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PCR for Capsular Typing of *Haemophilus influenzae*

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A PCR method for the unequivocal assignment of *Haemophilus influenzae* capsular type (types a to f) was developed. PCR primers were designed from capsule type-specific DNA sequences cloned from the capsular gene cluster of each of the six capsular types. PCR product was amplified only from the capsular type for which the primers were designed. Product was confirmed by using either an internal oligonucleotide or restriction endonuclease digestion. A total of 172 *H. influenzae* strains of known capsular type (determined genetically) comprising all capsular types and noncapsulate strains were tested by PCR capsular typing. In all cases the PCR capsular type corresponded to the capsular genotype determined by restriction fragment length polymorphism analysis of the *cap* region. When used in conjunction with PCR primers derived from the capsular gene *bexA*, capsulate, noncapsulate, and capsule-deficient type b mutant strains could be differentiated. PCR capsular typing overcomes the problems of cross-reaction and autoagglutination associated with the serotyping of *H. influenzae* strains. The rapid and unequivocal capsular typing method that is described will be particularly important for typing invasive *H. influenzae* strains isolated from recipients of *H. influenzae* type b vaccine.

Capsulate *Haemophilus influenzae* isolates express one of six chemically distinct capsular polysaccharides (types a to f) (17). Until the implementation of *H. influenzae* type b vaccination, more than 95% of *H. influenzae* disease was caused by *H. influenzae* type b (21), but as the incidence of *H. influenzae* type b disease decreases (1, 5), the relative importance of the other capsular types (a and c to f) and noncapsulate (NC) strains will increase. At present, serotyping by slide agglutination is the most commonly used method of identifying the capsular type of *H. influenzae* isolates. However, this method has been shown to be unreliable (6, 20, 23). Therefore, there is a need for an unequivocal *H. influenzae* capsular typing method. The detection of capsule type-specific genes would provide an unambiguous capsular typing scheme. This would be particularly valuable for the capsular typing of the *H. influenzae* organisms isolated from vaccine recipients.

A region of the chromosome, termed *cap*, contains the gene cluster necessary for capsule expression in *H. influenzae* (4). The *cap* loci in all six capsular types share a common organization consisting of three regions, regions 1 to 3; regions 1 and 3 are common to all types and flank region 2, which is capsule type specific (7, 14). A DNA-based method of differentiating the capsular types of *H. influenzae* has been developed (10). It relies on the hybridization of *capB* regions 1 and 3, which is contained in a DNA probe (pUO38), and produces type-specific patterns on Southern blots of electrophoretically separated *Eco*RI digests of total cellular DNA from capsulate *H. influenzae* isolates. Spontaneously occurring capsule-deficient mutants of type b strains (b^- strains) can also be identified. The usually duplicated *capB* locus can undergo a recombinational event resulting in a single copy of *cap* genes and the loss of a gene (*bexA*) necessary for capsule expression (11). These b^- strains are indistinguishable from NC strains by traditional serotyping techniques but can be differentiated by pUO38 capsular genotyping (10). Capsular genotyping has proved to be of use in the characterization of *H. influenzae* strains (8, 10), but the requirement for DNA extraction and Southern blotting and hybridization limits its usefulness to reference centers.

H. influenzae capsular genes have been successfully amplified by PCR. PCR primers designed from the *bexA* DNA sequence (12) by van Ketel et al. (22) have been used to distinguish capsulate *H. influenzae* isolates from NC strains. However, these primers (HI-I and HI-II) cannot be used to distinguish between different capsular types or between b^- and NC strains. We describe the application of PCR to the detection of type-specific DNA in all six capsular types of *H. influenzae* and the application of this technique to differentiate between b^- and NC *H. influenzae* isolates.

This work was presented as a poster at the 93rd General Meeting of the American Society for Microbiology, Atlanta, Ga., 1993 [6a].)

MATERIALS AND METHODS

Strains and culture media. *H. influenzae* RM125, RM135, and RM127 to RM130 were used. They are well-characterized laboratory transformants of the capsule-deficient strain Rd^-/b^-:02 to capsular types a to f, respectively (24, 25). One hundred seventy-two isolates comprising NC strains and all capsular types were selected from the Haemophilus Reference Laboratory, Oxford Public Health Laboratory, and Department of Paediatrics, Oxford University collections (Table 1). Twelve *Haemophilus* spp. and an *Actinobacillus* sp. control strain were selected from the Haemophilus Reference Laboratory and comprised clinical isolates and strains from the

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TABLE 1. *H. influenzae* strains characterized by PCR capsular genotyping

Strain (reference)	pUO38 genotype[a]	Type-specific PCR primers	HI-1 and HI-2 PCR primers	PCR genotype
RM125 (23)	$Rd^-/b^-/a^+$	a +	+	a
RM107 (4)	a (N)	a +	+	a
RM7193 (10)	a (K)	a +	+	a
RM7189 (10)	a (T)	a +	+	a
2 CI[b]	a (T)	a +	+	a
RM135 (23)	Rb^+/:02	b +	+	b
Eagan (2)	b (G)	b +	+	b
16 CI	b (S)	b +	+	b
4 CI	b (G)	b +	+	b
4 CI	b^- (S-ive)	b +	−	b^-
2 CI	b^- (G-ive)	b +	−	b^-
RM127 (23)	$Rd^-/b^-/c^+$	c +	+	c
RM6129 (13)	c (1)	c +	+	c
RM6132 (13)	c (1)	c +	+	c
1 CI		c +	+	c
RM128 (23)	$Rd^-/b^-/d^+$	d +	+	d
RM6137 (13)	d	d +	+	d
6 CI		d +	+	d
RM129 (23)	$Rd^-/b^-/e^+$	e +	+	e
RM1170 (22)	e	e +	+	e
13 CI	e	e +	+	e
RM130 (23)	$Rd^-/b^-/f^+$	f +	+	f
RM1176 (22)	f (F)	f +	+	f
11 CI	f (F)	f +	+	f
3 CI	f (O)	f +	+	f
10 CI	NC[c]	−	−	NC
91 CI	NC[d]	−	−	NC

[a] Nomenclature for capsular genotype as described by Musser et al. (15).
[b] CI, clinical isolate.
[c] NC strains subjected to PCR with capsule-specific primer pairs a to f.
[d] NC strains subjected to PCR with type b-specific primers alone.

National Collection of Type Cultures (NCTC) (Table 2). *H. influenzae* isolates were cultured on chocolate agar consisting of heated Columbia agar (Unipath, Basingstoke, United Kingdom) containing 5% horse blood.

Source of capsule type-specific DNA. The plasmids pAD2 (14), pJSK77, and pJSK68 (14) were used as sources of type a, c, and d capsule type-specific DNAs, respectively. The type b-specific DNA sequence was obtained from the plasmids pCAP1 and pCAP2 (3). Type e-specific DNA was obtained from pFTe3, and type f DNA was obtained from pFTf1 and pFTf4 (7).

TABLE 2. *Haemophilus* control strains

Species	Reference
Haemophilus aegyptius	Clinical isolate
Haemophilus aphrophilus	NCTC 5906
Haemophilus avium	NCTC 11297
Haemophilus ducreyi	Clinical isolate
Haemophilus haemolyticus	NCTC 10839
Haemophilus parahaemolyticus	Clinical isolate
Haemophilus parainfluenzae biotype I	Clinical isolate
Haemophilus parainfluenzae biotype II	Clinical isolate
Haemophilus paraphrophilus	NCTC 10557
Haemophilus pleurohaemolyticus	Clinical isolate
Haemophilus parapleurohaemolyticus	Clinical isolate
Haemophilus segnis	Clinical isolate
Actinobacillus pleuropneumoniae	ATCC 27088

TABLE 3. Capsule type-specific PCR primers

Capsular type	Primer name[a]	Primer (5′ to 3′)
a	a1	CTACTCATTGCAGCATTTGC
	a2	GAATATGACCTGATCTTCTG
	a3	AGTGGACTATTCCTGTTACAC
b[b]	b1	GCGAAAGTGAACTCTTATCTCTC
	b2	GCTTACGCTTCTATCTCGGTGAA
	b3	ACCATGAGAAAGTGTTAGCG
c	c1	TCTGTGTAGATGATGGTTCA
	c2	CAGAGGCAAGCTATTAGTGA
	c3	TGGCAGCGTAAATATCCTAA
d	d1	TGATGACCGATACAACCTGT
	d2	TCCACTCTTCAAACCATTCT
	d3	CTCTTCTTAGTGCTGAATTA
e	e1	GGTAACGAATGTAGTGGTAG
	e2	GCTTTACTGTATAAGTCTAG
	e3	CAGCTATGAACAAGATAACG
f	f1	GCTACTATCAAGTCCAAATC
	f2	CGCAATTATGGAAGAAAGCT
	f3	AATGCTGGAGTATCTGGTTC
a to f	HI-1	CGTTTGTATGATGTTGATCCAGAC
	HI-2	TGTCCATGTCTTCAAAATGATG
	HI-3	TGATGAGGTGATTGCAGTAGG

[a] EMBL/GenBank/DDBJ nucleotide sequence library accession numbers: a1, Z33384; a2, Z33386; a3, Z33385; b1, b2, and b3, X78559; c1 and c3, Z33387; c2, Z33388; d1, d2, and d3, Z33389; e1, Z33390; e2, Z33391; e3, Z33392; f1, Z33393; f2 and f3, Z33394.
[b] The type b sequence was from Brophy (3).

Sequencing of type-specific DNA. Sequencing of capsule type-specific DNA cloned into pBluescript or pUC was performed by the dideoxy chain termination method of Sanger et al. (19) by using the Sequenase version 2.0 DNA sequencing kit (United States Biochemicals, Cleveland, Ohio) with the M13 universal forward and reverse primers (British Biotechnology, Abingdon, United Kingdom). PCR product was sequenced directly by using the Cyclist *Exo*$^-$ *Pfu* DNA Sequencing kit (Stratagene Cloning Systems, La Jolla, Calif.) (9).

Design of PCR primers. Primers of approximately 20 bp in length were designed; they had a melting temperature in the region of 60°C. Two primers (primers 1 and 2) were chosen for each capsular type listed in Table 3. A third internal primer (primer 3) was also designed for each capsular type (Table 1). The primers used to amplify *bexA* DNA (primers HI-1 and HI-2) were designed from the *bexA* sequence (12) by van Ketel et al. (22) (Table 3). A third, internal primer (primer HI-3) was designed from the sequence of *bexA* (12) to generate a confirmatory product when used with primer HI-1, which was based on the primer HI-III designed by van Ketel et al. (22).

Preparation of total cellular DNA. Purified total cellular DNA was extracted from isolates of *H. influenzae* by the method of Pitcher et al. (16). Rapid DNA extraction, adequate for amplification by PCR, was performed by boiling a sweep of eight colonies for 3 min in 50 μl of sterile distilled water. The cell debris was pelleted by centrifugation at 12,000 × *g* for 3 min, and 40 μl of the supernatant was removed.

TABLE 4. Primer sets, products, and restriction endonucleases involved in PCR capsular typing

Capsular type	First-round primer pair	Primary product (bp)[a]	Second-round primer pair	Secondary product (bp)	Restriction endonuclease	Secondary restriction endonuclease product (bp)
a	a1–a2	250	a2–a3	180	*Taq*I	130, 50
b	b1–b2	480	b2–b3	370	*Eco*RI	270, 220
c	c1–c2	250	c2–c3	200	*Taq*I	210, 30
d	d1–d2	150	d2–d3	100	*Mse*I	80
e	e1–e2	1,350	e2–e3	1,160	*Taq*I	800, 450
f	f1–f2	450	f1–f3	400	*Taq*I	340, 120

[a] PCR product sizes are given to the nearest 10 bp.

PCR. The amplification of target DNA by PCR was performed on the basis of the description of Saiki et al. (18). The reaction volume was 25 μl and contained 10 mM Tris-hydrochloride (pH 8), 50 mM KCl, 3.5 mM MgCl, 0.01% gelatin, 0.2 mM deoxyribonucleotides (Pharmacia P-L Biochemicals Inc., Milwaukee, Wis.), 1 μM (each) oligonucleotide primer (British Biotechnology), and 0.5 U of *Taq* polymerase (Advanced Biotechnologies, London, United Kingdom). To this was added 1.5 μl of template DNA. The mixtures were processed for 25 cycles in a programmable thermal cycler (PHC3; Techne, Cambridge, United Kingdom). The cycling parameters consisted of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C. Following the final cycle all reactions were incubated for an additional 10 min at 72°C.

Detection and confirmation of PCR products. A total of 10 μl of the PCR mixture was electrophoresed through 1.5% agarose in Tris-Cl–borate–EDTA buffer and was visualized by ethidium bromide staining. The sizes of the amplified products were compared with those of a positive control amplified product and a 1-kb molecular mass marker ladder (Gibco-BRL, Uxbridge, United Kingdom) or DNA molecular mass marker VIII (Boehringer Mannheim, Lewes, United Kingdom). Confirmation of the primary product was performed by a second round of PCR with the third, internal primer and one of the first-round pair of primers for 15 cycles under the same conditions used in the first round. The template for this reaction was a 10^3 dilution of the primary product. Confirmation of the primary product was also performed by restriction endonuclease digestion.

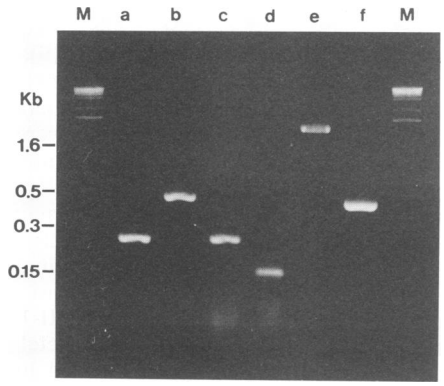

FIG. 1. Agarose gel electrophoresis of PCR products from *H. influenzae* capsular types a to f amplified with capsule type-specific primers. Lanes designated M contain 1-kb molecular mass markers. Lanes a to f contain PCR products from capsulate *H. influenzae* types a to f, respectively, each amplified with their respective specific PCR primers.

RESULTS

Between 200 and 360 bp of DNA sequence was obtained from the plasmids containing capsule type-specific DNA for each of the capsular types (data not shown) and was used to design the PCR primers. The three PCR primers designed for each of the capsular types are listed in Table 3 and are designated by the capsular type for which they were designed. They are termed 1, 2, or 3, where 3 is the primer internal to 1 and 2. The initial e3 primer designed from the sequence of pFTe3, a clone of RM129, failed to amplify product. A new e3 primer was designed from the sequence obtained by directly sequencing the e1-e2 PCR product of RM1170. RM1170 is a wild-type type e strain used as the source of the *capE* DNA used to create RM129.

Amplification of serotype-specific DNA from region 2 was first performed on total cellular DNA from the laboratory transformants in Rd^-/b^-/:02 to capsular types a to f. Each primer set amplified a product of the anticipated size from the capsular type for which it was designed (Table 4 and Fig. 1). In further experiments all six primer pairs failed to amplify product with the 12 *Haemophilus* spp. and the 1 *Actinobacillus* sp. listed in Table 2. For confirmation, the primary product was subjected to further PCR by using primer 3 with one of the first-round primers. These reactions produced specific products of the anticipated sizes (Table 4 and Fig. 2). Also, restriction endonuclease digestion of the primary products produced restriction fragments of the characteristic sizes for each of the capsular types (Table 4 and Fig. 3).

To ensure the reproducibility of the technique, 172 strains of *H. influenzae* were tested, including 163 clinical isolates (Table 1). Each primer set amplified product only from capsulate strains for which they were specifically designed. Sixty-five were capsulate and represented all six capsular types, and 107 were NC and represented a selection of strains. Six of these NC

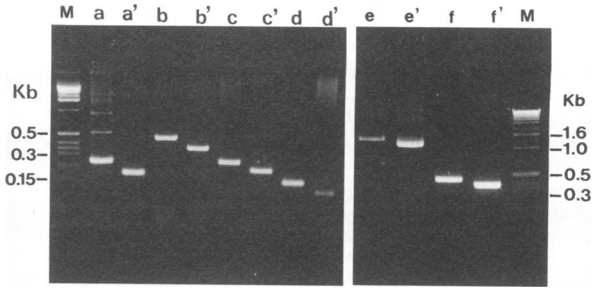

FIG. 2. Agarose gel electrophoresis of capsule type-specific primary and secondary PCR products from *H. influenzae* capsular types a to f. Lanes designated M contain a 1-kb molecular mass markers, lanes a to f contain the first-round capsule type-specific PCR products, and lanes a′ to f′ contain the respective secondary PCR products.

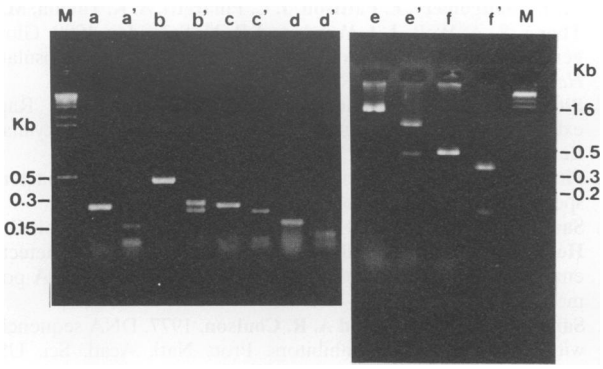

FIG. 3. Agarose gel electrophoresis of capsule type-specific primary products and their restriction endonuclease digestion products. Lanes labelled M contain molecular mass markers. Lanes a to f contain the first-round capsule type-specific PCR products, and lanes a' to f' contain the first-round products digested with restriction endonucleases. Products from types a, c, e, and f were digested with *Taq*I, products from type b were digested with *Eco*RI, and products from type d were digested with *Mse*I.

isolates were b^- strains, and the remainder (101 strains) were NC *H. influenzae.*

All capsular types produced PCR products of 343 bp when they were amplified with primers HI-1 and HI-2 as described previously (22). These products were confirmed by using a second round of PCR with primers HI-2 and HI-3, yielding a product of 181 bp (Fig. 4). The 101 NC *H. influenzae* isolates failed to produce product with HI-1 and HI-2, as did the 6 b^- isolates. However, b^- strains could be clearly recognized because they produced type b-specific product, but failed to yield product with the HI-1 and HI-2 primer set.

DISCUSSION

The technique of PCR capsular typing described here provides a rapid and unequivocal method for determining the capsular types of *H. influenzae* isolates. The method can be performed directly from colonies, and the capsule type specificity of the PCR products can be confirmed either by a second round of PCR or by restriction endonuclease digestion. Further characterization of the *cap* configuration by PCR amplification would be useful in the postvaccine era, and the development of such techniques is being pursued in the Public Health Laboratory, Oxford, laboratory.

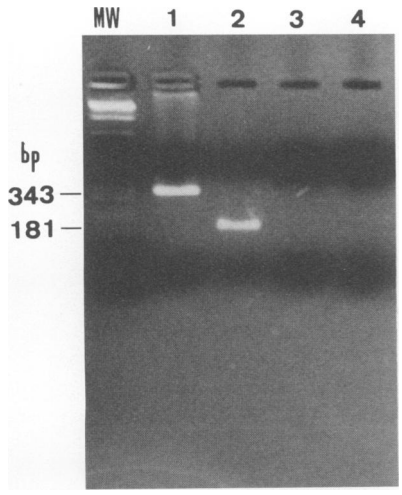

FIG. 4. Agarose gel electrophoresis of PCR products amplified from b and b^- strains of *H. influenzae* by using PCR primers HI-1, HI-2, and HI-3. Lane MW contains a 1-kb molecular weight marker. Lanes 1 and 3 contain primary products, and lanes 2 and 4 contain secondary PCR products amplified from a b and a b^- strain, respectively.

An unequivocal capsular typing method is particularly important for typing *H. influenzae* strains isolated from recipients of the *H. influenzae* type b vaccine. In this laboratory, a stepwise protocol that enables typing of >90% of isolates by two PCRs has been implemented. Strains are first amplified with HI-1 and 2 and type b-specific primers. By using these primers, capsulate and NC isolates can be differentiated; furthermore, type b, b^-, and NC strains can be recognized. For the rare isolates that are capsulate and not type b, five PCRs each with a type-specific primer set, sets a, c, d, e, and f, are performed. In this way the capsular genotypes of most *H. influenzae* isolates can rapidly be determined in comparison with the speed with which they could be determined by traditional methods. Also, this technique overcomes potential doubts which may arise from serological cross-reactions and allows potential vaccine failures to be characterized reliably. This is desirable for a precise assessment of the efficacies of *H. influenzae* type b vaccines.

Serologically, nontypeable strains of *H. influenzae* may have an NC or b^- capsular genotype. The latter have spontaneously descended from capsulate type b strains and may have been capsulate when they invaded the host. The role of b^- strains in the post-*H. influenzae* type b vaccine era is unclear. If they arise after invasion they would qualify as vaccine failures. However, b^- strains that arise before invasion may be an important source of NC invasive strains. In either event, an unequivocal typing method is important for detecting and determining the patterns of disease caused by these strains. In addition, b^- mutants potentially give confusing results when typed serologically. It has been shown that a large proportion of b^- strains cross-react with type-specific antisera and produce misleading results (6). There is a need to differentiate between b^- and NC *H. influenzae*, and as described here, this can be performed rapidly by using PCR capsular genotyping.

Capsular genotyping could also be applied to other species in which highly specific antisera need to be raised to differentiate the capsular types, such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Escherichia coli*. The development of a capsule type-specific PCR for these organisms would provide an alternative and unequivocal means of determining capsular type.

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