Prevalence, distribution and transfer of small β-lactamase-containing plasmids in Swedish *Haemophilus influenzae*  

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**Objectives:** The β-lactamase genes of *Haemophilus influenzae* are commonly positioned on large integrative and conjugative elements, but a group of *bla*TEM-carrying small plasmids (4000–6000 bp) with a common structural backbone have recently been characterized. In this study we investigated the epidemiological significance and potential for transfer of this group of small plasmids.

**Methods:** We developed a two-step PCR assay to screen for and type this group of resistance plasmids in *H. influenzae*. A large collection of respiratory isolates (*n* = 2845) from south Sweden, obtained from 2009 to 2011, as well as a collection of invasive Swedish *H. influenzae* from 1997 to 2010 (*n* = 310) was screened. The distribution of plasmid types among clinical isolates was investigated using multilocus sequence typing (MLST).

**Results:** In the collection, 15.8% of β-lactamase-producing isolates and 1.4% of total isolates possessed a small plasmid with the signature structure. The plasmids were genetically conserved and widely spread geographically. MLST revealed that the spread of small plasmids occurred by both clonal expansion and horizontal transfer. *In vitro* experiments suggested that one plasmid type, pN223, can transfer ampicillin resistance to susceptible *Escherichia coli*.

**Conclusions:** Small β-lactamase-encoding plasmids constitute a significant mechanism for β-lactam resistance in *H. influenzae* and can spread through clonal expansion of resistant clones as well as through horizontal plasmid transfer.

**Keywords:** antimicrobial resistance, clinical epidemiology, horizontal gene transfer, phylogenetic analysis

**Introduction**

The Gram-negative coccobacillus *Haemophilus influenzae* is the most common bacterial finding in infectious exacerbations of chronic obstructive pulmonary disease,¹ and a common cause of respiratory tract infections in children.² The first choice for treatment of *H. influenzae* infections is β-lactam antibiotics, predominantly ampicillin. In recent decades, resistance to ampicillin has become a clinical problem. Two main mechanisms of ampicillin resistance exist in *H. influenzae*: alterations of penicillin-binding proteins and production of β-lactamases.³ The dominant β-lactamase genes of *H. influenzae*, *bla*TEM, are most commonly (85%–95% of resistant isolates) found on large integrative and conjugative elements (ICEs).⁴

Since the mid-1970s, it has been known that β-lactam resistance in *H. influenzae* also can be mediated by β-lactamases on small plasmids.⁵ The main group of such small resistance plasmids has been characterized only recently, and consists of seven distinct plasmids (size 4000–6000 bp) with a similar structural backbone that includes the *bla*TEM β-lactamase and a common replicase-encoding gene.⁶,⁷ However, the distribution and clinical significance of such plasmids among *H. influenzae* is not clear.

The objective of this study was to investigate the prevalence, distribution and spread of the recently characterized *bla*TEM plasmids in a large collection of clinical *H. influenzae* (*n* = 3155). Even though the study was limited to the south of Sweden, five of the seven previously described plasmid types were identified. Multilocus sequence typing (MLST) not only revealed the expansion of two clones carrying small plasmids but also indicated horizontal transfer of plasmids.

**Materials and methods**

**Bacterial strains and culture conditions**

All *H. influenzae* used in this study were characterized by standard bacteriological techniques, including X and V tests. Isolates were stored at
Epidemiology of plasmids in *Haemophilus influenzae* JAC

—70°C and cultured on blood agar plates. The first collection consisted of 2845 consecutive nasopharyngeal *H. influenzae* from patients with respiratory tract infections in Skåne, Sweden from 2009 to 2011. All cultures were performed at the instigation of the treating physician. The indications for nasopharyngeal culture in Sweden are pneumonia in adults and otitis media in children, but the clinical diagnosis for each patient was not known. The second collection included 310 invasive isolates from blood or CSF from patients in Sweden between 1997 and 2010. Capsule typing was performed as described previously.8

### Plasmid screening and identification

All isolates were screened for β-lactam resistance using the current EUCAST method and breakpoints. Isolates positive in the β-lactam resistance screening were assessed for β-lactamase production using cefinase discs (bioMérieux, Marcy-l’Etoile, France). Total DNA from isolates positive using MEGA 5.20.10 The resulting phylogenetic tree was presented using [FigTree](http://haemophilus.mlst.net/). Sequences were trimmed and concatenated. The resulting sequences were aligned with the *H. influenzae* small plasmid sequences available in public databases (pA1606: JQ611726; pN223: JQ611727; pLF55: JQ670904; pLFH64: JQ670905; pLFH49: JQ670906; pA1209: JQ783055; pJ612: JQ783056). The analysis was performed with Geneious 6.1.4 created by Biomatters (http://www.geneious.com/).

### MLST

Isolates carrying small plasmids, as well as 60 ampicillin-susceptible controls, were sequence typed according to the *H. influenzae* MLST protocol (http://haemophilus.mlst.net/). Sequences were trimmed and concatenated manually. The concatenated sequences were aligned using ClustalX. A best model nucleotide substitution fit of the alignment was assigned as GTR+I+G. A maximum likelihood tree was constructed using MEGA 5.2.10 The resulting phylogenetic tree was presented using [FigTree](http://haemophilus.mlst.net/) software (Andrew Rambaut, University of Edinburgh, UK).

### Transformation of *Escherichia coli*

Transformation of *E. coli* DH5α was performed by electroporation or chemotransformation as previously described. Briefly, competent cells were mixed with 100 ng of each plasmid, and transformants were recovered on lysogeny broth agar plates supplemented with 50 mg/L ampicillin. The plasmids from resistant transformants were extracted using the Genelute plasmid extraction kit (Sigma–Aldrich, St Louis, MO, USA).

### Results and discussion

Of the 3155 clinical *H. influenzae* collected in south Sweden, 278 (8.8%) isolates (245 respiratory and 33 invasive isolates) were positive for β-lactamase production (Figure 1a) and subsequently analysed using PCR1. The β-lactamase gene was detected in all 278 isolates, while the rep gene was found in only 44. Thus, these 44 isolates carried a plasmid with a structure similar to the *H. influenzae* β-lactamase-carrying small plasmids. Of the isolates, 43 were non-typeable whereas 1 isolate was type f. Taken together, small

![Figure 1](https://example.com/f1.png)
Figure 2. MLST not only unveils the expansion of two clones but also indicates in vivo horizontal transformation of small resistance plasmids. The dendrogram is based on a maximum likelihood analysis of concatenated MLST sequences from 44 isolates in which a small resistance plasmid has been identified, as well as 60 ampicillin-susceptible controls. The plasmid types are indicated by colour in the dendrogram. Even though two distinct clusters are identified in the tree, it is clear that no plasmid type is limited to a single cluster of isolates. This strongly suggests in vivo horizontal transformation. The two clusters ST57 and ST836 are indicated.
in an early study (5%), but well in line with the two more recent studies (14% and 17% respectively). The epidemiological data from all three prior studies, however, was based on a very limited number of isolates. In our study we observed slight year-to-year variation (ranging from 13.1% to 20.5%) in plasmid prevalence (Figure 1c), but no evident trend. While 8.8% were β-lactamase positive and ampicillin resistant (BLPAR), 15.2% were β-lactam resistant and nitrocefin negative. This suggests that in the study period, chromosomal non-β-lactamase-mediated β-lactam resistance was more common than plasmid-encoded β-lactamases.

Using PCR2, the 44 purified plasmids from the clinical isolates yielded single PCR products of variable size (600–1300 bp). Five of the seven previously characterized small plasmids (Figure 1a) were identified, but despite the large number of isolates in our study, no new plasmid type with a similar structural backbone was identified. The pLFH49 and pA1209 plasmids were the most commonly found, in 16 and 13 isolates, respectively, whereas pLF55 and pA1606 were absent from our collection. The results imply that at least five of the seven small replicase-carrying resistance plasmids have spread worldwide.

Small β-lactamase-carrying plasmids in H. influenzae were demonstrated in the 1970s, and subsequently their capacity for in vitro transfer. However, the possibility of in vivo transfer of plasmids between strains remained unclear. The MLST phylogenetic analysis (Figure 2 and Table S2, available as Supplementary data at JAC Online) revealed the expansion of two clusters of plasmid-carrying isolates but also suggested in vivo transformation. One cluster (around ST57) was mainly composed of isolates carrying pLFH49 plasmids. The second cluster (around ST836) was mainly composed of isolates carrying pA1209 plasmids. Neither the ST57 nor the ST836 cluster was limited to one time period or geographical area, and there was no apparent relation between the patients infected with the clustering isolates. The analysis also showed that identified plasmids could be found in genetically distinct strains in the dendrogram, demonstrating that the plasmids can be transferred horizontally in vivo. This observation raises questions about the mechanism of the plasmid transfer.

Early work on small plasmids suggested that plasmid transmission occurs via conjugation, but this requires the formation of a mating channel encoded by a set of genes lacking in blaTEM plasmids. However, pLFH49 and pLFH64 possess genes encoding a mobilization protein (Mob), allowing transmission by conjugation if assisted by the mating channel from another genetic element. The absence of genes for Mob or T4SS in the three other plasmids (pN223, pA1209 and pJ612) renders them unlikely to be transmitted by conjugation. Although natural transformation is an important and well-studied mechanism in DNA exchange between strains of H. influenzae, its role in plasmid transfer was excluded on the observation that DNA exchange was unsuccessful in the presence of DNase. However, it is possible that DNA is sheltered from degradation by outer membrane vesicles (OMVs), even though the role of OMVs in vivo remains unclear.

To study the potential for spread of the identified resistance plasmids, all plasmid types were tested for transformation of a susceptible E. coli. One of the plasmids from the study (pN223), but no other, consistently transferred ampicillin resistance to E. coli. Furthermore, the plasmid could be extracted and identified from the E. coli transformants, suggesting the capacity of pN223 to replicate in an enterobacterium. Plasmid transfer from H. influenzae to other species, and vice versa, has been previously reported, indicating that H. influenzae could act as a bridge between species. Our finding clearly indicates that certain plasmids can transfer resistance elements between the two species, and this allows speculation on this being a possible route for extended-spectrum β-lactamase (ESBL) transfer to H. influenzae. It has been suggested that ESBLs may exist in H. influenzae, but go undetected by standard bacteriological techniques.

In conclusion, H. influenzae possessing small plasmids that carry blaTEM have spread worldwide, and account for 15.8% of the total β-lactamase-producing clinical H. influenzae in south Sweden. The small plasmids can transfer in vivo between H. influenzae strains, and one plasmid type can be transferred in vitro to E. coli. In order to prevent the process of small plasmid exchange and limit the spread of small plasmid-associated ampicillin resistance, the exact mechanism of transfer needs to be further studied.

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Supplementary data
Figure S1, Table S1 and Table S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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