory infections; therefore, the study of the prevalence of these bacteria and of their resistance patterns can provide useful indications for more rational antibiotic therapy of these infections.

In summary, this study shows that widespread immunization with a *Hib* conjugate vaccine is associated with a significant reduction in the number of *Hib* nasopharyngeal carriers among *Hib* immunized children at DCCs, and despite the high use of antibiotics for young children treatment in Brazil, the rate of antibiotic resistance of the children attending DCC is not different from that of the general population. Nevertheless, DCC's should be continuously monitored to detect elimination or replacement of *Hib* colonization.

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RESUMO

Colonização da nasofaringe por *Haemophilus influenzae* em crianças que freqüentam creches em Ribeirão Preto, Estado de São Paulo, Brasil

A colonização da nasofaringe por *Haemophilus influenzae* (*Hi*) foi estudada em 114 crianças saudáveis com menos de 3 anos de idade e que freqüentam creches (day-care centers DCC) em Ribeirão Preto, estado de São Paulo, Brasil. Para cada uma das cepas isoladas foram determinados o biótipo, o sorotipo (por antisoro específico e PCR) e a sensibilidade a 14 antibióticos. A freqüência de colonização por *Hi* foi de 72,0%. As cepas isoladas foram identificadas como pertencentes aos biótipos II (36,5%), I (21,5%), V (18,2%) e III (16,1%). A freqüência encontrada de cepas encapsuladas foi de 3,2% para o tipo f, 1,0% para o tipo b, 1,0% para o tipo d e 1,0% para o tipo e. A resistência para trimetoprim-sulfametoxazole e ampicilina foi de 46,2% e 10,7% respectivamente. Resistência múltipla foi encontrada em 14 (15,0%) das cepas analisadas. 13,9% das cepas analisadas eram produtoras de β -lactamase, e não foi recuperada nenhuma cepa β -lactamase negativa e ampicilina resistente. DCCs são considerados locais de risco, com um alto potencial de disseminação de microrganismos e por isto devem ser continuamente monitorados com a finalidade de detectar a eliminação da colonização da nasofaringe por cepas *H. influenzae* tipo b das crianças que freqüentam DCC, ou detectar a sua substituição por outro tipo de cepa.

Palavras-chave: *Haemophilus influenzae*, creches, colonização por *Hib*, anti*Hib* vacinação

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