

# Multilocus Sequencing Typing of Invasive *Haemophilus influenzae* strains Isolated in Portugal in the Pre-vaccination Period (1989-2001)



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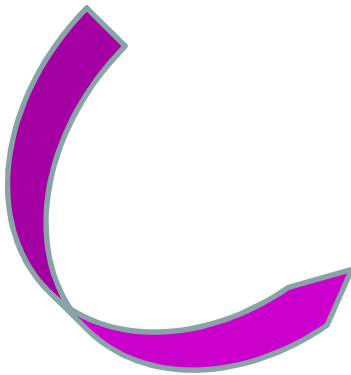
National Institute of Health, Lisbon, Portugal





# Aim of this work

- MLST characterization of Portuguese *H. influenzae* isolates (70 strains) from invasive infection, before introduction of Hib vaccine in NIP

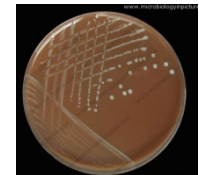


STs from pre-vaccination period isolates (1989-2001) will be compared to STs from pos-vaccination period isolates (2002-2010)



# Introduction / *H. influenzae*

- ✿ *Haemophilus influenzae* can cause life-threatening (invasive) infections in children and adults: pneumonia, bacteremia, meningitis...
- ✿ Six capsular types, a-f, have been identified. Non-capsulated *H. influenzae* (NCHi) have also been described
- ✿ *H. influenzae* serotype b (Hib), has been a major cause of morbidity and mortality, especially in children ( $\leq 5$  years old)





# Introduction /Hib vaccine

- ✿ In Portugal, Hib vaccination was implemented in the National Immunization Program (NIP) in June 2000, for all children of pre-school age ( $\leq 5$  years old)
- ✿ The vaccine schedule is three doses (2, 4, 6) months of age, and a booster dose at 18 months
- ✿ The introduction of Hib conjugate vaccine has led to the nearly extinction of Hib disease
- ✿ In turn an increase in non-Hib disease was observed, especially NCHi, in all age groups



# Introduction /MLST



- ✿ *H. influenzae* MLST: unambiguous characterization of both encapsulated and non-encapsulated isolates
- ✿ Initially, the database (<http://haemophilus.mlst.net>) contained the allelic profiles of 131 isolates published by Meats *et.al.*. Further isolates have been added
- ✿ Recently: retirement of Brian Spratt - transfer of MLST databases from Imperial College London to Oxford:K. Jolley and M. Maiden have kindly agreed to host them at PubMLST.org  
<http://pubmlst.org/hinfluenzae>



# Methods

- DNA extraction by boiling procedure (pure culture of *H. influenzae* on PVX chocolate agar)
- Amplification of the 7 housekeeping genes using primers described by Meats *et al*, 2003
- Sequencing with only one primer for each allele (exception for new sequences types): *adk* (R), *atpG* (F), *frdB* (R), *fuck* (F), *mdh* (F), *pgi* (R), *recA* (R)
- *frdB* primers were substitute for the ones described by Schouls *et al*, 2005





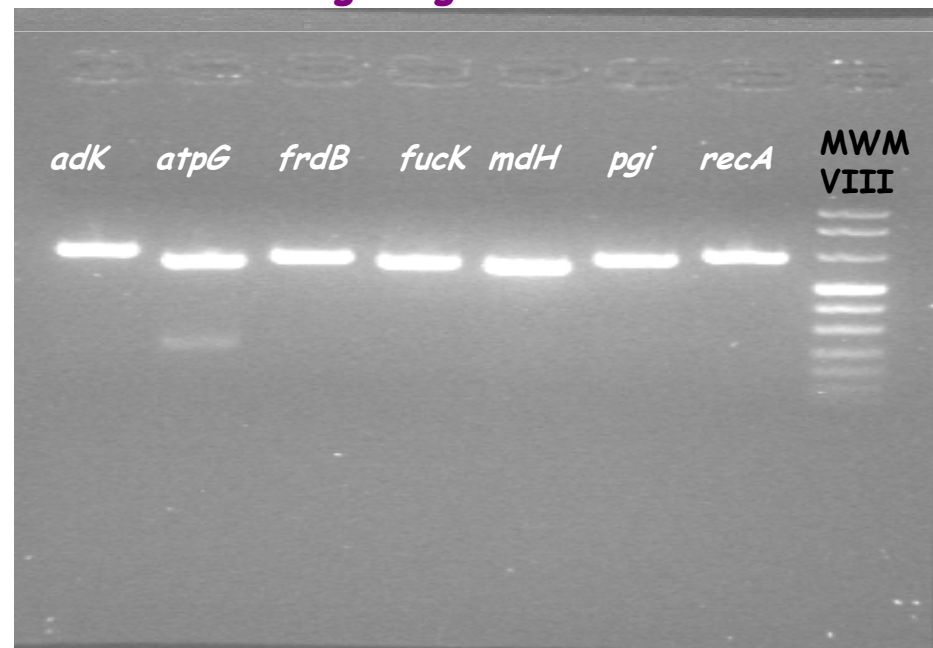


# Methods (Problems)

- ✿ Amplification protocol with HotStar Taq Polimerase (Quiagen): Frequently no amplification of *atpG*
- ✿ Amplification protocol with BIO-X-ACT Short DNA Polymerase (Bioline)

Amplification of the 7 loci  
gel agarose 2%

489bp-345bp





# Analysis

- ✿ The allelic profile of an *H. influenzae* strain was obtained after sequencing internal fragments of 7 house-keeping genes: *adh*, *atpG*, *frdB*, *hcr*, *mdh*, *pgi*, *recA*
- ✿ For each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST)
- ✿ Two or more isolates with the same ST are characterized in the same clonal complex (CC)

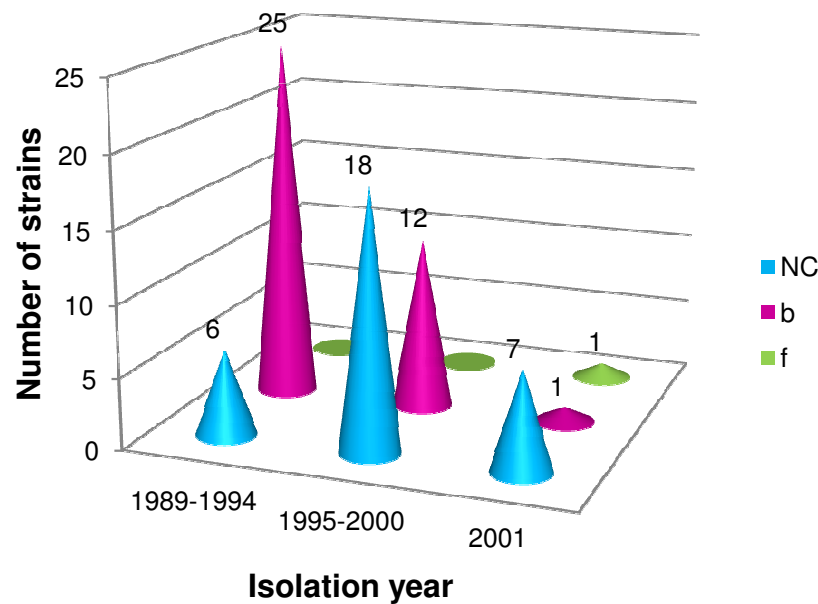




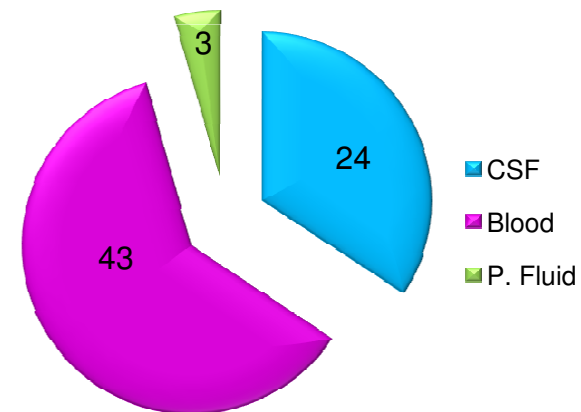
# Strain characterization

70 strains were randomly chosen among invasive isolates  
1989-2001

Serotype vs Isolation year

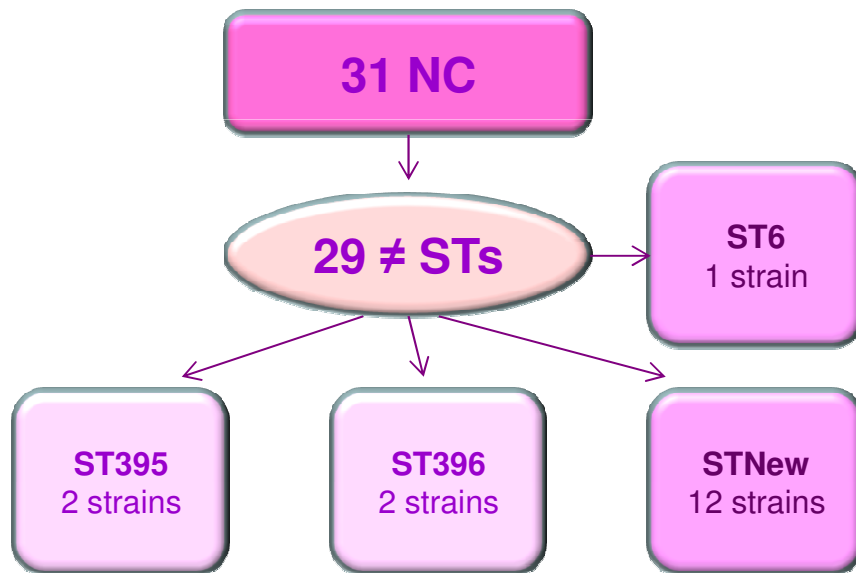


Isolation Product

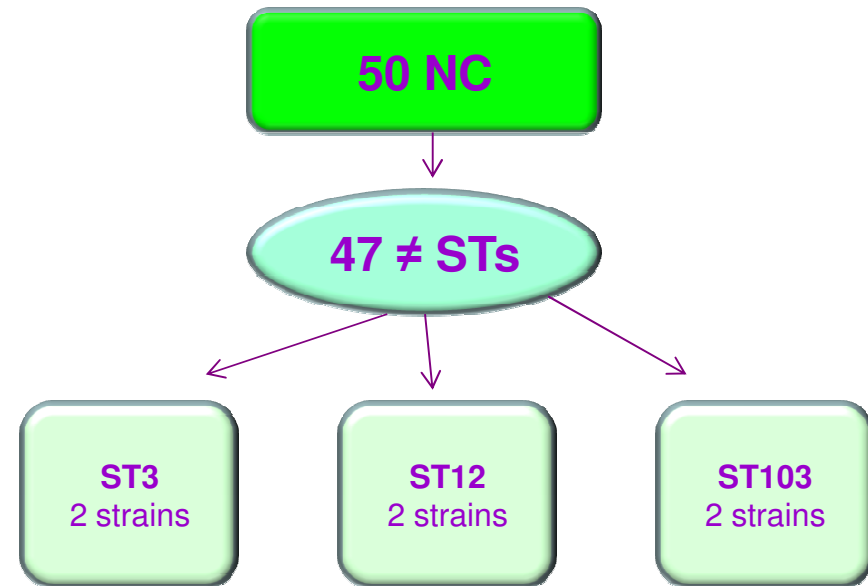


# Results pre-vaccine vs pos-vaccine

1989-2001: 70 isolates

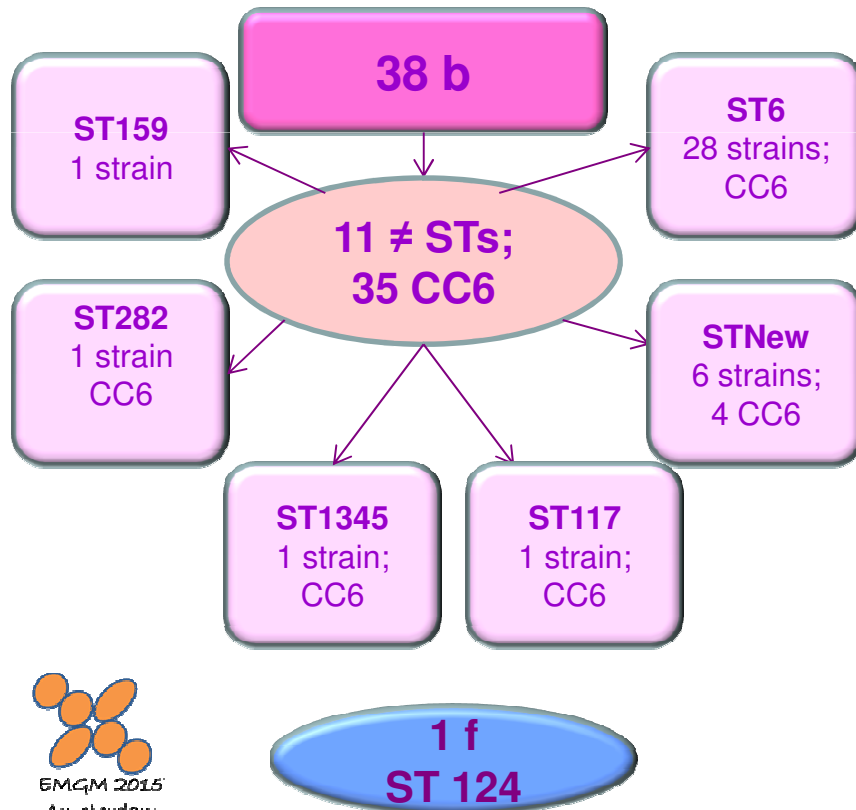


2002-2010: 68 isolates

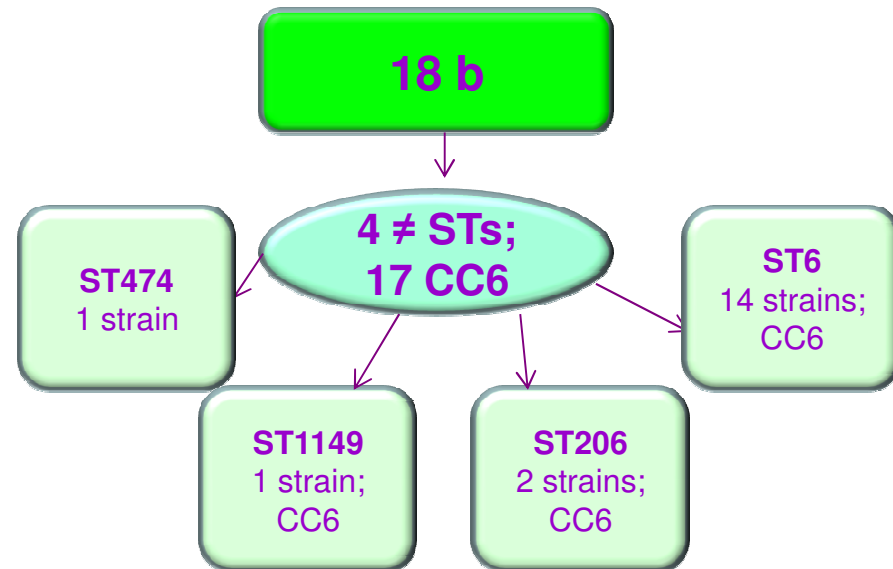


# Results pre-vaccine vs pos-vaccine

1989-2001: 70 isolates

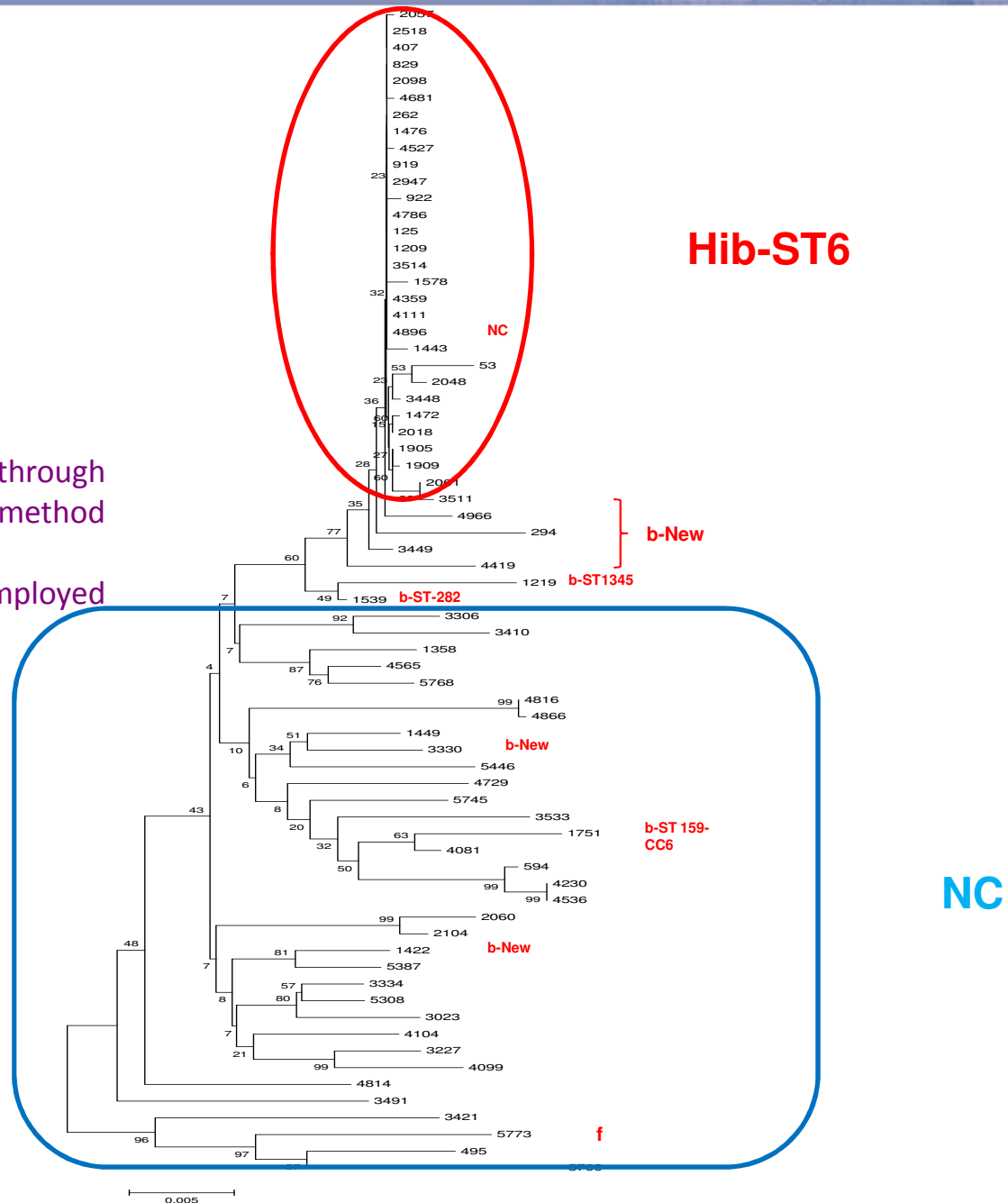


2002-2010: 68 isolates

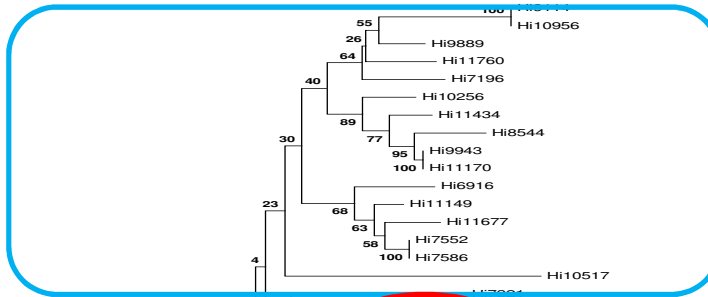


Pre-vaccination: 1989-2001  
(n=70)

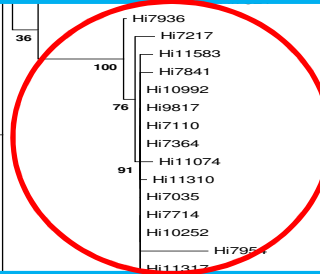
Phylogenetic estimations were conducted through MEGA5 by using the neighbor-joining method with bootstrapping (1000 replicates). The Kimura two-parameter method was employed to estimate the evolutionary distances.



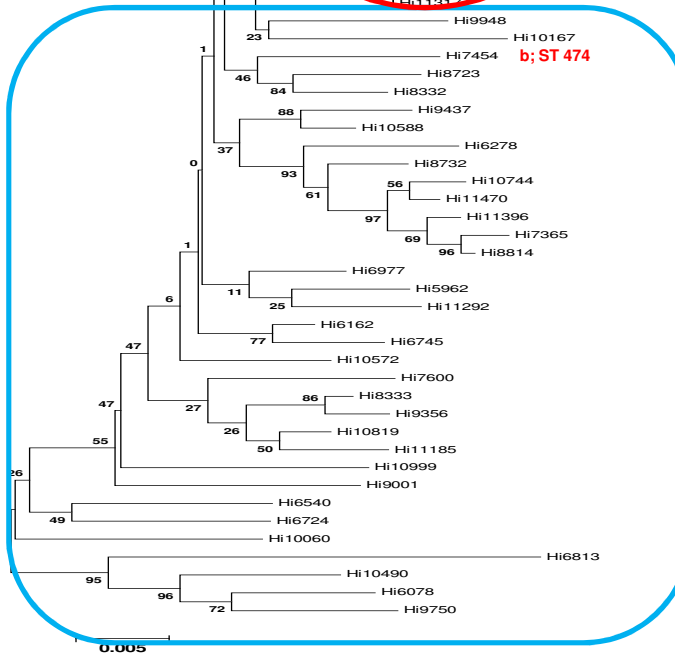
Pos-vaccination: 2002-2010  
(n=68)



NC



Hib-ST6



NC



# Discussion

Our MLST results showed:

- ✿ NCHi: highly genetic diversity in both periods
- ✿ Hib: highly clonal, in both periods

Strains from pre and pos-vaccination periods:

- ✿ Higher percentage of antibiotic resistance strains, including (Bla+) in pre-vaccination period especially due to MR Hib strains
- ✿ Decline in serotype b with concomitant increase of Nc, in pos-vaccination period
- ✿ non-b serotypes (a, d, and f) were characterized in pos-vaccination period







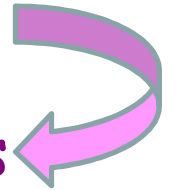
# MLST: advantages and limitations

- ✿ automated technology
- ✿ highly unambiguous
- ✿ portable and reproducible
  
- ✿ MLST combines advances in sequencing and bioinformatics to establish population genetics
- ✿ data can be used to investigate evolutionary relationships among bacteria



# MLST: advantages and limitations

- ✿ MLST is an expensive technique
- ✿ Although it has a good discriminatory power sometimes this is not enough to differentiate isolates, due to the sequence conservation in housekeeping genes
- ✿ Limitation of its use in epidemiological investigations
- ✿ This can be overcome by comparing MLST results with PFGE patterns (technique with a more powerful discriminatory power)





# Conclusions

The majority of episodes of invasive disease occurring in Portugal are now due to

- ✿ fully susceptible
- ✿ highly diverse
- ✿ non-capsulated strains

*Given the evolving dynamics of this pathogen and the increase in non-type b capsulated isolates, continuous surveillance is needed*





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Thanks Very Much for your  
attention

