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Characterisation of invasive *Haemophilus influenzae* isolates in Slovenia, 1993–2008

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Abstract The objectives of our study were to describe the epidemiology of invasive *Haemophilus influenzae* disease from 1993 to 2008 in Slovenia, a country with routine *H. influenzae* serotype b (Hib) conjugate vaccination since the year 2000. A total of 292 isolates of *H. influenzae*, recovered from a normally sterile site, were collected in the study period. The isolates were serotyped by slide agglutination and antibiotic susceptibility was determined. One hundred and eight isolates received after the year 2000 were serotyped by slide agglutination and by polymerase chain

reaction (PCR) capsule typing, and both methods were compared. After the introduction of the routine Hib vaccination, the incidence of *H. influenzae* disease in children under the age of 5 years has decreased by 87.6% and type b was replaced by non-typeable *H. influenzae* as the predominant serotype. The proportion of serotype b decreased from 85.3% in the pre-vaccination period to 13.0% in the vaccination period and the proportion of non-capsulated isolates increased from 12.0 to 85.2%. The study of genetic relatedness by pulsed-field gel electrophoresis (PFGE) demonstrated that the isolates of serotypes b and f were genetically homogeneous within the serotype. The results of our national surveillance showed that the vaccine has been very efficient in preventing Hib invasive disease in Slovenia. Nevertheless, we see a need for further monitoring of invasive *H. influenzae* infections at a national level.

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Introduction

Haemophilus influenzae is an important respiratory pathogen in both children and adults and, until the implementation of vaccination, in many countries, it was also one of the three leading causes of bacterial meningitis in young children. *H. influenzae* serotype b (Hib) was an important cause of paediatric morbidity and mortality. Hib conjugate vaccine has produced a dramatic decline in the incidence of invasive Hib disease over a very short period of time [1–4]. As a consequence, non-capsulated isolates are predominant in invasive *H. influenzae* disease in countries with Hib vaccination programmes [5, 6]. In countries without Hib vaccination, *H. influenzae* remains a major cause of meningitis in very young children and increasing rates of antibiotic resistance have also been reported [7–9].

In 1993, the national surveillance project, which followed the occurrence of invasive diseases caused by *Neisseria meningitidis*, *H. influenzae* and *Streptococcus pneumoniae* in Slovenia was initiated. After the introduction of regular Hib vaccination at the end of 1999, the main cause of vaccine-preventable bacterial invasive diseases in Slovenia became *S. pneumoniae* and *N. meningitidis* [10]. Nevertheless, the laboratory surveillance of invasive *H. influenzae* is needed in order to monitor the effectiveness of immunisation programmes and vaccines. The objective of this study was to characterise the epidemiology of invasive *H. influenzae* in Slovenia before and after Hib conjugate vaccine was introduced. In this study, we also evaluated two methods to identify the serotype, slide agglutination and molecular capsule typing, since accurate results are very important where *H. influenzae* has become a very rare cause of invasive disease.

Materials and methods

Bacterial isolates

All invasive isolates of *H. influenzae*, recovered from normally sterile sites, isolated in all Slovenian microbiological laboratories were collected at the National Institute of Public Health (NIPH) as a part of national surveillance system. Since 1993, all invasive isolates of *H. influenzae* isolated from children (0–14 years of age) have been collected and since 1996, the isolates from adult patients have also been collected.

From 1993 to 1999, in the so-called pre-vaccination period, 184 isolates were collected; of these, 160 were from children (0–14 years of age) and 24 were from adult patients (≥ 15 years of age). From 2000 to 2008, 108 isolates were collected; of these, 31 were from children and 77 were from adult patients. Altogether, 292 isolates were included in the study. The isolates were derived from 135 (46.2%) female and 157 (53.8%) male patients. Altogether, the isolates were derived from the following clinical specimens: blood only (155 isolates), cerebrospinal fluid only (51), both blood and cerebrospinal fluid (73), pleural aspirates (8) and puncture joint exudates (5). Isolates were regrown on chocolate agar (Merck KGaA, Darmstadt, Germany) and on Levinthal agar (Merck KGaA, Darmstadt, Germany) and incubated at 35°C for 24 h in 5% CO₂. Isolates were routinely serotyped by slide agglutination and stored at –70°C.

Serotyping

Isolates were serotyped by slide agglutination using the Phadebact Haemophilus Test (Bactus AB, Huddinge,

Sweden) and specific *H. influenzae* capsular antisera (BD, Maryland, USA). From the year 2000 onwards, all results were reviewed and molecular capsule typing by polymerase chain reaction (PCR) was done. Both methods were compared and Cohen's Kappa test was used for the statistical evaluation.

Bacterial DNA isolation and PCR

Isolation of bacterial DNA for PCR was done using the MagNA Pure Instrument and the MagNA Pure LC DNA Isolation Kit III (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Each isolate was tested for the presence of the *ompP2*, *bexA* and *capB* genes by PCR. Capsulated isolates (isolates positive for *bexA* and negative for *capB*) were further tested for other *cap*-specific genes. Strains that lacked both the type b-specific and *bexA* gene regions were designated as non-typeable (NT). Strains that carried the type b-specific gene region and lacked *bexA* were designated as Hib⁻. Sequence-specific primers were previously described; for *cap* genes by Falla et al., *ompP2* primers by Hobson et al. and *bexA* primers by van Ketel et al. [11–13]. Amplification reactions were performed in a total reaction volume of 50 μ l with 2 μ l of template, 0.2 mM dNTP, 0.4 μ M each primer and 0.01 U/ μ l Top Taq DNA polymerase (Qiagen, Hilden, Germany). PCR conditions were 3 min at 94°C, 30 s at 94°C, 30 s at 52°C, 1 min at 72°C, for 35 cycles, 10 min at 72°C and 4°C hold. PCR products were analysed on 1% agarose gel containing 0.1 μ g/ml ethidium bromide. The following strains were used as positive controls; ATCC 9006 (capsule type a), ATCC 10211 (capsule type b), ATCC 9007 (capsule type c), ATCC 9008 (capsule type d), ATCC 8142 (capsule type e) and ATCC 9833 (capsule type f).

Susceptibility testing

Susceptibility testing was performed for 108 isolates from 2000 to 2008. Susceptibilities to ampicillin, ampicillin–sulbactam, azithromycin, tetracycline, trimethoprim/sulfamethoxazole, cholamphenicol, ciprofloxacin, cefaclor and cefuroxime were tested by the disc diffusion method following the recommendations of the Clinical Laboratory Standards Institute (CLSI) [14]. The minimum inhibitory concentrations (MICs) for ampicillin, amoxicillin–clavulanic acid, cefotaxime and ceftriaxone were determined with the Etest (AB Biodisk, Solna, Sweden). Both methods were performed on Haemophilus Test Medium (HTM) agar (Oxoid, Hampshire, United Kingdom) under incubation at 35°C in 5% CO₂ for 16–18 h (disc diffusion) or 20–24 h (Etest). Standard quality control strains, *H. influenzae* ATCC 49247, ATCC 49766 and ATCC 10211, were included in each run. Beta-lactamase production was

detected using a Cefinase™ disc (BBL, Becton Dickinson, MD, USA).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of the selected isolates was performed using the commercial kit Genepath Group 1 (Bio-Rad, Hercules, USA), which included digestion with restriction enzyme *SmaI*. PFGE was run in 0.5× TBE buffer on 1% agarose (Bio-Rad, Hercules, USA) in the Gene Path@System, programme 12 (18.5 h, 14°C at 6 V/cm with a ramped pulse time of 1–17 s). After electrophoresis, the gels were stained for 30 min with 0.5 mg of ethidium bromide per litre. *Staphylococcus aureus* NCTC 8352 strain was used for a quality control. DNA bands were visualised on the Gel Doc XR system with the Quantity One Software (Bio-Rad, Hercules, USA). The software Fingerprinting II (Bio-Rad, Hercules, USA) was used to generate unweighted pair group method with arithmetic mean (UPMGA) dendrograms of fragment patterns with the dice coefficient (optimisation and position tolerance settings of 1.0%). PFGE-based clusters were defined as isolates with ≥80% genetic relatedness.

Results

Incidence and serotype distribution

In Slovenia, compulsory vaccination for Hib was introduced at the end of 1999. In the period 1993 to 1999,

invasive *H. influenzae* was as frequent as invasive disease caused by *S. pneumoniae* in children under the age of 15 years [10]. After the year 2000, the incidence of invasive *H. influenzae* has rapidly fallen to, on average, 1 per 100,000 children (0–14 years of age) per year and no case of invasive *H. influenzae* type b disease has been reported in children under the age of 15 years since 2002 (Table 1).

After the introduction of the routine Hib vaccination, we observed a dramatic decline of invasive *H. influenzae* disease in children under the age of 5 years (Fig. 1). In the pre-vaccination period (1993–1999), the average incidence of *H. influenzae* invasive disease per year in the age group 0–1 was 28.3/100,000, and for children 2–4 years of age, it was 20.9/100,000 (together 24.6/100,000). In the period after the vaccine introduction, the average incidence of *H. influenzae* invasive disease per year for children 0–1 year of age was 4.2/100,000 and for children 2–4 years of age, it was 1.9/100,000 (together 3.05/100,000). The introduction of the routine vaccination made significant changes in the epidemiology of invasive *H. influenzae*, as we observed an 8-fold decrease in the incidence of *H. influenzae* invasive disease in the children population under the age of 5 years (from 24.6/100,000 to 3.05/100,000 or a 87.6% decrease).

Besides the decline of invasive *H. influenzae* disease in children, we also observed the change in serotype distribution. Before the vaccine introduction, the majority of all 184 cases were type b (157; 85.3%), followed by non-capsulated or NT isolates (22; 12.0%) and serotype f (4; 2.2%). After the vaccine introduction, most of the 108 cases were caused by NT (92; 85.2%), followed by serotype b (14; 13.0%) and serotype f (2; 1.9%). In the case of isolates

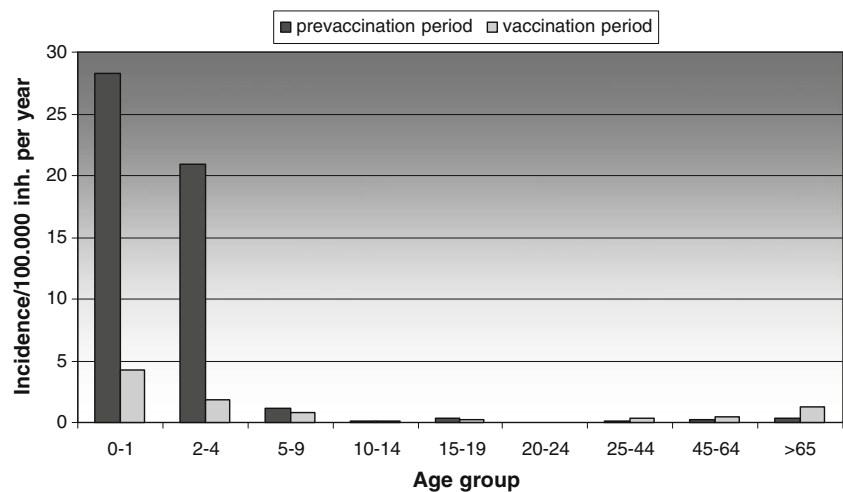
Table 1 Annual incidence of invasive disease caused by *Haemophilus influenzae* (type b, other capsulated and non-capsulated) in children (0–14 years of age) in Slovenia, 1993–2008

Year	Incidence of invasive <i>H. influenzae</i> /100,000 children (0–14years of age)			
	Hib	Other capsulated	Non-capsulated	Total
1993	3.7	0.3	0.0	4.0
1994	7.6	0.0	0.3	7.9
1995	3.1	0.0	0.0	3.1
1996	5.3	0.0	0.9	6.2
1997	9.4	0.0	0.9	10.3
1998	7.0	0.3	0.3	7.6
1999*	6.8	0.0	0.0	6.8
2000*	1.9	0.0	0.6	2.5
2001	0.4	0.0	1.2	1.6
2002	0.0	0.0	0.3	0.3
2003	0.0	0.0	0.9	0.9
2004	0.0	0.0	0.6	0.6
2005	0.0	0.0	0.6	0.6
2006	0.0	0.0	0.7	0.7
2007	0.0	0.0	1.4	1.4
2008	0.0	0.4	1.4	1.8

*Compulsory Hib vaccination was initiated at the end of 1999 and in 2000

Hib = *H. influenzae* serotype b

Fig. 1 Incidence of invasive *Haemophilus influenzae* disease per year according to age groups in the pre-vaccination period (1993–1999) and in the vaccination period (2000–2008) in Slovenia



from children 0 to 5 years of age in the period 1993 to 1999, the majority of 151 cases were type b (142; 94.0%), followed by NT (7; 4.6%) and serotype f (2; 2.2%). In the vaccine period in the population of children 0 to 5 years of age, there were only 23 cases of invasive *H. influenzae* disease; most of them were NT (16; 70%), followed by type b (6; 26%) and serotype f (1; 4%) (Fig. 2). In the period 2000–2001, three children with Hib meningitis were previously vaccinated. Two of them were vaccinated with only one dose. The onset of diseases was 1 to 3 months after Hib vaccination. One child with cancer who received four doses of vaccine developed Hib meningitis five months later.

Analysis of isolates by serotyping and PCR typing

One hundred and eight isolates received in the period from 2000 to 2008 were serotyped by slide agglutination and by PCR capsule typing. By slide agglutination, there were two isolates type a, 16 isolates type b, two isolates type c, five

isolates type d, three isolates type f and 80 isolates were NTHi (non-typeable *H. influenzae*). All 80 isolates originally serotyped as NT were confirmed by PCR capsule typing. Twelve of twenty-eight isolates reported as encapsulated by slide agglutination (two Hia, two Hic, five Hid, one Hif and two Hib) were shown to be NT by PCR. None of the 108 tested isolates were Hib⁻ (Table 2).

The compared methods showed good agreement (Cohen's Kappa test 0.745). The rate of discordance was 11.1% (12/108). Nevertheless, there was some important disagreement, especially the misclassification of two NT isolates as serotype b. Capsule type d isolates were found to have the highest discrepancy between the two tested methods, since all five isolates were misclassified. Moreover, the PCR capsule typing has the advantage of detecting the capsule-deficient Hib⁻, but in our case, there was no such an example. Therefore, we believe that PCR capsule typing should be used as a gold standard, since slide agglutination results can be misinterpreted in the case

Fig. 2 Serotype distribution in invasive *H. influenzae* isolates from children aged less than 5 years in the period 1993 to 2008 in Slovenia. *Vaccine introduction

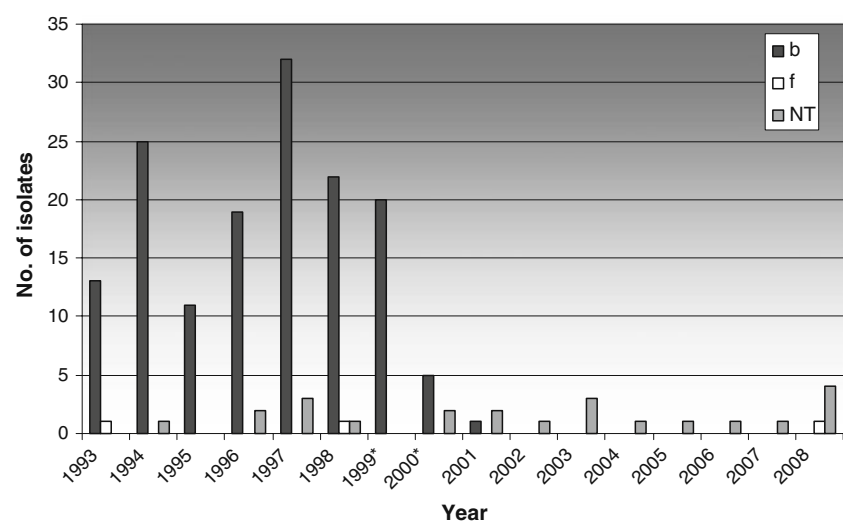


Table 2 Comparison of typing of 108 invasive *H. influenzae* isolates by slide agglutination and by PCR capsule typing in the period 2000 to 2008 in Slovenia

Serotype by SA	PCR capsule type								Total	
	a	b	c	d	e	f	NT	b ⁻		
a							2		2	
b		14					2		16	
c							2		2	
d							5		5	
e							0		0	
f						2	1		3	
NT								80	80	
Total	0	14	0	0	0	2		92	0	108

SA = slide agglutination; NT = non-typeable

of non-specific agglutination, cross-reactions or auto-agglutination.

Antimicrobial resistance

A total of 108 invasive isolates of *H. influenzae*, collected in the period 2000–2008 in Slovenia, were investigated. Out of these, 14 isolates (13%) were non-susceptible to ampicillin. Production of beta-lactamase was the principal mechanism of ampicillin resistance, occurring in 10.2% of isolates (11/108) (MIC \geq 256 mg/l). Beta-lactamase production was tested also in the period 1993 to 1999, and it occurred in 13.6% of isolates. One isolate was defined as beta-lactamase-negative ampicillin-resistant (BLNAR); it was also resistant to amoxicillin–clavulanic acid, cefuroxime and cefaclor. Twelve percent (13/108) of isolates were intermediate to tetracycline, 2.8% (3/108) were intermediate or resistant to cefuroxime, 9.0% (10/108) were resistant to trimethoprim/sulfamethoxazole and 6.5% (7/108) were intermediate or resistant to cefaclor. All isolates were susceptible to cefotaxime, ceftriaxone, azithromycin, cholamphenicol and ciprofloxacin.

PFGE analysis

In this study, all of the encapsulated isolates within a particular serotype showed high genetic homogeneity. Among all 14 Hib isolates from the post-vaccine era, nine isolates belonged to one clonal cluster and three isolates to the second clonal cluster (Fig. 3). The six isolates with serotype f (four from the pre-vaccine period, two isolated since the year 2000) were clonally highly related. Among 11 isolates carrying the beta-lactamase, we observed no clustering, only three NT isolates were clonally related. Among the tested 23 NTHi isolates, genetic diversity was observed (results not shown).

Discussion

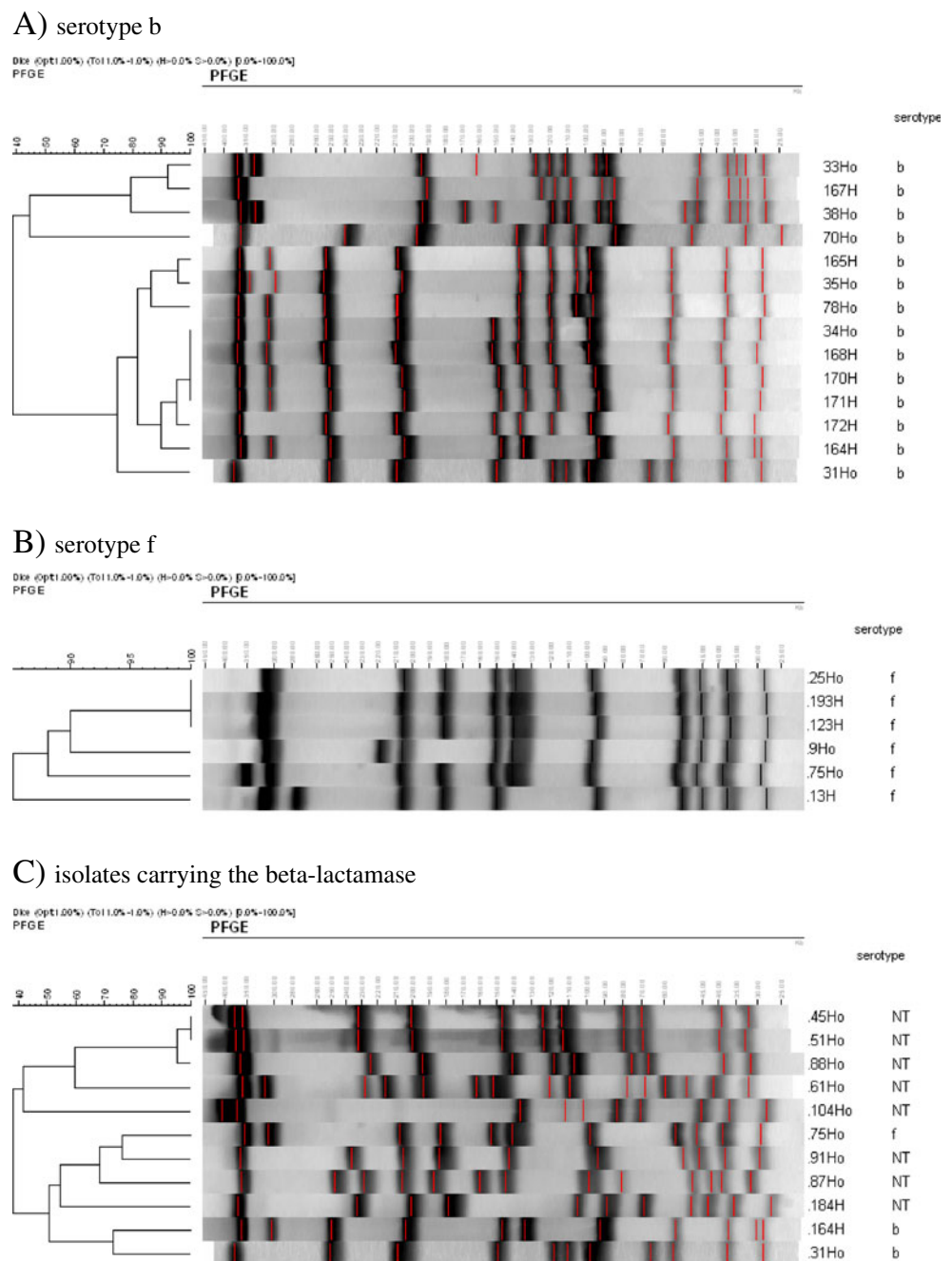
Surveillance of invasive *H. influenzae* disease in the Hib vaccine era is important for monitoring the effectiveness of Hib conjugate vaccines, as well as for monitoring the epidemiology of invasive *H. influenzae*. Prior to the introduction of Hib conjugate vaccine, this pathogen was an important cause of serious paediatric invasive disease. Because of the introduction of Hib conjugate vaccine, disease due to invasive *H. influenzae* has become rare. This vaccine also prevents nasopharyngeal colonisation, which partly explains such effectiveness [2].

In some countries, the re-emergence of *H. influenzae* type b in vaccinated children has been reported [2, 15–18]. Recent studies suggest that NT strains are now responsible for most of the *H. influenzae* disease [1, 5, 15, 19]. Concern about a possible rise in the incidence of invasive infection with non-serotype b capsulate isolates after the widespread Hib vaccination exists. Indeed, reports regarding an expansion of *H. influenzae* type f clone were published [20, 21]. The study by Cerquetti et al. documented the emergence of invasive *H. influenzae* type e disease [22]. Therefore, the need for surveillance to monitor developments concerning replacement as vaccination becomes prevalent worldwide and accurate serotyping to enable reliable results is of great importance [5, 20].

In Slovenia, a country with a population of approximately 2 million (~280,000 children under 15 years of age), the epidemiologic situation of invasive *H. influenzae* diseases has been constantly monitored since 1993 [10]. According to the law on communicable diseases, some vaccine-preventable diseases are compulsory notified directly to the NIPH. All medical laboratories are sending isolates to the reference laboratory at the NIPH for serotyping and resistance determination. Via this project, all invasive strains of *H. influenzae* were intentionally collected and the evaluated results reflect the national level.

We demonstrated that the introduction of the Hib vaccine in Slovenia at the end of 1999 led to changes in invasive *H. influenzae* epidemiology. The incidence of invasive *H. influenzae* disease in children aged less than 5 years decreased 8-fold and reached an average of 3 per 100,000 per year in the vaccination era. We also showed the change in the serotype distribution of invasive *H. influenzae*: the proportion of serotype b decreased from 85.3% in the pre-vaccination era to 13.0% in the vaccination era and the increase of proportion of NT isolates from 12.0 to 85.2% was noted. The results coincide with data published for Portugal [5]. In both periods, we identified only a few isolates of serotype f. We detected only one case of vaccine failure receiving four doses and, moreover, no case of Hib has been observed in the children aged less than 5 years since the year 2001. We also observed no case of capsule-

Fig. 3a–c Dendrograms based on pulsed-field gel electrophoresis (PFGE) *Sma*I restriction pattern analysis of invasive *H. influenzae* isolates using the unweighted pair group method with arithmetic mean (UPMGA). The dice band-based similarity coefficient, with a band position tolerance of 1% and optimisation of 1%, was used for clustering. **a** Serotype b. Dendrograms based on PFGE of 14 *H. influenzae* serotype b invasive isolates. **b** Serotype f. Dendrograms based on PFGE of six *H. influenzae* serotype f invasive isolates. **c** Isolates carrying the beta-lactamase. Dendrograms based on PFGE of 11 *H. influenzae* beta-lactamase-positive isolates



deficient Hib⁻. As a result of the decline in Hib invasive disease caused by the introduction of vaccination, NTHi has become the most important cause of *H. influenzae* invasive disease in Slovenia.

In surveillance, it is very important to carry out reliable serotyping, especially to discriminate between Hib and other serotypes or NTHi. Because Hib has become a rare cause of invasive disease, the need to identify the serotype correctly has increased. Therefore, we compared the standard slide agglutination serotyping with molecular capsule typing by PCR. Several studies have favoured PCR capsule typing [23–26]. In our study, the rate of

discordance was 11.1% (12/108). The most common error was the misidentification of NTHi as capsulated (in most cases as type d) by slide agglutination. Considering the facts that serotyping by slide agglutination is less accurate, it is unable to detect Hib⁻ and invasive *H. influenzae* infections have become rare—we recommend the PCR capsule typing for confirming the serotype result. Because of the low numbers of invasive *H. influenzae* isolates, mistyping might have a big influence on the surveillance results.

Antibiotic resistance in *H. influenzae* was first detected in 1972 with an ampicillin-resistant isolate and, since then,

the resistance to several other antibiotics has developed. Therefore, it is also important to monitor the antibiotic susceptibility in invasive *H. influenzae*. Our results show that resistance to ampicillin was the most frequently detected resistance. The main mechanism of resistance was beta-lactamase production (11 out of 14 ampicillin-resistant isolates), as was the case in other studies [27, 28]. In Slovenia, BLNAR isolates of invasive *H. influenzae* were very rare. Indeed, we detected only one isolate in the period 2000 to 2008. These data coincide with multi-centre European studies, where considerable variation from different countries, from 1.5 to 33.9%, was observed [29]. There is also some evidence that the prevalence of ampicillin resistance of *H. influenzae* in Europe may be decreasing due to a reduction in the number of beta-lactamase-positive isolates, whereas the prevalence of BLNAR strains is relatively stable [29, 30]. In Spain, however, an increase of BLNAR strains has been reported [31]. In Japan and South Korea, they are confronted with high rates of BLNAR strains [7, 28, 32]. In Slovenia, we did not observe any increase in ampicillin resistance and, according to the PFGE results, ampicillin-resistant isolates were not clonally related.

The analysis of genetic relatedness of invasive *H. influenzae* from Slovenia showed that isolates of serotype b were mostly clonally related. This observation coincides with other studies [33–35]. The *H. influenzae* type f isolates also showed very high degree of homogeneity, which is in concordance with previously published data [20]. On the other hand, most of the NT isolates did not cluster and the high levels of diversity indicated different clonal origins. A clone of serotype f was genetically unrelated to Hib or NT isolates, as expected [5, 36].

In summary, data from our surveillance strongly indicate that, after the introduction of routine vaccination, the incidence of *H. influenzae* invasive disease and its epidemiological pattern have changed remarkably. Therefore, we see a need for further monitoring of invasive *H. influenzae* infections at the national level.

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