Comparison of usefulness of randomly amplified polymorphic DNA and amplified-fragment length polymorphism techniques in epidemiological studies on nasopharyngeal carriage of non-typable *Haemophilus influenzae*

Ewa Augustynowicz,¹ Anna Gzyl,¹ Leszek Szenborn,² Dorota Banys,² Grzegorz Gniadek¹ and Janusz Ślusarczyk¹

Correspondence

Ewa Augustynowicz
eaugustynowicz@pzh.gov.pl

¹Department of Sera and Vaccine Evaluation, National Institute of Hygiene, Chocimska 24 St, 00-791 Warsaw, Poland

²Department of Infectious Diseases of Children, Medical University, Wrocław, Poland

Randomly amplified polymorphic DNA (RAPD) and automated amplified-fragment length polymorphism (AFLP) techniques with fluorescently labelled primers were used to type non-serotypable *Haemophilus influenzae* (NTHI) isolates. Eighty-seven isolates from healthy children attending day-care centres or living at orphanages in southern Poland were investigated. Through comparison of the AFLP data with RAPD analysis, it has been concluded that the discriminatory power of AFLP for NTHI typing is higher than RAPD. Generally, the NTHI isolates analysed were highly heterogeneous, as detected with a *HindIII*/*TaqI* AFLP genotyping scheme on intra/inter similarity levels of 94 and 96 % using Pearson's correlation coefficient. The range of similarity values found for isolates from children permanently residing at a particular day-care centre was much wider than that for isolates from orphanages. AFLP can efficiently access NTHI strain diversity and can monitor their turn-over for comparative typing in local and inter-local epidemiological investigations.

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INTRODUCTION

Non-serotypable *Haemophilus influenzae* (NTHI) strains commonly colonize the upper respiratory tracts of children under 4–5 years of age and are implicated in otitis media, sinusitis, bronchitis and conjunctivitis manifestations (Cerquetti *et al.*, 2000; Peltola, 2000; Urwin *et al.*, 1996). The carriage of NTHI and other capsular types (a and c–f) strains may increase in frequency and its relative clinical importance might grow as a consequence of the reduction of *H. influenzae* type b (Hib) carriage and its infections as a result of vaccination against Hib (Hargreaves *et al.*, 1996; Leaves & Jordens, 1996; Omikunle *et al.*, 2002; Urwin *et al.*, 1996). Additionally, a decrease in Hib carriage has been suspected to increase infections with other species such as *Streptococcus pneumoniae* or *Haemophilus parainfluenzae* (Peltola, 2000). In Finland, implementation of Hib vaccination has been positively correlated with an increase in paediatric pneumococcal disease (Leaves & Jordens, 1996). Thus, selective vaccination pressure is capable of inducing

changes in serotype distribution in a given population and, moreover, NTHI might replace Hib strains as the predominant cause of *H. influenzae* disease. Monitoring of *H. influenzae* carriage is a potent method of evaluating the importance of serotypes after vaccine implementation (Pettigrew *et al.*, 2002; Smith-Vaughan *et al.*, 1998).

Encapsulated *H. influenzae* strains are known to be successfully subclassified by serotyping, biochemical testing, outer-membrane protein typing, lipopolysaccharide analysis, multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (Fuste *et al.*, 1996; Gazagne *et al.*, 1998; Meats *et al.*, 2003; Moor *et al.*, 1999; Spinola *et al.*, 1986; St Geme *et al.*, 1994; van Belkum *et al.*, 1994). All published data recognize encapsulated *H. influenzae* population structure as clonal (Moor *et al.*, 1999; Musser *et al.*, 1988; Omikunle *et al.*, 2002). Furthermore, most invasive Hib disease worldwide has been found to be caused by a limited number of bacterial clones (Saito *et al.*, 1999; Smith-Vaughan *et al.*, 1998). Compared with encapsulated strains, non-encapsulated *H. influenzae* are less well characterized and are thought to be more diverse (Fuste *et al.*, 1996). Most studies directed towards recognizing links between closely related but not identical NTHI strains have been conducted by application

Abbreviations: AFLP, amplified-fragment length polymorphism; DCC, day-care centre; Hib, *H. influenzae* type b; NTHI, non-typable *H. influenzae*; RAPD, randomly amplified polymorphic DNA.

of MLEE or randomly amplified polymorphic DNA (RAPD) techniques (Fuste *et al.*, 1996; Jordens *et al.*, 1993; van Alphen *et al.*, 1997).

This study presents the first report on amplified-fragment length polymorphism (AFLP) differentiation performed on a representative number of NTHI isolates, coupled with estimation of their genetic relationship. The presence of the *cap* locus among pharyngeal isolates of NTHI was also examined. Some aspects of the effect of bacterial persistence in hosts who differ in levels of close contacts and on the genetic diversity of the *H. influenzae* population are discussed.

METHODS

Description of the group. In total, 87 *H. influenzae* isolates obtained from healthy children who attended day-care centres (DCC) or orphanages and their teachers. DCCs refer to childcare institutions in which children are resident during the time that their parents are at work, generally between 7 a.m. and 4 p.m. Orphanages refer to special care institutions in which children waiting for adoption or without parental supervision reside permanently. From January to May 2002, public health nurses from six DCCs and three orphanages in different regions of the western part of Poland collected nasopharyngeal swabs from healthy 3- to 5-year-old children. In collecting samples, nurses swept a cotton-tipped applicator over the nasopharynx and then immediately placed the swab in transport medium (Medlab). The material was processed on the same day in a microbiological laboratory. All individuals tested were not vaccinated against Hib and none of the children was acutely ill. The Ethical Committee of the Medical Academy in Wroclaw approved the research protocol.

Bacterial isolates and culture media. All *H. influenzae* isolates were grown on chocolate agar plates at 37 °C in 5% CO₂ or in brain heart infusion broth supplemented with NAD (2000 µg l⁻¹; Sigma) and haemin (2000 µg l⁻¹; Sigma). Isolates were identified as *H. influenzae* on the basis of Gram reaction, cell morphology, lack of β-haemolysis and NAD and haemin requirements. Isolates were assigned to biotypes on the basis of standard tests for indole, urease and ornithine decarboxylase production. The lack of agglutination with antiserum (Difco) determined the non-typable character of the isolates. Isolates were kept as frozen stocks at -70 °C in broth containing 15% glycerol, usually after fewer than five passages from the primary culture plate.

In the study, *H. influenzae* ATCC 49766, *H. influenzae* type b ATCC 10211 and 24 Hib strains (isolated from cerebrospinal fluid from children with meningitis) were used as controls.

Isolation of total DNA. Bacteria were grown on brain heart infusion broth supplemented with NAD and haemin to an OD₆₅₀ of the culture suspension of 0.25.

DNA for AFLP analysis was prepared according to the method of Bolduc *et al.* (2000). Two millilitres of overnight culture of *H. influenzae* was centrifuged at 5000 g for 10 min at 4 °C, resuspended in 3 ml 50 mM Tris/HCl, 20 mM EDTA (pH 8.0) and recentrifuged. The cell pellet was resuspended in 4 ml 50 mM Tris/HCl, 2 mM EDTA (pH 8.0) containing 1 mg lysozyme and incubated for 30 min at 4 °C. Addition of 1 mg proteinase K and 0.3 ml 10% SDS was followed by incubation of the lysate with agitation for 4 h at 56 °C. Next, 1 ml 10% lauroylsarcosine was added. Chromosomal DNA was purified with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with absolute ethanol overnight at -20 °C. DNA was harvested by centrifugation at 12 000 g

for 20 min, washed with 70% ethanol and resuspended in 100 µl TE buffer with DNase-free RNase (0.25 mg ml⁻¹) for 1.5 h at 37 °C. DNA for RAPD typing was extracted by use of the QIAamp Mini kit (Qiagen).

The concentration of the DNA was measured by electrophoresis of samples on 1% agarose gel with a diluted preparation of λ phage DNA of known concentration.

Confirmation of serotype and detection of capsule genes by PCR.

All NTHI and Hib isolates were subjected to PCR as described by Falla *et al.* (1994), using primers directed to the *bexA* region and the type b-specific region. Primers H1 (5'-CGTTTGTATGATGTTGATCCA GAC-3'), H2 (5'-TGTCATGCTCTTCAAAATGATG-3'), b1 (5'-GCGAAAGTGAACCTTATCTCTC-3') and b2 (5'-GCTTACGCT TCTATCTCGGTGAA-3') were used. A product of 343 bp obtained from amplification with primers H1 and H2 confirmed capsulation, and generation of a 480-bp product with primers b1 and b2 indicated specific capsulation.

Typing by the RAPD method. RAPD differentiation was performed by using Ready-to-Go RAPD analysis beads (Amersham Pharmacia Biotech). Primers I (5'-GGTGCGGGAA-3'), II (5'-GTTTCGCTCC-3'), III (5'-GTAGACCCGT-3'), IV (5'-AAGAGCCCGT-3'), V (5'-AACGCGCAAC-3') and VI (5'-CCCCTCAGCA-3') were used. Forty-five cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 1 min were applied in a Biometra thermal cycler. Aliquots of amplified PCR products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide. Electrophoresis was carried out in TAE buffer (40 mM Tris/acetate, 1 mM EDTA). Gels were photographed with the Gel Doc 1000 Gel Documentation system (Bio-Rad). Stored patterns were analysed with the Windows version of GelCompar software version 3.10 (Applied Maths) after conversion of the data into the TIF format. Electrophoresis gels were normalized according to external standards contained in each fifth lane (1 kb ladder, Gibco).

Typing by the AFLP method. Firstly, five DNAs isolated from representatives of the NTHI strain collection were used in screening analysis to evaluate the potential to obtain distinctive AFLP patterns. Seven different AFLP sets were used, *HindIII*/*TaqI*, two different *ApaI*/*TaqI* sets, *HindIII*, *MfeI*/*BglII*, *PstI*/*TaqI* and *EcoRI*/*MseI*, based on different enzyme restriction/specific adaptor ligation and primer-specific amplification with/without selective bases complementary to nucleotides flanking the restriction sites. They were previously found to work with species other than *H. influenzae* (Grady *et al.*, 1999; Huys *et al.*, 1996; Kokotovic *et al.*, 1999; McLauchlin *et al.*, 2000; van Eldere *et al.*, 1999; Vos & Kuiper, 1998). Characteristics of AFLP sets are presented in Table 1. Sequences of adaptors/primers and thermal conditions were applied as described previously but different conditions of restriction, ligation and amplification reactions were applied. Briefly, in all tested AFLP sets, 200 ng *H. influenzae* DNA was digested with the following restriction enzymes: *HindIII* (BioLabs)/*TaqI* (Gibco-BRL), *ApaI* (BioLabs)/*TaqI*, *HindIII*, *MfeI* (BioLabs)/*BglII* (BioLabs), *PstI* (Eurogentec)/*TaqI* and *EcoRI* (Gibco-BRL)/*MseI* (BioLabs), according to the suppliers' recommendations. Samples were heat-treated to inactivate restriction enzyme activity. Next, adaptors (sequences shown in Table 1; synthesized by Invitrogen) were ligated to the DNA restriction fragments by T4 DNA ligase (BioLabs) according to the supplier's recommendations. For rare-cutting enzymes, adaptors were used at a concentration of 5 pmol µl⁻¹, and, for frequent-cutting enzymes, at 50 pmol µl⁻¹. After completion of ligation, samples were heat-inactivated, diluted 10-fold in distilled water and refrigerated at -20 °C until the start of the PCR. Restriction fragments tagged with specific adaptors were selectively amplified with particular primers (Table 1). PCR was performed in a volume of 20 µl in a Biometra thermal cycler. Each amplification reaction mixture contained 5 µl 10-fold-diluted ligation reaction sample, 12 ng Cy5-labelled primer, 60 ng

Table 1. Characteristics of enzyme restriction/specific adaptors and primers used successfully in this study

Adaptors	Cy-5-labelled primer			Unlabelled primer		Reference
	Name	Sequence (5'-3')	Name	Sequence (5'-3')	Name	
HindIII/TaqI set	AD1HindIII	CTCGTAGACTGCGTACC	HindIII+1	GACTGCGTACCAGCTTA	TaqI+1	van Eldere <i>et al.</i> (1999)
	AD2HindIII	AGCTGGTACGCAGTC				
	AD1TaqI	GACGATGAGTCTCTGAC				
	AD2TaqI	CGGTCAGGACTCAT				
MfeI/BglII set	AD1BglII	CGGACTAGACTACTGTC	BglII+0	GAGTACACTGTCGATCT	MfeI+0	Kokotovic <i>et al.</i> (1999)
	AD2BglII	GATCGACAGTGTACTCTAGTC				
	AD1MfeI	AATCCAAAGAGCTCTCCAGTAC				
	AD2MfeI	TAGTACTGGAGAGCTCTTGG				
PstI/TaqI set	AD1PstI	CTCGTAGACTGCGTACATGCA	PstI+0	GACTGCGTACATGCAG	TaqI+3	Vos & Kuiper (1998)
	AD2PstI	TGTACGCGAGTCTAC				
	AD1TaqI	GACGATGAGTCTCTGAG				
	AD2TaqI	CGCTCAGGACTCAT				

unlabelled primer, 120 µM each of dATP, dGTP, dCTP and TTP, 0.3 U AccuTaq LA DNA polymerase (Sigma), 3.0 mM MgCl₂, 50 mM Tris/HCl, 15 mM ammonium sulphate and 0.1 % Tween 20.

After completion of the PCR, 5 µl of each reaction mixture was mixed with 3 µl loading dye (Amersham Pharmacia), denatured at 90 °C for 3 min and applied to the gel. Selectively amplified fragments were separated through a ReproGel High Resolution (Amersham Pharmacia) in 0.5× TBE buffer on an ALFexpress DNA sequencer. Separation was done at 1500 V, 60 mA, 25 W for 420 min at 55 °C. In order to evaluate intra- and inter-gel differences and to identify levels, a fluorescein-labelled molecular size marker in the size range of 50 to 500 bp and an external reference strain were used as external size markers (ALFexpress Sizer 50–500). ALFexpress Sizer 300 added to each sample was used as an internal size marker in each lane. Stored fluorograms were analysed with the Windows version of the GelCompar software version 3.10 (Applied Maths) after conversion to TIF format. Separate electrophoresis gels were normalized according to the internal standards contained in each lane (300 bp). The lane resolution was reduced, excluding the primer front and fragments larger than 500 bp. Dendrograms for cluster analysis were based on similarity matrices calculated from Pearson's product-moment correlation coefficient and the UPGMA algorithm (Everitt, 1993). Statistical significance of overall similarities obtained for dendrograms within the seven institutions under study was evaluated by Wilcoxon's test.

RESULTS

Selection of isolates for examination

All of the *H. influenzae* isolates ($n = 87$) used in the study were obtained from the nasopharynx of healthy children who were not vaccinated against Hib and who attended six DCCs and three orphanages. The isolates were previously identified by conventional methods including serotyping and this was confirmed by PCR using primers for type b-specific sequences (primers b1 and b2). Additionally, primers H1 and H2, directed to *bexA*, were used to identify encapsulation genes. Thus, among the isolates tested, 9 of 87 (10.8 %) and 74 of 87 (89.2 %) were respectively identified as Hib and NTHI isolates. An additional four isolates from teachers were classified into the NTHI group. The *H. influenzae* isolates under study are detailed in Table 2.

Analysis of RAPD

For fingerprinting analysis, six short (10 bp) primers were used for amplification. The *H. influenzae* strain-specific DNA arrays consisted of approximately 8–15 bands, with a fragment length distribution in the range of 0.1–2.0 kb. Of the primers tested, primer VI was found to have greater potential to produce informative DNA profiles. The levels of similarity for patterns obtained for a single strain with primer VI were at least 97 % within the same gel and at least 90 % between gels. Generally, a high level of diversity was found for NTHI isolates; these were clustered into a few clonal groups within one clonal division. The overall genetic similarity of the NTHI isolates within the dendrogram, as defined by Pearson's product-moment correlation coefficient, was 57.3 %. At the 70 % level of similarity, five clusters were found, designated A–E (with 24, 21, 6, 13 and 8 isolates, respectively) (Fig. 1). All nine Hib isolates under study were

Table 2. Characteristics of 87 nasopharyngeal *H. influenzae* isolates investigated in this study

DCC, Day-care centre; O, orphanage; –, not tested; X, non-typable by RAPD; XX, non-typable by AFLP.

Isolate	Serotype/genotype	Age of child (years)	Location	RAPD type	AFLP type		
					<i>HindIII/TaqI</i>	<i>MfeI/BglII</i>	<i>PstI/TaqI</i>
1W	NTHI	4.5	DCC1	A	CIc	UB1	III
2W	NTHI	5	DCC1	A	CIb	UB1	III
3W	NTHI	5	DCC1	A	CIc	UB3	III
4W	NTHI	5	DCC1	A	CIc	UB1	–
6W	Hib	4.5	DCC1	X	XX	XX	XX
7W	NTHI	4.5	DCC1	B	CIc	UB1	III
8W	NTHI	4	DCC1	A	CIc	UA2	III
9W	NTHI	4	DCC1	B	CIc	UB1	III
10W	NTHI	4	DCC1	X	XX	XX	XX
11W	NTHI	4.5	DCC1	A	CIc	UB1	III
12W	Hib	4	DCC1	X	XX	XX	XX
13W	NTHI	5	DCC1	A	CIb	UB1	III
14W	NTHI	3.5	DCC1	A	CIa	UB1	III
15W	NTHI	3.5	DCC1	A	CIc	UB3	III
17W	NTHI	5	DCC1	A	CIc	UB1	–
18W	NTHI	3.5	DCC2	A	CIa	UA2	III
20W	NTHI	3.5	DCC2	B	CIc	UB1	III
22W	NTHI	2.5	DCC2	A	CIc	UA2	III
24W	NTHI	4	DCC2	–	CIc	–	–
26W	NTHI	3	DCC2	A	CIc	UB1	III
27W	NTHI	3	DCC2	A	CIa	–	–
30W	NTHI	2	DCC3	D	CIc	UB1	III
32W	NTHI	2	DCC3	A	CIc	UB1	III
33W	NTHI	3	DCC3	A	CIc	UB1	III
34W	NTHI	2	DCC3	C	CIc	UB3	III
35W	NTHI	2	DCC3	D	CIb	UC	III
37W	Hib	3	DCC3	X	XX	XX	XX
38W	NTHI	2.5	DCC3	C	CIa	UA1	III
39W	Hib	3.5	DCC3	X	XX	XX	XX
40W	Hib	3.5	DCC3	X	XX	XX	XX
41W	NTHI	3	DCC3	D	CIb	UB1	III
42W	NTHI	3.5	DCC3	A	CIc	UA2	III
43W	Hib	2.5	DCC3	X	XX	XX	XX
44W	NTHI	3.5	DCC3	D	CIb	UC	III
45W	NTHI	2	DCC3	D	CIb	–	–
46W	NTHI	Teacher	DCC3	D	CIId	UB2	I
47W	NTHI	0.58	DCC4	D	CIId	UB2	I
48W	NTHI	4	DCC4	D	CIId	UB2	–
49W	NTHI	4.5	DCC4	A	CIc	–	III
50W	NTHI	3.5	DCC4	C	CIa	UA1	III
51W	NTHI	4	DCC4	D	CIId	UA3	I
53W	NTHI	3.5	DCC4	–	CIb	UB2	I
54W	NTHI	4	DCC4	D	CIId	UB2	I
55W	NTHI	4	DCC4	D	CIId	UB2	I
56W	NTHI	0.67	O7	B	CIc	UB3	III
57W	NTHI	0.67	O7	A	CIc	UA2	III
58W	NTHI	0.5	O7	B	CIc	UB3	III
59W	NTHI	0.5	O7	B	CIc	UA2	III
61W	NTHI	2	O7	B	CIb	UB3	III
62W	NTHI	2	O7	D	CIb	UB3	II
64W	NTHI	Teacher	O7	B	CIc	UB3	III

Table 2. cont.

Isolate	Serotype/genotype	Age of child (years)	Location	RAPD type	AFLP type		
					<i>HindIII/TaqI</i>	<i>MfeI/BglII</i>	<i>PstI/TaqI</i>
66W	NTHI	1	O8	B	CIIb	UB3	III
67W	NTHI	0.25	O8	A	CIIc	UB3	III
68W	NTHI	0.42	O8	B	CIIb	UA2	III
69W	NTHI	0.17	O8	B	CIIb	UA2	III
70W	NTHI	1.25	O8	B	CIIc	UA2	III
71W	NTHI	2	O8	A	CIIb	UB3	III
72W	NTHI	2	O8	B	CIIc	UD3	III
73W	NTHI	1.25	O8	C	CIIb	UA2	II
74W	NTHI	1.5	O8	B	CIIb	UA2	III
75W	NTHI	3.5	O8	C	CIIb	UA2	–
76W	NTHI	2	O8	B	CIIb	–	–
77W	NTHI	2	O8	B	CIIb	UA2	III
78W	NTHI	3.5	O8	B	CIIc	UA2	III
81W	Hib	1	DCC9	X	XX	XX	XX
83W	NTHI	1.17	DCC9	A	CIIc	UB3	III
85W	Hib	1.5	DCC9	X	XX	XX	XX
86W	NTHI	2	DCC9	D	CIIb	UB3	III
89W	NTHI	2	O8	–	CIIb	UA2	III
94W	NTHI	2	DCC10	B	CIIc	UB3	III
95W	NTHI	2	DCC10	A	CIIc	UB3	III
97W	NTHI	1	DCC10	B	CIIb	UB1	III
101W	NTHI	Teacher	DCC10	B	CIIc	UB3	III
102W	NTHI	Teacher	DCC10	–	CIIc	UA2	I
104W	NTHI	2	O6	E	CIIId	UB2	I
105W	NTHI	1	O6	E	CIIId	UB2	I
106W	NTHI	2	O6	E	CIIId	UA3	I
107W	NTHI	1	O6	E	CIIId	UA3	I
108W	NTHI	1	O6	B	CIIId	UA3	I
109W	NTHI	0.67	O6	E	CIIId	UA3	I
111W	NTHI	0.83	O6	E	CIIId	UA3	I
112W	NTHI	0.25	O6	E	CIIId	UA3	–
113W	NTHI	1.5	O6	E	CIIId	UA3	I
114W	Hib	1.5	O6	X	XX	XX	XX
120W	NTHI	2.5	DCC10	A	CIIc	UB3	III
125W	NTHI	4	DCC10	–	CIIc	UB3	III
126W	NTHI	5.5	DCC10	–	CIIa	UA2	III

found to represent a single and identical RAPD pattern and were classified by cluster analysis into a single branch of the dendrogram with a 94 % similarity level. No band variation was observed in RAPD band patterns obtained for DNA isolated from seven passages of a single strain or with DNA content in the range 100–500 ng.

Screening of AFLP sets

Preliminary screening of seven different AFLP schemes tested revealed that one *Apal/TaqI* set and the *HindIII* set were not useful for NTHI typing, as no banding patterns were observed. In the case of the other *Apal/TaqI* set and the *EcoRI/MseI* set, identical bands patterns were observed for all isolates tested, revealing no potential for discrimination. Of

the successful sets, in the *PstI/TaqI* and *MfeI/BglII* sets, a larger number of bands (40–60) within profiles was obtained in comparison with the *HindIII/TaqI* set (16–37 bands). Bands were more equally distributed within profiles for the *HindIII/TaqI* set.

For the *PstI/TaqI* and *MfeI/BglII* sets, in comparison with the *HindIII/TaqI* scheme, much lower inter- and intra-gel similarity values were found. Generally, overall intra-/inter-gel similarity levels for the external reference strain were respectively 84/86, 75/81 and 94/96 % for the *PstI/TaqI*, *MfeI/BglII* and *HindIII/TaqI* sets. In the *PstI/TaqI* and *MfeI/BglII* AFLP schemes, lower levels of similarity were associated with difficulties in gel normalization with the internal reference (300 bp marker) included separately in each sample, which

Fig. 1. UPGMA dendrogram of RAPD patterns of NTHI isolates. Characteristics of isolates are listed in Table 2. Levels of linkage are expressed as Pearson's product-moment similarity coefficient.

was often masked with several bands generated in the area close to the internal 300 bp control. Thus, gel normalization in these cases was based exclusively on external reference markers. Overall genetic similarities defined by Pearson's product-moment correlation coefficient for banding patterns of NTHI isolates reached values of 26.5, 30.2 and 35.4 %, respectively, for the *Pst*I/*Taq*I, *Mfe*I/*Bgl*II and *Hin*-dIII/*Taq*I AFLP sets. In the case of the *Mfe*I/*Bgl*II set, the

overall similarity level was 30.2 % and three clusters, UA (27 isolates), UB (43 isolates) and UC (two isolates), were identified. Within each of clusters UA and UB, three subclusters, UA1, UA2 and UA3 (with two, 17 and eight isolates, respectively) and UB1, UB2 and UB3 (16, eight and 19 isolates), were defined. In the case of the *Pst*I/*Taq*I scheme, the overall similarity level was 26.5 % and four clusters, I (15 isolates), II (two isolates), III (46 isolates) and IV (five

isolates), were differentiated. In the case of the *HindIII/TaqI* AFLP scheme, the overall similarity was 35.4 % and two main clusters, CI (nine isolates) and CII (68 isolates), were discriminated. Cluster I comprised subclusters CIa, CIb and CIc, respectively containing three, four and two isolates. Within cluster II, three, 17, 33 and 15 isolates made up subclusters IIa, IIb, IIc and IId. High discriminatory power,

coupled with the highest identity level among successful AFLP schemes, identified the *HindIII/TaqI* system as a first method of choice for NTHI strain genotyping (Fig. 2).

DNA isolates obtained from seven subcultures of two different isolates of NTHI repeated over time were used to compare specific pattern reproducibility. The band patterns

Fig. 2. UPGMA dendrogram of AFLP patterns (*HindIII/TaqI* set) of NTHI isolates. See Fig. 1 for further details.

did not change after seven passages in any of the isolates tested (data not shown). Similarly, the quantity of DNA in the range 100–500 ng did not induce band variation in the AFLP profile of a single strain. For both the *Pst*I/*Taq*I and *Mfe*I/*Bgl*II sets, identical banding profiles were observed for Hib strains, and, for the *Hind*III/*Taq*I set, no profiles were observed for Hib strains. The *Hind*III/*Taq*I set has been chosen as a first-choice AFLP screening scheme for NTHI typing.

*Hind*III/*Taq*I AFLP versus RAPD

The overall genetic similarity of the NTHI isolates genotyped with the *Hind*III/*Taq*I AFLP set, as defined by Pearson's product-moment correlation coefficient, was 35.4 %, which allowed discrimination of patterns with much higher discriminatory power than in the case of RAPD. The dendrogram of RAPD profiles classified all NTHI isolates with 54 % overall similarity, with two main RAPD clusters containing 50 and 21 isolates, respectively subdivided into three and two subclusters (Table 2). In contrast to the overall similarity value of 54 % for RAPD, AFLP with *Hind*III/*Taq*I identified six different clusters, containing three, four, two, two, one and 65 isolates. Thus, *Hind*III/*Taq*I AFLP presented superior discriminatory power in comparison with the RAPD scheme applied.

AFLP clustering according to institution

For isolates from eight institutions, dendrograms were constructed for NTHI isolates differentiated with the *Hind*III/*Taq*I set. Generally, a much narrower range of highly related overall similarity values was obtained for orphanages (54–67.4 %) (not statistically significantly different, however; $P = 0.07$) in comparison with the overall similarities found within isolates from DCCs (30–47 %) (Fig. 3). Overall genetic similarity values for isolates genotyped with the RAPD technique did not reveal any closer relationship between isolates from orphanages (64–70 %) and DCCs (45–88 %) (Fig. 3).

DISCUSSION

Implementation of vaccination against Hib has generally reduced Hib invasive infections and rates of carriage (Peltola, 2000; Urwin *et al.*, 1996). It is suspected that, in future, NTHI and *H. influenzae* of other capsular types might fill the 'empty' Hib ecological niche in Hib-vaccinated populations, resulting in growing importance of carriage and disease caused by these types (Leaves & Jordens, 1996). The small but progressive increase in serious disease due to NTHI has prompted researchers to examine the molecular epidemiology of the bacterium (Cerquetti *et al.*, 2000; Falla *et al.*, 1993; Jordens *et al.*, 1993). Studies that have allowed recognition of relationships in the circulating strain population structure were directed towards monitoring their frequency and turnover (Cerquetti *et al.*, 2000; Faden *et al.*, 1995). Several proposed protocols based on different tools, e.g. outer-membrane protein typing, MLEE, PFGE, RAPD or multi-

Fig. 3. Distribution of overall similarity values obtained for NTHI isolates within institutions tested. DCC, Day-care centre (open circles); O, orphanage (filled circles).

locus sequence typing, have been used to evaluate NTHI strain relationships (Fuste *et al.*, 1996; Gazagne *et al.*, 1998; Gilsdorf, 1998; Moor *et al.*, 1999; Saito *et al.*, 1999; St Geme *et al.*, 1994; van Belkum *et al.*, 1994, 1997). Of these, some were aimed at finding differences among NTHI strains isolated from patients with persistent infections and from healthy humans (Cerquetti *et al.*, 2000; van Alphen *et al.*, 1991, 1997).

In this study, we describe AFLP as a new tool for generating detailed genotyping data that might be useful for studying relationships between isolates with a discriminatory power much higher than previously described for the RAPD technique. Several AFLP systems, differing in restriction enzyme/adaptors/primers and conditions and in respect of their usefulness in NTHI epidemiological studies, were tested and compared with the discriminatory capacity of RAPD. The NTHI collection examined comprised 87 *H. influenzae* isolates from the nasopharynx of healthy, unvaccinated 1- to 5-year-old children (and their teachers) attending DCCs and orphanages in Poland during January to May 2002. The isolates under study had been characterized by capsular typing with primers designed from the *bexA* DNA sequence (Falla *et al.*, 1994). Generally, in comparison with Hib, NTHI isolates were found to predominate in 90 % in positive swabs taken from the nasopharynx of individuals tested. Thus, the level of NTHI isolation agrees with studies performed previously, in which NTHI isolates predominated at frequencies of 93–99 % in carriers and 87 % in aboriginal infants (Smith-Vaughan *et al.*, 1998; Trotter *et al.*, 1989).

In order to study the genetic relationships among NTHI isolates collected from different environmental backgrounds, RAPD and AFLP methods were used. As results from the

RAPD technique have generally been found to be very labile depending on the procedures and reagents used (Heersma *et al.*, 2001; Mori *et al.*, 1999; Olive & Bean, 1999), a standardized methodology was applied, including commercially available kits for DNA isolation and RAPD amplification to overcome some problems with reproducibility. General approaches were included for reliable and accurate intra- and inter-gel comparison of banding patterns for the two methods (Desai *et al.*, 1998; Janssen, 2001; Savelkoul *et al.*, 1999). Additionally, reproducibility was tested by generation of multiple subcultures on which DNA extraction, RAPD and AFLP procedures (also, on different amounts of DNA) were performed. Intra-/inter-gel reproducibility experiments performed with external references found identity levels for RAPD and for the *HindIII/TaqI* AFLP set reaching values of 97/96 and 96/94 %, respectively. Of the seven sets screened, the *HindIII/TaqI* set was chosen as the first-choice method because it showed the highest levels of identity of the three AFLP schemes that were successful in NTHI genotyping. The overall genetic similarity defined by Pearson's product-moment correlation coefficient was respectively 57.3 and 35.4 % for RAPD and AFLP (*HindIII/TaqI* set). Although RAPD can provide data very rapidly, its discriminatory power was much lower in comparison with AFLP.

The non-typable isolates tested appeared to be much more heterogeneous, at a low level of clonality, supporting previously published studies regarding the panmictic population structure (Faden *et al.*, 1995; Falla *et al.*, 1993; Leaves & Jordens, 1996; Omikunle *et al.*, 2002). Similarly, our data confirmed those published previously on the limited diversity and clonal population structure of Hib-type strains (Fuste *et al.*, 1996; Gazagne *et al.*, 1998; Gilsdorf, 1998; Musser *et al.*, 1988; Saito *et al.*, 1999). All Hib isolates comprising our control group, typed by RAPD and AFLP techniques, were found in a single clade of the dendrograms for both methods. One hypothesis to account for the clonality of Hib, supported by experimental data, is that the capsule acts as a barrier to exogenous DNA (Musser *et al.*, 1988). AFLP patterns were used to investigate whether certain isolates were circulating more frequently within institutions of different environmental backgrounds (DCCs, orphanages) that vary in the intensity of shared living conditions and contact among individuals. The overall similarity values of AFLP dendrograms (*HindIII/TaqI* scheme) constructed for isolates originating from particular DCCs were lower than those obtained for isolates originating within particular orphanages. No trend was observed in RAPD discriminatory potential. The closer genetic relationship detected among NTHI isolates originating at DCCs, where children are in contact only during the day in the working hours of their parents, was suspected to be much more related to the higher frequency of contact in orphanages, due to permanent, and thus closer, cohabitation in the same environment. Although a high level of diversity has generally been found for NTHI, more frequent transmission of closely related strains among children living together in

orphanages can support the idea of an association between transmission rates and environmental factors dependent on contact intensities. It seems that increased transmission rates and repeated passage through human hosts living more closely together might result in more 'clonal' relationships among isolates (Peerbooms *et al.*, 2002). Larger numbers of isolates and institutions need to be compared in order to confirm or reject this hypothesis.

With application of the AFLP technique presented here, understanding of the population structure of NTHI, especially when Hib vaccination is introduced or continued, might provide insights into the emergence of newly acquired clones and/or follow the expansion of clones, to elucidate transmission routes in the community and the reasons for vaccine failure, if the latter occurs. The authors of this study postulate AFLP to be more accurate than the previously described RAPD for definition of bacterial strain differences and for tracking genetic relatedness.

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