Genomic DNA fingerprinting of clinical *Haemophilus influenzae* isolates by polymerase chain reaction amplification: comparison with major outer-membrane protein and restriction fragment length polymorphism analysis

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Summary. Non-capsulate strains of *Haemophilus influenzae* were genotyped by analysis of variable DNA segments obtained by amplification of genomic DNA with the polymerase chain reaction (PCR fingerprinting). Discrete fragments of 100–2000 bp were obtained. The reproducibility of the procedure was assessed by comparing: (i) the fingerprints of 16 colonies of a single *H. influenzae* strain; (ii) isolates obtained from individual sputum samples (a total of 57 *H. influenzae* isolates from three cystic fibrosis patients); and (iii) 17 isolates collected during an outbreak of *H. influenzae* infection in a local pulmonary rehabilitation centre. The discriminatory power of the method was demonstrated by showing that the PCR fingerprints of eight unrelated *H. influenzae* strains from sputum samples of patients with chronic obstructive pulmonary disease (COPD) and 32 strains from cystic fibrosis patients were all different. These 40 isolates also differed with respect to their restriction fragment length polymorphisms (RFLP) and major outer-membrane protein (MOMP) composition. Twelve MOMP antigenic strain variants from sputum samples of five COPD patients had identical PCR fingerprints and RFLPs. It was concluded that PCR fingerprinting is a reliable and reproducible method for genotyping non-capsulate strains of *H. influenzae*. The discriminatory power of PCR fingerprinting was similar to that of RFLP analysis, but the results of PCR fingerprinting were easier to interpret.

Introduction

Haemophilus influenzae is found exclusively in man. Non-capsulate strains cause acute and persistent infections of the mucous membranes and colonise the nasopharynx of a high percentage of healthy individuals.¹ Throat cultures of children can remain positive for a particular *H. influenzae* strain for several months.^{2,3} In patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), *H. influenzae* is isolated frequently from sputum samples.^{4,5} However, the availability of high-resolution bacterial typing assays is a pre-requisite for the study of *H. influenzae* epidemiology.

Non-capsulate *H. influenzae* isolates are charac-

terised commonly by major outer-membrane protein (MOMP) analysis with SDS-PAGE. This method is highly discriminatory because of strong inter-strain variability.^{6–9} However, MOMP patterns have been shown to change during persistent *H. influenzae* infections in patients with COPD.^{10,11} Antigenic diversity of lipopolysaccharides (LPS), which may also be used to characterise non-typable *H. influenzae* strains,¹² is difficult to interpret because LPS undergoes phase variation at high frequency.¹³ Of the genotypic methods, multilocus enzyme electrophoresis,¹⁴ rRNA gene restriction pattern analysis¹⁵ and DNA restriction fragment length polymorphism (RFLP) analysis have been applied successfully to differentiate isolates.^{10,11,16} However, since multilocus enzyme electrophoresis is very laborious, rRNA gene restriction pattern analysis has a low discriminatory power, and RFLP analysis results in over-complex banding patterns, there is a clear need for a novel discriminatory typing method.

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Fig. 1. Analysis of the reproducibility of PCR fingerprinting of *H. influenzae* strains with a combination of the ERIC1R and the ERIC2 primers: **a**, fingerprints obtained from 16 colonies from a single strain cultivated on one chocolate agar plate; **b**, fingerprints obtained from 19 independent *H. influenzae* isolates from primary cultures of sputum samples from a CF patient; **c**, fingerprints of 13 *H. influenzae* strains collected from 13 COPD patients during an outbreak in a pulmonary rehabilitation clinic. The four righthand lanes show PCR fingerprints from non-related *H. influenzae* isolates.

A potential alternative procedure for the rapid detection of genetic diversity is now available.^{17–19} For many prokaryotic and eukaryotic micro-organisms, it has been shown that fingerprints can be generated after amplification of genomic DNA with random primers in the polymerase chain reaction (PCR).^{20–24} The genetic basis of the variation in such PCR fingerprints is two-fold. Firstly, DNA sequence variations can be detected by a combination of short, arbitrarily chosen primers and low-stringency hybridisation conditions during the PCR. Secondly, a PCR assay can be aimed at hypervariable regions of the genome surrounded by repetitive sequences that are recognised by specific PCR primers.^{23, 25, 26}

In the present study, genomic DNA amplified by PCR with primers for the enterobacterial repetitive intergenic consensus (ERIC) sequence²⁶ was used to genotype non-capsulate *H. influenzae* isolates. The discriminatory power of PCR fingerprinting was compared with RFLP genotyping and MOMP analysis. The reproducibility and specificity of PCR fingerprinting was analysed with epidemiologically well-defined *H. influenzae* isolates, obtained from COPD and CF patients, which had been characterised previously by RFLP and MOMP analyses.^{10, 11} In ad-

dition, the PCR fingerprints of *H. influenzae* strains were compared with those of other *Haemophilus* species.

Materials and methods

Bacterial strains and DNA isolation

Non-capsulate *H. influenzae* strains were isolated either from sputum samples obtained from COPD patients during an epidemiological study of persistent *H. influenzae* infections in these patients,^{4, 10, 11} or from sputum samples from COPD patients in a pulmonary rehabilitation centre during a local outbreak of multi-resistant *H. influenzae* infection.²⁷ *H. parainfluenzae* strains were isolated from sputum obtained from COPD patients or from throat swabs of healthy carriers. *H. haemoglobinophilus* and *H. haemolyticus* strains were obtained from I. I. Falsen, Department of Clinical Microbiology, University of Göteborg, Sweden. All haemophili were inoculated on to chocolate agar plates from stocks stored at -70°C in nutrient broth supplemented with glycerol 15% v/v, and were then incubated for 16 h at 37°C in a humid

Fig. 2. PCR fingerprints obtained with primers ERIC1R and ERIC2 from clinical *H. influenzae* isolates and strains belonging to other *Haemophilus* spp: **a**, strains from a selected group of CF patients (1, 3, 6, 11, 18 and 20) and COPD patients (P, T, K, D and M; see table); **b**, eight *H. parainfluenzae* strains (Hp1–8); two *H. haemolyticus* strains (Hh1–2) and one *H. haemoglobinophilus* strain (Hhg); **c**, *H. influenzae* antigenic strain variants from COPD patients T (d1–d4), K (16a and 16b), D (7a and 7b) and M (21a and 21b).

atmosphere enriched with CO₂ 5%. For the analysis of MOMP, *c.* five colonies were inoculated into 10 ml of brain heart infusion broth and incubated overnight at 37°C with shaking at 120 rpm. The resulting cells were harvested by centrifugation.⁹ For DNA isolation, bacterial growth was scraped from the plates and resuspended in 2 ml of phosphate-buffered saline (PBS) pH 7.0, to a density of *c.* 10⁹ cells/ml. DNA was isolated from 250 µl of this suspension by the guanidium isothiocyanate protocol described previously.^{24,28} The DNA concentration was estimated by comparing a small DNA sample stained with

ethidium bromide with known amounts (10–500 ng) of bacteriophage lambda DNA. Finally, DNA was dissolved at a concentration of *c.* 10 ng/µl in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

MOMP analysis and RFLP determination

MOMP typing was by SDS-PAGE as described by Lugtenberg *et al.*²⁹ and modified by van Alphen *et al.*⁹ RFLP fingerprinting after restriction endonuclease treatment of total genomic DNA was as described previously.¹¹

PCR fingerprinting

The PCR reaction buffer consisted of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM MgCl₂, gelatin 0.01 % w/v and Triton X100 0.1 % v/v. Each reaction included primers ERIC1R and ERIC2^{24,26} (50 pmol each), deoxyribonucleotide triphosphates (final concentration of 0.2 mM each), sample DNA (c. 50 ng), 0.5 U of *Taq* DNA polymerase (Sphaero Q, Leiden, The Netherlands). The arbitrary primer BG2 (50 pmol/reaction)—5'-TACATTCGAGGACCCCT-AAGTG-3'—was included as an alternative for one of the ERIC primers in some experiments. The PCR was initiated with a 5-min denaturation period at 94°C, followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 25°C) and enzymic chain extension (4 min at 74°C). Finally, an additional 10-min DNA extension step at 74°C was included. The PCR reactions were performed with a Model 60 Thermocycler (Biomed, Theres, Germany). DNA fingerprints were visualised by ethidium bromide staining of agarose 1–3 % w/v gels run in 40 mM Tris-acetate, pH 7.8, 2 mM EDTA, and were then photographed with Polaroid Polapan film. Results were analysed only if control samples without extraneous sample DNA failed to yield amplified DNA. Analysis of the PCR fingerprinting data was performed blind with respect to the RFLP and MOMP typing data.

Results

General characteristics, reproducibility and stability of the PCR fingerprints of non-capsulate *H. influenzae* isolates

PCR with primers ERIC1R and ERIC2 produced complex banding patterns on agarose gels (figs. 1 and 2), with fragments of 100–c.2000 bp in size. In contrast, PCR with the arbitrary primer BG2 in combination with one of the ERIC primers resulted in fewer bands in trial experiments with 10 strains of *H. influenzae*. Therefore, PCR with primers ERIC1R and ERIC2 was chosen for further analysis.

To assess whether reproducible banding patterns were generated by PCR fingerprinting, 16 colonies from a pure culture of a single *H. influenzae* strain obtained from the sputum of a CF patient were analysed. Fig. 1a shows that the 16 corresponding fingerprints appeared to be monomorphic. Minor differences in the staining intensity of the lanes probably resulted from differences in template DNA concentration as a consequence of variations in the original colony size. Next, PCR fingerprinting was performed on multiple *H. influenzae* isolates obtained from the primary cultures of sputum from three individual CF patients. Fig. 1b shows that 19 isolates from one primary sputum culture of one CF patient had identical DNA fingerprints. These isolates also had identical MOMP profiles and RFLP patterns (results not shown). Similar results were obtained with

19 isolates from the primary culture of sputum from a second CF patient. A third CF patient was infected by two apparently different *H. influenzae* strains, based on analysis of DNA patterns and MOMP profiles. These 19 isolates fell into two distinct groups (three and 16 isolates, respectively). Similar results were obtained when the isolates from the three CF patients were analysed on different days.

The stability of the PCR fingerprints was examined by comparing 17 isolates from a local outbreak of infection caused by multi-resistant non-capsulate *H. influenzae* in a pulmonary rehabilitation centre.²⁷ Thirteen isolates from different individuals were studied. Fig. 1c shows that these strains, shown previously to have identical MOMP patterns,²⁷ also had identical PCR fingerprints. The PCR fingerprints, MOMPs²⁷ and RFLPs (results not shown) of these 13 identical strains differed clearly from those of the four unrelated strains also obtained during the local outbreak, as exemplified by the PCR fingerprints in the four righthand tracks of fig. 1c.

Diversity of PCR fingerprints of unrelated non-capsulate *H. influenzae*

The variation in PCR fingerprints was investigated with six non-capsulate *H. influenzae* strains obtained

Table. Characteristics of *H. influenzae* strains from patients with chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF)

Patient*	Strain no.	PCR pattern†	RFLP type‡	MOMP type‡
CF1	41/z	A	§	§
CF3	42/z	B	§	§
CF6	43/z	C	§	§
CF11	44/z	D	§	§
CF13	22/z	E	§	§
CF18	23/z	F	§	§
CF20	26/a	G	§	§
COPD P	t1	H	I	1a
	t2	H	I	1b
COPD T	d1	I	II	2a
	d2	I	II	2b
	d3	I	II	2c
	d4	I	II	2d
COPD K	16a	J	III	3a
	16b	J	III	3b
COPD D	7a	K	IV	4a
	7b	K	IV	4b
COPD M	21a	L	V	5a
	21b	L	V	5b
	22	M	VI	6

* Different isolates from individual patients were obtained from samples taken at intervals of at least 1 month.

† Different PCR fingerprints are represented by capital letters, each of which indicates a unique DNA banding pattern.

‡ All isolates from CF patients had different RFLP and MOMP types (indicated by §). Different RFLP patterns of isolates from COPD patients are indicated by Roman figures. MOMP patterns of non-related strains are indicated by numbers; antigenic strain variants are indicated by a lower case letter following the number. The latter data are taken from Groeneveld *et al.*¹⁰

Fig. 3. Major outer-membrane protein (MOMP) patterns of seven non-related *H. influenzae* isolates from CF patients (table).

from five COPD patients and seven strains from seven CF patients. The six isolates from COPD patients were known to have different RFLP and MOMP patterns,^{10,11} as summarised in the table. The seven CF isolates also had different RFLP (results not shown) and MOMP patterns (fig. 3). PCR fingerprints of the COPD and CF isolates revealed marked differences between all isolates (fig. 2a). The PCR fingerprints of additional non-related COPD ($n = 2$) and CF ($n = 25$) *H. influenzae* isolates appeared also to be mutually different, indicating that this typing method was completely discriminatory.

The PCR fingerprints of *H. parainfluenzae* (fig. 2b, lanes 1–8), *H. haemolyticus* (fig. 2b, lanes 9 and 10) and *H. haemoglobinophilus* (fig. 2b, lane 11) isolates showed wide diversity.

Analysis of antigenic strain variants of *H. influenzae* by PCR fingerprinting

A total of 12 clinical isolates from five COPD patients was analysed. These isolates had been shown previously to be antigenic strain variants,^{10,11} and are listed in the table. These strain variants have identical RFLP patterns, but vary in MOMP composition, mainly in MOMP P2 and P5.^{10,11} In four of five COPD patients studied, the PCR fingerprints of the

strain variants were identical (patients P, K, D and M). The PCR fingerprints of *H. influenzae* strain variants d1–d4 from patient T were identical, except for a difference in intensity in one of the bands (fig. 2a). The PCR fingerprints of the antigenic strain variants from four of the COPD patients are shown in fig. 2c. *H. influenzae* strains different by PCR fingerprinting were also different by RFLP analysis.

Discussion

PCR fingerprinting of non-capsulate *H. influenzae* isolates offers the possibility of a faster and easier high-resolution genotyping method for epidemiological studies. Fig. 1 demonstrates that the DNA fingerprints obtained with the ERIC primers²⁶ were reproducible for epidemic isolates and individual colonies of a single strain. Differences in DNA fingerprints were observed also for various *H. parainfluenzae* isolates, and between *H. influenzae*, *H. parainfluenzae* and other *Haemophilus* spp. (fig. 2a and b).

The epidemiological findings from PCR fingerprinting of *H. influenzae* isolates were in full agreement with those derived from RFLP analysis (table), indicating that both methods were equally discriminatory. Moreover, as both these techniques focus on different parts of the bacterial genome, it is likely that variations reflect random genomic differences. An advantage of PCR fingerprinting over RFLP analysis is the ease with which the results may be interpreted.

The value of PCR fingerprinting for studying the clinical epidemiology of *H. influenzae* infections is illustrated in fig. 1c for multi-resistant non-capsulate *H. influenzae* isolates obtained during a local outbreak of infections in a pulmonary rehabilitation centre. Furthermore, the observation that antigenic strain variants from the sputum of COPD patients^{10,11} had identical PCR fingerprints (table; fig. 2) indicates that PCR fingerprinting is also suitable for genotyping sequential *H. influenzae* isolates obtained during persistent and recurrent infections.

In conclusion, PCR fingerprinting of genomic DNA is a simple, reliable method for genotyping non-capsulate *H. influenzae* isolates. Combined with MOMP analysis, PCR fingerprinting allows the identification of antigenic strain variants appearing during persistent infections. PCR fingerprinting and RFLP analysis may be combined to facilitate identification of closely related isolates as they focus on different parts of the chromosome. Although the epidemiological findings from PCR fingerprinting are similar to those from RFLP data, PCR fingerprints are easier to interpret.

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