

# Oropharyngeal Colonization by Nontypeable *Haemophilus influenzae* Among Healthy Children Attending Day Care Centers

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*Haemophilus influenzae* colonizes the upper respiratory tract and can spread causing otitis and sinusitis. This work aimed to study the oropharyngeal carriage rate in healthy <5-year-old children attending day care centers in Oviedo, Spain in two consecutive years (January to March 2004–2005). The carriage rate was 42% (400/960) and highly variable among centers (range, 12% to 83%). Isolates were mainly identified as nontypeable *H. influenzae* (NTHi, 99%). Epidemiologically, 127 different genotypes were identified by PFGE with a minimum of two genotypes per center. One hundred fourteen children (12%) were included in both studies and none of them harbored the same strain over a period of time. The isolates only showed resistance to cotrimoxazol and ampicillin, presenting a shift in the level of ampicillin reduced susceptibility, showing a predominance of PBP3 mutations in 2004 and a predominance of  $\beta$ -lactamase production in 2005. This study proved the great genetic variability of NTHi isolates that present similar genotypic patterns in both years with no long-term carriage of the same strain.

## Introduction

THE FASTIDIOUS GRAM-NEGATIVE coccobacilli *Haemophilus influenzae* form part of the indigenous nasopharyngeal microbiota and can also cause acute respiratory infections.<sup>1,8,15,18</sup> The presence or absence of a polysaccharide capsule segregates this bacterial species in two well-defined groups; a group of encapsulated strains and a second group of nonencapsulated strains, commonly referred as nontypeable *H. influenzae* (NTHi).<sup>1,15,18</sup>

NTHi isolates were initially associated with asymptomatic colonization; nevertheless, these unencapsulated bacteria are also pathogenic and frequently identified as the etiologic agent of otitis media, sinusitis, conjunctivitis, chronic bronchitis, and community acquired pneumonia.<sup>1,8,15</sup>

The pathogenesis of NTHi starts with an initial colonization of the upper respiratory tract, followed by the bacterial migration to other neighboring parts where the bacterial pathogen initiates an inflammatory response.<sup>15,18,22</sup> Strain transmission occurs frequently within households and it has also been observed at very high rates among children sharing the same Day Care Center (DCC).<sup>1,15</sup>

Many day care colonization studies investigate *Streptococcus pneumoniae* carriage, but information on *H. influenzae* colonization of young children is scarce and has not been reported from DCCs in Spain. On this ground, the objectives of this study were to investigate the colonization rate and level of antimicrobial resistance in the major DCCs and schools from Oviedo, Spain.

## Materials and Methods

### Study design and children selection

Two prospective point-prevalence studies were conducted in DCCs and schools from Oviedo (Spain) between January and March of 2004 and 2005. Informed written consent for participation in the study was obtained from the children's parents. The study was approved by the Ethics Committee from the Hospital Universitario Central in Asturias (Spain).

Children between 1 and 5 years were recruited from 16 DCCs (age 1–3) and three public schools (age 3–5). The exclusion criteria were falling out of the age range, respiratory infection, absence in the center on the sampling day, and inability to obtain the sample. Every child presented a

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parental filled in questionnaire on the children's illnesses and antimicrobial consumption in the 6 months previous to the study.

#### Sample collection

Oropharyngeal swabs were collected by a trained nurse and preserved in the STGG medium (3% Tryptone Soya Broth, 0.5% glucose, 2% skim milk, and 10% glycerol). Sterile cotton-tipped wooden swabs were wiped across the respiratory tract mucosa lining at the rear of the oropharynx with care not to touch the teeth, gums, or tongue. Bacterial identification was performed by standard microbiological methods.<sup>3</sup> Serotyping was achieved by latex agglutination with the Phadebact<sup>®</sup> Haemophilus Test (Bactus AB, Huddinge, Sweden) and by polymerase chain reaction (PCR) as previously described by Falla *et al.*<sup>9</sup> Species differentiation between *H. influenzae* and *H. haemolyticus* was performed by PCR detection of *iga*, *fucK*, and *lgtC* genes as described by Binks *et al.*<sup>4</sup>

#### Antimicrobial susceptibility testing

Bacterial susceptibility was determined by standard disc diffusion with the following antibiotics (Oxoid, Madrid, Spain): ampicillin, amoxicillin-clavulanic acid, cefotaxime, ciprofloxacin, chloramphenicol, sulfamethoxazole-trimethoprim, and tetracycline. Susceptibility was defined according to the CLSI guidelines.<sup>5</sup> The  $\beta$ -lactamase activity was determined by the chromogenic cephalosporin test using nitrocefin as a substrate and following the manufacturer's directions (BD, Madrid, Spain). Ampicillin and amoxicillin-clavulanic acid minimum inhibitory concentrations (MICs) were performed by e-test on all the isolates with a disk inhibition zone  $\leq 28$  mm for both antibiotics; these strains were selected for a molecular characterization of the mutations in the PBP3 by sequencing an inner region of the *fstI* gene, as described by Dabernat *et al.*<sup>6</sup>

#### Pulsed-field gel electrophoresis

Molecular typing of *H. influenzae* was performed by pulsed-field gel electrophoresis (PFGE). Genomic DNA embedded in agarose plugs was restricted with *SmaI*, and fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad, Madrid, Spain) as previously described.<sup>17</sup>

#### Statistical analysis

All the statistical analyses were performed using the SPSS v.16.0 (SPSS, Inc., Chicago, IL) software package. Differences were evaluated using the Fisher's exact test or chi-squared test with Yate's correction. A *p*-value of  $<0.05$  was considered significant. Logistic regression analysis was used to identify the independent risk factors for *H. influenzae* carriage. Variables with *p*  $<0.05$  in the univariate analysis and those found in previous studies were included in the multivariate analysis.

## Results

#### Sample collection

Sixteen DCCs and 3 schools participated in the study. A total of 960 oropharyngeal swabs were examined, with 482 healthy children included in 2004 and 478 children in 2005. An average of 25 samples was obtained per center (range, 6 to 59), which remained constant in both studies.

#### Questionnaire analysis

Data from the questionnaires were only used as an indicator of global health. Sixty-seven percent of the children had been healthy for the 3 months before the sampling period. On the other hand, 24% of the children had otitis media, 5% asthma, 2% sinusitis, and 2% respiratory tract infections or allergies.

TABLE 1. BASELINE CHARACTERISTICS OF THE STUDY CHILDREN POPULATION ACCORDING TO THE *HAEMOPHILUS INFLUENZAE* OROPHARYNGEAL CARRIAGE

Characteristics	2004 (n=481)				2005 (n=476)			
	Total (n=481)	HINF-carriage (n=206)	HINF-non carriage (n=275)	p*	Total (n=476)	HINF-carriage (n=194)	HINF-non carriage (n=282)	p*
Age $\leq 36$ months	400 (83.2%)	161 (78.2%)	239 (86.9%)	<b>0.01</b>	356 (74.8%)	149 (76.85)	207 (73.4%)	0.40
Sex								
Male/female	258/223	112/94	146/129	0.78	256/220	99/95	157/125	0.32
center								
DCC/school	338/143	138/68	200/75	0.17	344/132	142/52	202/80	0.71
center size								
>35 children	408 (84.8%)	181 (87.9%)	227 (82.5%)	0.11	382 (80.3%)	145 (74.7%)	237 (84.0%)	<b>0.01</b>
>40 children	323 (67.2%)	151 (73.3%)	172 (62.5%)	<b>0.01</b>	267(56.1%)	106 (54.6%)	161 (57.1%)	0.59
Antibiotic consumption								
Previous 6 months	310 (64.4%)	136 (66.3%)	174 (63.5%)	0.52	188 (39.5%)	108 (55.7%)	173 (61.3%)	0.35
Previous month	131 (27.2%)	53 (25.7%)	78 (28.4%)	0.53	193 (40.5%)	45 (23.2%)	77 (39.7%)	0.33
Sampling day	37 (7.7%)	9 (4.4%)	28 (10.2%)	<b>0.02</b>	187 (39.3%)	5 (2.6%)	21 (7.4%)	<b>0.03</b>
Previous otitis media	116 (24.1%)	43 (20.9%)	73 (26.5%)	0.16	110 (23.1%)	38 (19.6%)	72 (25.5%)	0.15
History of asthma	23 (4.8%)	8 (3.9%)	15 (5.5%)	0.16	23 (4.8%)	8 (4.1%)	15 (5.3%)	0.55
Brothers	249 (51.8%)	104 (50.5%)	145 (52.7%)	0.63	251 (52.7%)	106 (54.6%)	145 (51.4%)	0.49

\*Bold: statistically significant.

DCC, Day Care Center; HINF, *Haemophilus influenzae*.



### Molecular characterization of ampicillin-resistant isolates

Among the 400 strains isolated in both point-prevalence studies, 76 isolates showed reduced susceptibility to ampicillin; 96% were resistant due to  $\beta$ -lactamase production, and 4% (negative for  $\beta$ -lactamase) presented reduced ampicillin susceptibility.

A molecular characterization of mutations in the PBP3 was performed on 67 strains that showed an inhibition zone  $\leq 28$  mm for ampicillin and amoxicillin-clavulanic acid. Eighteen of those isolates were already resistant to ampicillin due to  $\beta$ -lactamase production and were mainly strains isolated during 2005 (5 and 13, in 2004 and 2005, respectively).

Sixty-nine percent of the tested isolates (46 out of 67) showed mutations at the *ftsI* gene; among them, 10  $\beta$ -lactamase-producing isolates also showed mutations in the *ftsI* gene (Table 2), and 36 isolates with a reduced susceptibility to ampicillin only presented mutations in the *ftsI* gene. The remaining 21 isolates with reduced susceptibility did not have any mechanism of ampicillin resistance.

### Discussion

Clinical and epidemiological factors associated to *H. influenzae* infections have changed in the last 20 years.<sup>1</sup> Before vaccine development, *H. influenzae* serotype b (Hib) was the most common cause of meningitis in young children,<sup>11</sup> but the introduction of the Hib conjugate vaccine reduced the nasopharyngeal carriage of this serotype.<sup>1</sup> As expected, we did not isolate any Hib, possibly because in Spain, the vaccine was incorporated to the national immunization schedule in 1998.<sup>16</sup>

Although NTHi is considered less virulent than Hib, it can be responsible for severe diseases, especially among children with previous comorbidities.<sup>11</sup> Several studies have been performed to establish the *H. influenzae* carriage rate after vaccine introduction,<sup>2,7,10,12-14,19-21</sup> although to our knowledge, no studies have been reported from Spain. Longitudinal studies performed on one or two selected DCCs give information about the dynamics of colonization, but fail to give an overall view of the situation in similar centers of the same geographical area. Our work presents two consecutive point-prevalence studies in 19 centers, and we observed that children sampled in both occasions did not harbor the same strain. With this approach, we could examine different colonization behaviors while targeting a big and heterogeneous group, although still vulnerable to the seasonality and environmental factors.

In addition, we have incorporated into the study, 5-year-old children from three public schools and found that, despite the typical differences between both centers (*i.e.*, age range, hours in the classroom, number of children enrolled), there was no association between the type of center and the level of colonization.

Other risk factors such as gender and respiratory problems showed no direct association with *H. influenzae* colonization, in agreement with previous works.<sup>7,14,19,21</sup> However, risk factors such as age, size of the center, and antibiotic consumption on the sampling day were linked to *H. influenzae* colonization. The age factor ( $> 36$  months) has been associated to higher colonization levels only in the first

year of study, probably because the study was unintentionally biased toward this age range as a result of the randomness of the sample. In the same way, in 2004, centers with a high number of children enrolled had an increased colonization level. Those results suggest that children's colonization is variable and cross-sectional studies have to be interpreted with caution, as their results cannot be contrasted.

By contrast, taking antibiotics on the sampling day showed a reduced colonization in both years, suggesting that antibiotic uptake produces a rapid effect on *H. influenzae* colonization. A study by Barbosa-Cesnik *et al.*<sup>2</sup> also suggested that children taking antibiotics on the day of culture were less likely to carry NTHi strains, and Raymond *et al.*<sup>19</sup> isolated less *H. influenzae* strains in children who received an antibiotic treatment over the 15 days previous to sample collection.

In our study, the carriage rate associated to *H. influenzae* was kept stable during the two point-prevalence studies, ranging from 43% to 41% in both consecutive years. The prevalence of *H. influenzae* was similar or lower than other colonization studies conducted on worldwide centers.<sup>2,7,10,13,14,19,20</sup> Recently, Carvalho *et al.*<sup>7</sup> reported NTHi colonization in 32% of the children from DCCs in a large Brazilian city with a frequent antibiotic use ( $> 80\%$ ) within the studied population,<sup>7</sup> while studies in the United States showed a carriage rate of 64%.<sup>2,10</sup>

Antimicrobial susceptibility was high probably due to the low range of antimicrobial agents given to treat infections in children. Raymond *et al.*<sup>19</sup> reported resistance to ampicillin (56%), cotrimoxazol (25%), and tetracycline (24%) in isolates from a French orphanage, while in northern Taiwan, a high-level resistance to several antimicrobial agents was found.<sup>21</sup> However, data on antimicrobial susceptibility among healthy young children are scarce, as most epidemiological studies only identify the presence or absence of  $\beta$ -lactamase-producing isolates. In our current work, the rate of ampicillin resistance due to  $\beta$ -lactamase production was very low (24% in 2005 and 13% in 2004) compared to other worldwide studies that ranged between 35% and 45% of the *H. influenzae* isolates.<sup>2,6,7,12,19,21</sup> Despite this low prevalence, ampicillin resistance increased significantly in the second year where we observed a twofold increment in the  $\beta$ -lactamase-producing strains. The isolate characterization was completed with a determination of the PBP3 mutations associated to ampicillin resistance. So far, the studies on amino acid modifications in the transpeptidase domain of the PBP3 were hardly ever performed on samples from young healthy children. In our study, 69% of the tested isolates showed mutations at the *ftsI* gene, which represented 12% of the *H. influenzae* isolates (46/400) and were detected twice as often in strains isolated in 2004 (Table 2); those mutations alone were not enough to confer ampicillin resistance, but they were associated to a reduced susceptibility phenotype (MIC 1 to 3  $\mu\text{g/ml}$ ).

Overall, this study provides an overview of the colonization diversity within a whole community from a country where there is scarce information on this subject. The study has determined the carriage rate of *H. influenzae* in children attending DCCs and schools in a large geographic area, with emphasis to the level of ampicillin resistance detected in the



isolates. We have shown that more than a tenth of the children were colonized with isolates presenting mutations in the PBP3, together with a shift in the level of ampicillin-reduced susceptibility with a predominance of PBP3 mutations in 2004 and a predominance of  $\beta$ -lactamase production in 2005.

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### Disclosure Statement

No competing financial interests exist.

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