Carriage of *Haemophilus influenzae* among Brazilian children attending day care centers in the era of widespread Hib vaccination

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**Abstract**

*Haemophilus influenzae* type b vaccine was introduced into the Immunization Program of Brazil in 1999 and no study has evaluated the impact of Hib vaccination in *H. influenzae* carriage so far. In June 2010, Brazil introduced the 10-valent pneumococcal nontypeable *H. influenzae* (NTHi) conjugate vaccine (PHiD-CV). We investigated the prevalence of encapsulated *H. influenzae* and NTHi isolates in nasopharyngeal samples of 1192 children attending day-care centers in Goiânia, central Brazil. *H. influenzae* carriage rate was 32.1% and 38.4% of them carried β-lactamase TEM-1 gene. Serotype f (4.6%) was the most frequent encapsulated isolate, type b was recovered in only 0.7% and carriage rate of NTHi was 23.3%. Recurrent acute otitis media and NTHi were independently associated with colonization by β-lactamase producing *H. influenzae*. Changes in frequency of *H. influenzae* carriage isolates should be carefully monitored to assess the impact of the PHiD-CV on NTHi carriage in young children.

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1. Introduction

Prior to the introduction of *Haemophilus influenzae* type b (Hib) conjugate vaccines for infants, Hib was the leading bacterial cause of meningitis among children younger than five years of age worldwide [1]. Widespread use of Hib conjugate vaccines rapidly reduced the burden of Hib meningitis and invasive Hib infections [1–3]. In many countries with routine infant immunization against Hib disease, serotypes other than type b and non-typeable (or unencapsulated) *H. influenzae* (NTHi) have overtaken Hib among invasive isolates [4–6]. Hib vaccination was introduced into the Brazilian Immunization Program at 2, 4, and 6 months of age, without booster dose, since July 1999. A sustained high coverage of vaccination (around 95%) has been maintained and invasive diseases due to type b sharply fell in the vaccine target population soon after the Hib vaccination [7,8].

The capsule of *H. influenzae* is a virulence factor that plays a role in invasiveness [9]. Unencapsulated NTHIs isolates more commonly colonize the human nasopharynx and are associated with noninvasive diseases including acute otitis media (AOM) and pneumonia, although NTHI are increasingly isolated from patients with blood stream infections [5,10,11]. Colonization of the nasopharynx is likely the initial event that can lead to AOM, pneumonia and invasive infection [12]. Few studies have examined nasopharyngeal colonization with *H. influenzae*. In 2010, Brazil became the first country to introduce into its national immunization program a novel pneumococcal conjugate vaccine containing 10 serotype-specific polysaccharides, eight of which are conjugated to protein D, a conserved membrane protein from *H. influenzae* (PHiD-CV) [15]. A similar vaccine containing 11 pneumococcal polysaccharides and the protein D carrier reduced
carriage of *H. influenzae* in 42.6% and also NTHi colonization and NTHi-associated otitis media in clinical trials [16,17]. As part of a study of nasopharyngeal colonization with *Streptococcus pneumoniae* conducted among children attending day-care centers (DCCs) in Goiânia, Brazil [18], we measured the prevalence of colonization with encapsulated and NTHi prior to the introduction of PHID-CV as part of the national program. Additionally, the study examined variables associated with carriage of β-lactamase positive *H. influenzae*. This data will provide a baseline to assess the impact of the novel 10-valent pneumococcal conjugate vaccine on carriage of *H. influenzae* among young children in Brazil.

### 2. Materials and methods

#### 2.1. Study population

Between August and December 2005, demographic data and nasopharyngeal (NP) swabs were collected from children aged 2–59 months in 62 of the 70 municipal DCCs in the municipality of Goiânia (population 1,201,007), capital of the central Brazilian state of Goiás. All day-care attendees had received three doses of Hib vaccine. The number of children sampled per DCC was proportional to the number of children attending the center. We calculated that 1100 children would be necessary to estimate prevalence of *H. influenzae* colonization with 95% confidence, assuming 20% of NTHi carriage [14,19], with a design effect = 1.5. Socio-demographic characteristics (age of child, sex, household size, number of siblings, and participant siblings) were obtained by parent interview. Information on history of fever or hospitalization in the previous 3 months and history of AOM were also collected as proxies for antibiotic use and consequently potential risk factors for carriage of non-susceptible *H. influenzae*.

The study protocol was approved by the Regional Ethical Committee of the Federal University of Goiás and written informed consent was obtained from each child’s parents or legal representative.

#### 2.2. Laboratory methods

NP specimens were collected and placed into Stuart transport medium tubes (Transwabs, Medical Wire, Corsham, UK). Swabs were transferred to the Laboratory of Bacteriology of the Federal University of Goiás and processed immediately. Bacterial isolation and identification was performed according to the WHO recommendations [20]. Methods for the characterization of nasopharyngeal carriage of *S. pneumoniae* have been previously described [21]. For identification of *H. influenzae*, nasopharyngeal specimens were inoculated on chocolate agar supplemented with bacitracin (300 µg/mL) and incubated in 5–7% CO₂ at 37°C for 24–48 h. Gram staining was performed for those colonies with suggestive morphology of *H. influenzae*. Identification of *H. influenzae* was confirmed by the satellitism test with *Staphylococcus aureus* (ATCC 23922) co-culture as well as by biochemical tests (fermentation of glucose, xylose, lactose, mannose, sucrose; ornithine decarboxylase, indole production and urease test).

DNA was extracted by the boiling method [22] from one colony per plate of confirmed *H. influenzae* cultures. Molecular identification of *H. influenzae* was done by amplification of the P6 gene [23], while the presence of capsule was confirmed by amplification of the *bexA* gene. Capsulated *H. influenzae* typing was performed by multiplex PCR [22]. Screening of capsule-deficient type b strains (b− phenotype) was performed by PCR of the b capsule gene for all *H. influenzae* strains negative for the amplification of the *bexA* gene. This phenotype is genetically similar to type b, however b− isolates do not produce PRP capsule [24]. Screening for β-lactamase genes was carried out with TEM-1 and ROB-1 primers to amplify the *blaTEM* and *blablaROB* genes, respectively [25]. These genes are the most frequent and clinically relevant in β-lactamase production by *Haemophilus* [26]. Standard strains used as positive controls in PCR reactions were Hia ATCC9006, Hib ATCC33533, Hic ATCC9007, Hid ATCC9332, Hie ATCC8142 and Hif ATCC9833.

#### 2.3. Data analysis

Age was stratified into <24 months and ≥24 months groups. History of three episodes of AOM in 6 months or more than three episodes in 12 months was defined as recurrent AOM. Values of 95% confidence interval (95% CI) of the prevalence of *H. influenzae* and NHTi carriage were adjusted by DCCs. Logistic regression was used to analyze risk factors associated with carriage of β-lactamase positive *H. influenzae*. Results were presented as odds ratios (OR) with 95% CI. All variables with p-values less than 0.10 in univariate analyses were included in a multivariate model to identify factors independently associated with carriage of *H. influenzae* containing the β-lactamase TEM-1 gene. Significant variables were selected based on likelihood ratio tests [27]. A probability level of 0.05 (two-tailed) was used to determine statistical significance.

### 3. Results

A total of 1276 children between 2 and 59 months of age attending 62 out of 70 public DCCs in Goiânia were recruited for swab collection. Eighty-four children were excluded from the analysis because they had taken an antibiotic in the previous 7 days. Therefore, 1192 children were available for the study. The median number of children sampled per DCC was 16.5 (range 7–50). The median age of the participants was 39 months (range 4–59), and 645 (54.1%) were male. A total of 129 children were siblings of other study participants. Overall, 80.5% of the children had taken antibiotics (76% amoxicillin) at least twice in the last six months. History of antibiotic use in the previous 3 months was more frequent among children ≥24 months compared to those <24 months (p = 0.003). A total of 137 (11.5%) children had attended other DCCs and 10.3% had been admitted to hospital in the previous six months. A significant association was found between fever or hospitalization in the previous three months and antibiotic use in the previous three months (p < 0.01). The annual median household income was US$2643 which is below the Brazilian poverty line.

#### 3.1. Colonization with *H. influenzae*

The prevalence of *H. influenzae* nasopharyngeal colonization among the 1192 day-care attendees was 32.1% (95% CI 25.5–38.8). Colonization by DCC ranged from 20.0% to 62.5%. Encapsulated and NTHi isolates accounted for 8.8% (95% CI 7.3–10.6) and 23.3% (95% CI 17.3–29.4), respectively. No significant association was found between carriage rates of NTHi ≥ 0.068, beta-lactamase production (p > 0.05), and different nurseries (p > 0.05) among siblings. Among encapsulated organisms, the most frequent serotype was f (4.6%), which predominated in older children. Serotype a (2.0%) was more frequent in young children, aged between 4 and 11 months. Type B was only recovered from 8 children (0.7%) (Fig. 1). Among NTHi, 17 (1.4%) of 278 were type b mutants (b−). The age distribution of capsular types showed that for all *H. influenzae* types the number of isolates increased with the age-group. However, isolates of type f and NTHi predominated in older children (≥36 months of age).
3.2. Factors associated with presence of β-lactamase gene

Of the 383 children colonized with *H. influenzae*, 47 (38.4%) were colonized with organisms possessing the TEM-1 β-lactamase gene. The prevalence of TEM-1 was significantly higher in NTHi (43.2%) compared to the typeable strains (25.7%). No *H. influenzae* isolate carried the ROB-1 gene.

β-Lactamase positive isolates predominated in children above 11 months of age (Fig. 2). Colonization with NTHi (*p = 0.001*) and recurrent AOM (*p = 0.029*) were associated with colonization of β-lactamase producing *H. influenzae* (Table 1). After adjustment in the multivariate model, colonization with NTHi (OR, 1.84; 95% CI, 1.06–3.18; *p = 0.021*) and recurrent AOM (OR, 3.14; 95% CI 1.18–8.33; *p = 0.06*) remained independently associated with the risk of β-lactamase positive *H. influenzae* carriage. There was no interaction between recurrent AOM and NTHi (*p = 0.06*).

3.3. Colonization with *Haemophilus influenzae* and *Streptococcus pneumoniae*

Of the 383 children colonized with *H. influenzae*, 235 (61.4%) were co-colonized with *S. pneumoniae*, while 451 (78.6%) of 574 children not colonized with *H. influenzae* carried *S. pneumoniae* in their nasopharynx. Carriage of *H. influenzae* was negatively associated with carriage of *S. pneumoniae* (OR, 0.43; 95% CI 0.32–0.58; *p < 0.001*).

4. Discussion

In this cross-sectional study of children attending DCCs in a large Brazilian city, the overall prevalence of *H. influenzae* carriage was similar to or lower than that reported among children attending DCCs, living in orphanages and pre-schools students from studies conducted in Europe and North America after the introduction of Hib conjugate vaccines [14,28]. Also, the prevalence of NTHi colonization in the current study (23%) was lower than that observed in children in USA (64%) and France (40%) after Hib vaccination [14,28]. Frequent antibiotic use (mainly amoxicillin) may have reduced the overall prevalence of *H. influenzae* colonization, despite exclusion of 7% of the original sample of children who had received antibiotics in the previous 7 days. Seasonal differences in *H. influenzae* carriage may also play a role; sampling in Goiânia was conducted from August to December (corresponding to winter and spring time in the southern hemisphere), while *H. influenzae* and *S. pneumoniae* colonization in Brazil is reportedly higher during May to July, following the rainy season [29]. We could not rule out the possibility of niche replacement by non-typeable *H. influenzae* as a result of pneumococcal seven-valent conjugate vaccine to explain the higher rates of NTHi in countries where this vaccine has been extensively used. Another point is that carriage studies have used different methodologies. We characterized only a single *H. influenzae* colony recovered from each child and used PCR to identify capsular type, which may be more accurate than slide agglutination methods [30]. Carriage studies using similar methodologies are needed to compare prevalence of *H. influenzae* in different settings and populations.

Few carriage studies have examined factors associated with reduced susceptibility of *H. influenzae* to β-lactams. In the current study, reduced susceptibility to β-lactam antibiotics was not measured directly. However, presence of the TEM-1 gene is highly predictive of β-lactam resistance, with positive predictive values ranging from 94% to 100% [26,28]. Frequent exposure to antibiotics exerts selective pressure favoring β-lactamase-producing *H. influenzae* strains. We found that history of antibiotic use was more frequent among older children. NTHi strains being more common colonizers of the nasopharynx have more antibiotic exposure and were more likely than encapsulated organisms to possess the TEM-1 gene. Plasmid-mediated production of TEM-1 or ROB-1 β-lactamase is the most common mechanism of *H. influenzae* resistance [31]. The global distribution of these enzymes is highly variable with ROB-1 being found almost exclusively in North America—Canada, USA, Mexico [26]. In our study all *H. influenzae* strains that produced β-lactamase were TEM-type.

The clinical relevance of β-lactamase-producing *H. influenzae* is not well known, although it was found to be associated with...
recurrent acute otitis media in our study. NTHi is one of the most common causes of recurrent AOM in childhood [32–34]. The relative proportion of AOM cases caused by NTHi has increased since the introduction of the 7-valent pneumococcal conjugate vaccine in developed countries [32,35,36]. AOM is the main reason for ambulatory and emergency room visits by children in both developing and developed countries, contributing to high rates of antibiotic prescription [37,38]. β-lactams, especially ampicillin, are the most frequently prescribed antibiotics for children treated as outpatients in Goiânia, where the study was conducted [33]. Therefore, β-lactamase-producing NTHi would complicate the management of AOM, an association already reported in children failing first-line AOM therapy in other studies [32,35,36].

The low rate of Hib carriage in this survey (<1%) is not a surprise, even though day-care attendees are at increased risk for H. influenzae colonization. In Brazil, Hib carriage rates before the routine vaccination ranged from 4.6% to 7.3% [39,40]. Hib vaccination coverage in Goiânia is maintained close to 100% [41]. In England, the prevalence of Hib carriage was lower than 2% in children after vaccination [42]. In USA, studies in attendees four years after vaccination detected a 46% decrease in type b Hi colonization in children who had received the conjugate vaccine [43]. Hib conjugate vaccines induce high levels of mucosal anti-Hib antibody, which reduce Hib carriage in the nasopharynx as well as transmission of Hib in the community [9,44,45]. Although anti-Hib antibodies are serotype-specific, reduction of Hib carriage as a result of vaccination could create an ecological niche for other H. influenzae or other bacteria [2]. However, the prevalence of nasopharyngeal colonization with encapsulated H. influenzae other than serotype b in this study was low, similar to reports from Spain, France and USA [14,19,28]. Serotype f was the most commonly identified H. influenzae type, from just 4.6% of the children attending day care. Serotype f has been associated with increased invasive infections in United States after Hib immunization [40,46]. Finally, we may have underestimated the prevalence of colonization with encapsulated H. influenzae by characterizing only one single colony from each child’s sample culture [11].

In conclusion, this survey of H. influenzae carriage among children attending public day-care centers in a major Brazilian city provides a baseline to evaluate the effect of the PHiD-CV containing protein D from H. influenzae on NTHi carriage. Clinical trials are underway to evaluate the effectiveness of this vaccine on AOM. In the Pneumococcal Otitis Efficacy Trial (POET) with the 11-valent pneumococcal vaccine, a 35.6% protection against AOM by NTHi was observed [16]. In addition, a reduction of 41.4% on NTHi nasopharyngeal colonization in the vaccine recipients was found compared with the control group [17]. With the introduction of this novel pneumococcal vaccine into the routine childhood immunization schedule in Brazil, changes in frequency not only in pneumococcal but also in NTHi carriage and disease should be monitored. Whether the PHiD-CV will protect against NTHi carriage and disease is an exciting issue; if so, it would be an additional bonus to vaccination, since the vaccine was primarily designed to prevent pneumococcal disease.

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