

Multilocus sequencing typing as a tool to investigate childhood *Haemophilus influenzae* invasive disease in Portugal

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INTRODUCTION

Haemophilus influenzae is an important cause of serious childhood invasive disease including meningitis and septicaemia. The presence or absence of a polysaccharide capsule divides the bacteria into two different groups. Encapsulated *H. influenzae* strains are classified into six serotypes: a-f (1).

Before the introduction of conjugate vaccine against *H. influenzae* type b (Hib), this serotype was the major responsible for invasive infection, especially in young children. However, the implementation of Hib vaccine in the National Vaccination Program, in the year 2000, turned to a drastic reduction of Hib invasive disease, which is now due to non encapsulated strains (NChi), with encapsulated strains being rarely characterized (2,3).

Multilocus Sequencing Typing (MLST) is a powerful method that allows a precise and unambiguous characterization of *H. influenzae* genotypes. It combines advances in sequencing and bioinformatics to establish population genetics. It's also increasingly applied as a routine typing tool that enables international comparisons of isolates (4,5,6).

A partnership between the Reference Laboratory for *H. influenzae* of the National Institute of Health and the Society for Paediatric Infectious Diseases was established in 2010, with the objective of characterizing invasive infection in children, at both clinical and laboratory level.

The objective of the present study was to genotype isolates by MLST to characterize the disease.

METHODS

Haemophilus influenzae strains

➤ A total of 41 *H. influenzae* strains were isolated from children (≤ 18 years old) with invasive disease, from 2010 to 2015, evolving paediatricians and clinicians in hospitals all over the country.

Methods

➤ Serotyping was performed by PCR amplification of *bexA* gene; capsulated strains (a-f) were identified by amplification of capsule-specific genes using primers and conditions described in the literature (7).

➤ MLST was performed by amplifying and sequencing internal fragments of the 7 housekeeping genes (*adhk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi* and *recA*) (5).

➤ Sequences were analyzed and submitted to the MLST website <http://pubmlst.org/hinfluenzae/> for assignment of sequence type (ST). To display the allelic distances between the obtained STs, we applied the goeBURST algorithm implemented in the PHYLOViZ platform.

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RESULTS

Figure 1. Distribution of *Haemophilus influenzae* isolates by age groups

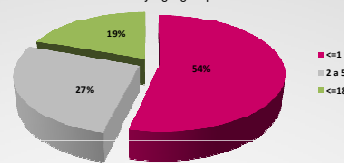


Figure 2. Source of *Haemophilus influenzae* isolates

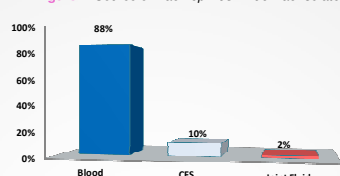


Figure 3. Capsulation status of invasive *Haemophilus influenzae* isolates: 2010 - 2015

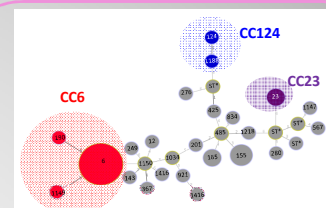
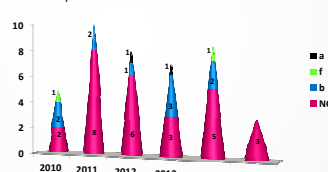


Figure 4. MLST-based representation of genetic diversity among *H. influenzae* invasive disease isolates - this study. MLST sequence types (STs) are indicated by numbers within circles (*means a novel ST), where the size of the circles is proportional to the number of isolates from each ST and different colors mean different capsular types. Connecting lines are labeled with the number of allelic differences between STs. A clonal relationship is observed in each of capsulated strains, with serotypes a, b and f being assigned to three different clonal complexes (CC), while high genetic diversity is observed among NC isolates.

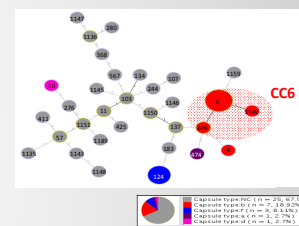


Figure 5. MLST-based representation of genetic diversity among *H. influenzae* invasive disease isolates - previous study: 2002-2009. A clonal relationship is observed within capsulated strains, with 6 of the 7 Hib assigned to CC6 and the three serotype f strains assigned to CC 124, while high genetic diversity is observed among NC isolates.

DISCUSSION AND CONCLUSION

Results of this study reveals differences in invasive infection after the introduction of Hib vaccine:

- younger children are mostly affected (less than 1 years old) (Figure 1);
- septicaemias predominate over meningitis cases (Figure 2);
- most cases are due to NC strains (27/41; 66%); 14 isolates (34%) are capsulated and characterized as follow: two serotype a (5%), 10 b (24%) and two f (5%) (Figure 3);

➤ MLST typing shows high genetic variability among NC isolates: 24/27 (89%) are characterized in different sequence types (STs), with four new STs, represented by previously unidentified allele combinations. In opposition, capsulated isolates are very clonal: all 10 Hib are assigned to CC6 (eight strains ST6, one ST 1149, one ST 190), the two Hia strains are assigned to CC 23 (ST 23) and the two Hif belong to CC124 (ST 124 and ST 1188) (Figure 4).

➤ Comparing these MLST results with the ones obtained in another study performed in our laboratory (3), with strains isolated from invasive disease in children from 2002 to 2009 (Figure 5), we also observed a genetic diversity among NC strains. In capsulated strains there were a few differences: one of the 7 serotype b was characterized as ST4, an ST that is not included in CC6; the serotype a strain was assigned to ST 474, again a ST that is not included in CC23. We also described in that period the first invasive serotype d strain, isolated from a child with meningitis (8).

➤ Our results, that shows a genetic diversity of NC isolates in opposite to a greater clonality of capsulated strains are in accordance with studies from other researchers (5,9,10,11).

➤ Although MLST has a good discriminatory power, the changes in the housekeeping genes may be too slow to afford enough discrimination within each of serotypes, which limits its use in epidemiological investigations. This can be overcome if MLST is based on more variable genes known to play a role in virulence (9,12).

➤ In resume, invasive disease among Portuguese children is nowadays due to NC and highly genetically diverse strains. Nevertheless, we are concerned about Hib disease despite the higher vaccine coverage.

In order to monitor the evolving dynamics of this pathogen and the epidemiology of invasive disease ongoing surveillance is needed.

In a near future we should considered the whole genome sequence analysis as a tool to achieve the true magnitude of this problem.