Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification

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Summary. Two primer sets were chosen for the detection of *Haemophilus influenzae* in cerebrospinal fluid by polymerase chain reaction (PCR) DNA amplification. One primer set was selected from sequences encoding a capsulation-associated protein and reacted with target DNA from all 15 capsulate *H. influenzae* strains (all serotypes) examined. The other primer set was selected from the DNA sequence of a gene encoding for outer-membrane protein P6 and reacted with the 15 capsulate and 10 non-capsulate strains of *H. influenzae* tested. This primer set also reacted with the closely related species *H. haemolyticus* and *H. aegyptius*, and with two of nine *H. parainfluenzae* strains. In reconstruction experiments, PCR DNA amplification was able to detect as few as five *H. influenzae* cells when 40 cycles of amplification were used. Two hundred cerebrospinal fluid (CSF) samples collected consecutively from patients suffering from meningitis were investigated by PCR; 40 were culture-positive for *H. influenzae* and 39 of these were also clearly positive in the PCR test with both primer sets. Contamination occurred to some extent with 40 cycles of amplification but was completely eliminated when the number of cycles was reduced to 35. We conclude that the two primer sets are appropriate for the detection of *H. influenzae* by PCR, each having its own specificity. When these two primer sets are used, PCR is a technique of equivalent sensitivity to culture for the detection of *H. influenzae* in CSF.

Introduction

In-vitro DNA amplification by the polymerase chain reaction (PCR)¹ is a new technology that has considerable implications for diagnostic microbiology because of its potentially exquisite specificity and sensitivity. Besides its application in the pre-natal diagnosis of genetic defects, published reports on the adaptation of PCR to the diagnosis of infectious diseases have focused initially on the detection of viral agents.^{2–4} More recently, PCR assays have been reported for the detection of *Legionella pneumophila*,⁵ leptospires,⁶ *Mycobacterium tuberculosis*⁷ and parasites such as *Toxoplasma gondii*⁸ and others. The pronounced sensitivity to contamination is a distinct drawback of PCR assays.⁹

Comparison of the sensitivity and specificity of PCR assays with well established microbial detection techniques is needed to determine the clinical usefulness of the new technology. For the diagnosis of *Haemophilus influenzae* meningitis, culture is well established and easily performed. Additional

information, i.e., the clinical presentation, the properties of the cerebrospinal fluid (CSF) and latex-based antigen detection assays, makes this illness a narrowly defined entity. Therefore, we chose this infectious disease to study the value of a PCR assay with two sets of primers as a diagnostic method. One primer set was selected from the nucleotide sequence encoding the outer-membrane lipoprotein P6, present in both capsulate and non-capsulate *H. influenzae* strains examined^{10,11} and would be expected to react with all *H. influenzae* strains. The second primer set was selected from sequences within the gene encoding the capsulation-associated Bex A protein,¹² a protein most probably involved in the intracellular transportation of the capsular polysaccharide, and would be expected to react only with capsulate *H. influenzae* strains.

The specificity of our primer sets for different species and types of *Haemophilus* and the sensitivity of the assay on isolated DNA and bacterial cells are described. We further report the results of the PCR assay as a diagnostic tool on 200 consecutive samples of CSF from patients with meningitis.

Materials and methods

Bacterial strains and CSF samples

Strains and CSF samples were submitted by bacteriologists throughout the Netherlands to the Netherlands Reference Laboratory for Bacterial Meningitis, which identified and serotyped the strains. Reference strains of *H. influenzae* (various subtypes) were obtained from Dr D. M. Granoff and have been described previously.¹³ The strains used are listed in table I.

Two hundred samples of CSF from patients suffering from meningitis were tested consecutively by PCR with a strict set of protocols, essentially as described by Kwok and Higuchi.⁹ Most CSF samples tested by PCR were supernates obtained after centrifugation of the clinical samples and, generally, no data were available on the numbers of bacteria cultured from the samples.

Preparation of DNA

For bacterial strains, DNA was extracted from overnight cultures as described earlier.¹⁴ For staphylococci an additional incubation with lysostaphin (Sigma) 250 µg/ml was included; for streptococci a treatment with mutanolysin (Sigma) 20 µg/ml was added. DNA was liberated from CSF by incubating the specimen for 10 min at 95°C followed by centrifugation (10 000 *g* for 1 min) and placing the tubes on wet ice. A 25-µl volume of each cerebrospinal fluid supernate was used for the PCR assay. The sensitivity of this procedure was

Table I. Results of PCR DNA amplification of *Haemophilus* spp. and other bacteria by two primer sets

Strain	Number tested	Reaction with primer set*	
		1 (BexA)	2 (P6)
<i>H. influenzae</i> serotype b	10	+	+
<i>H. influenzae</i> serotype a	1	+	+
<i>H. influenzae</i> serotype c	1	+	+
<i>H. influenzae</i> serotype d	1	+	+
<i>H. influenzae</i> serotype e	1	+	+
<i>H. influenzae</i> serotype f	1	+	+
<i>H. influenzae</i> non-typable	10	—	+
<i>H. parainfluenzae</i>	7	—	—
<i>H. parainfluenzae</i>	2	—	+
<i>H. aphrophilus</i>	3	—	—
<i>H. paraphrophilus</i>	3	—	—
<i>H. segnis</i>	1	—	—
<i>H. haemolyticus</i>	3	—	+
<i>H. aegyptius</i>	3	—	+

**E. coli* (2 strains), *S. aureus* (1), *N. meningitidis* serogroups A,B,C (3), *Str. pneumoniae* (2), *Str. agalactiae* (3), *S. typhimurium* (2), *L. monocytogenes* (2), and *S. epidermidis* (2) and 10 other genera of bacteria, not commonly involved in meningitis, gave negative reactions with both primer sets.

compared with the method described by Boom *et al.*¹⁵ In experiments with serial dilutions of *H. influenzae* cells the same heating technique was used.

Oligonucleotide primers

Primer set 1 was derived from the published sequence for the Bex A protein, a capsulation-associated protein.¹² Oligonucleotides were selected from this sequence by excluding homologous sequences of similar proteins of *Escherichia coli* and *Salmonella typhimurium*.¹² The amplified region contains the nucleotide numbers +277 to +619 of the protein-coding nucleic-acid sequence, which results in a length of the amplified product of 343 base-pairs. The oligonucleotides for primer set 1 were:

HI—I: 5'-CGTTTGTATGATGTTGATCCAGACT-3'
 HI—II: 5'-TGTCCATGTCTTCAAATGATG-3'
 HI—III (probe; nucleotide numbers +445 to +475):
 5'-GTGATTGCAGTAGGGGATTGCGC-
 TTTCAG-3'

Primer set 2 was derived from the published sequence for the outer-membrane protein P6 of *H. influenzae*.¹⁰ Oligonucleotides were selected from these sequences by excluding homologous sequences of a similar protein in *E. coli*.¹⁶ The amplified region contains the nucleotides +103 to +375 of the protein-encoding nucleic-acid sequence, making the length of the amplified products 273 base-pairs. The oligonucleotides for primer set 2 were:

HI—IV: 5'-ACTTTTGGCGGTTACTCTGT-3'
 HI—V: 5'-TGTGCCTAATTTACCAGCAT-3'
 HI—VI (probe; nucleotide numbers +217 to +243):
 5'-GCATATTAAATGCAACACCA-
 GCTGCT-3'

The oligonucleotides were custom synthesised by Eurosequence (Groningen, The Netherlands) in an Applied Biosystems DNA Synthesizer 380 B.

Amplification and detection of *H. influenzae* DNA

The amplification procedure with both primer sets was based on the description by Saiki *et al.*¹ and performed in a final volume of 100 µl. The reaction mixture contained 10 mM Tris-hydrochloride (pH 8.3), 50 mM KCl, 2 mM MgCl₂, gelatin 0.001%, 200 µM each of the deoxyribonucleotides, approximately 0.35 µM each of the oligonucleotides, Taq polymerase (Perkin-Elmer Cetus) 2.5 units, to which were added samples containing isolated *H. influenzae* DNA 10 ng, isolated DNA of other bacteria 50 ng, or 25 µl of CSF. The mixtures were processed in a programmable DNA thermal cycler (Perkin-Elmer Cetus) and subjected to 35 or 40, if indicated, cycles of amplification. One cycle consisted of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. After the final cycle, the tubes were incubated for a further 8 min at 72°C. Electrophoresis was carried out on 10 µl of amplified sample in agarose 2% gels with a 123-bp DNA ladder

(BRL, Bethesda Research Laboratory) as a mol.-wt marker. DNA was transferred on to nylon filters (Zetaprobe; Bio-Rad) under alkaline conditions (0.4 N NaOH). Hybridisation was done at 55°C overnight in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium-citrate), sodium dodecyl sulphate (SDS) 0.5%, Ficoll-polyvinylpyrrolidone-BSA 0.1% (5× Denhardt's solution), 100 µg/ml of sheared, denatured salmon sperm DNA and ³²P-5'-labelled probe oligonucleotides HI-III for primer set 1 and HI-VI for primer set 2. After hybridisation, the blots were washed twice with 2× SSC and SDS 0.1% at room temperature for 10 min and twice with 2× SSC and SDS 0.5% at 55°C for 30 min. Membranes were finally exposed to radiographic films for 16 h in cassettes.

Results

Specificity of PCR with the two primer sets

The results of experiments performed to analyse the specificity of primer sets 1 and 2 for *Haemophilus* spp. are listed in table I. Primer set 1, which amplifies a sequence within the gene encoding the capsulation-associated Bex A protein reacted with capsulate *H. influenzae* strains (serotypes a-f). Primer set 2 (outer-membrane protein P6 derived) reacted with both capsulate and non-capsulate strains of *H. influenzae* and with strains of *H. haemolyticus* and *H. aegyptius*, which are closely related to *H. influenzae*.¹⁷ Two of the nine strains of *H. parainfluenzae* examined also reacted with primer set 2 and hybridised with probe HI-VI. All other genera of bacteria examined gave negative reactions with both primer sets (table I).

Human (placenta) DNA did not react with primer set 1, but amplification with primer set 2 resulted in three faintly visible bands of incorrect sizes, that did not hybridise with probe HI-VI.

The reaction of both primer sets with different types of *H. influenzae* is shown in fig. 1. Lower signal intensities were noticed with primer set 1 for some serotypes other than serotype b and for the *H. influenzae* serotype b strain 17H, presented in lane 4. This *H. influenzae* b subtype 17H strain is known to belong to division II of *H. influenzae*,¹⁸ a small cluster of *H. influenzae* strains distantly related to the much more common division I strains. The lower signal intensities were attributed to the imperfect matching of the oligonucleotides to their target DNA.

Sensitivity of PCR with the two primers

The sensitivities of the PCR with both primer sets were investigated with serial dilutions of

Fig. 1. PCR DNA amplification of strains of *H. influenzae* (10 ng DNA) with primer set 1 (Bex A) (upper panel) and primer set 2 (P6) (lower panel). Lane 1: 123 bp DNA ladder (mol. wt marker); 2: *H. influenzae* type b Minna ST 1H; 3: *H. influenzae* type b 649 ST 1L; 4: *H. influenzae* type b Rab ST 17H; 5: *H. influenzae* type a (ref); 6: *H. influenzae* type b (ref); 7: *H. influenzae* type c (ref); 8: *H. influenzae* type d (ref); 9: *H. influenzae* type e (ref); 10: *H. influenzae* type f (ref); 11 and 12: *H. influenzae* type b (Netherlands isolates); 13-22: *H. influenzae* non-typable (Netherlands isolates).

isolated DNA and with bacterial cells of an *H. influenzae* serotype b strain with 40 amplification cycles. Serial dilutions of DNA gave, reproducibly, a lower limit of detection of 0.05 pg for primer set 1 and 0.01 pg for primer set 2. The lowest numbers of bacterial cells detected, as determined by serial dilution, plating of samples on agar and counting of colonies, were, respectively, 50 cfu for primer set 1 and 5 cfu for primer set 2 (fig. 2). On visual inspection, Southern blot analysis did not appreciably increase sensitivity compared with agarose gel electrophoresis for both primer sets (fig. 2). Comparison of the heating technique used with the

Fig. 2. Sensitivity of PCR (40 cycles) on serially diluted bacterial cells of *H. influenzae* type b (ref). Lanes 1–6: primer set 1 (Bex A); 7–12: primer set 2 (P6). Lanes 1 and 7: 5×10^4 cfu; 2 and 8: 5×10^3 cfu; 3 and 9: 5×10^2 cfu; 4 and 10: 50 cfu; 5 and 11: 5 cfu; 6 and 12: 0.5 cfu. Upper panel: agarose gel 2% electrophoretic analysis. Lower panel: Southern blot analysis with HI–III (lanes 1–6) and HI–VI (lanes 7–12) as probes for hybridisation.

method described by Boom *et al.*,¹⁵ which makes use of chaotropic agents and silica particles, revealed that both DNA purification methods were equally sensitive in our tests (data not shown). With the method described by Boom *et al.*,¹⁵ the yield of DNA recovered after purification was approximately 50%.¹⁵

PCR on CSF specimens

A total of 200 consecutive CSF samples (generally supernates) were tested by PCR with both primer sets. The culture results of these samples are listed in table II. Initially, 40 cycles of amplification were used. This resulted in 5% positive PCR signals with one or both primer sets in CSF samples from which bacteria other than *H. influenzae* or no bacteria were cultured. Those CSF samples gave

faint, hardly visible bands of correct sizes that hybridised with the probes. The difference in band intensities between truly and most likely PCR false-positive CSF samples after 40 cycles of amplification is shown in fig. 3.

From these results and the data from Kwok⁹ we assumed that the false-positive reactions were due to contamination. Therefore, after 50 consecutive samples, the protocol for the following 150 samples was switched to 35 cycles of amplification, which resulted in some reduction of band intensity in *H. influenzae* culture-positive samples but also in the complete disappearance of signals in other CSF samples. All CSF samples that were positive for *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Str. agalactiae*, *Listeria monocytogenes*, *Staphylococcus aureus*, *S. epidermidis* and other genera of bacteria on culture and those with negative culture results gave negative results in the PCR test with both primer sets. From the 40 CSF samples that

Table II. Culture results of 200 consecutive CSF samples from patients suffering from meningitis

Bacterial species	Number of isolates
<i>H. influenzae</i>	40
<i>N. meningitidis</i>	47
<i>Str. pneumoniae</i>	16
<i>Str. agalactiae</i>	3
<i>L. monocytogenes</i>	1
<i>S. aureus</i>	3
<i>S. epidermidis</i>	4
Other species	16
Culture negative	70
Total	200

Fig. 3. PCR (40 cycles) DNA amplification of 10 CSF samples with primer set 1 (Bex A). Lane 1: 123 bp DNA ladder (mol. wt marker); 2–11: amplified CSF. The culture results of these samples were: lane 2: *N. meningitidis*; 3: *N. meningitidis*; 4: *E. coli*; 5: *Str. pneumoniae*; 6: *N. meningitidis*; 7–11: *H. influenzae* type b.

were culture-positive for *H. influenzae*, serotype b was isolated from 38 and non-typable strains from two. Thirty-nine of the CSF samples that were culture-positive for *H. influenzae* were PCR positive; 37 gave positive results with both primer sets and two, the samples from which non-typable *H. influenzae* strains were isolated, were positive only with primer set 2. From the PCR-negative, culture-positive sample, only a single colony of *H. influenzae* type b was grown. On the other hand, some of the other CSF samples were known to be also culture-positive but with only a few colonies of *H. influenzae*, yet were, nevertheless, PCR-positive, producing clearly visible bands of correct sizes on agarose gels with both primer sets.

Discussion

PCR DNA amplification is a technology that can be adapted easily to identify micro-organisms involved in infectious diseases. The exquisite specificity and sensitivity make the technique very attractive as a diagnostic method. Unfortunately, it is reported to be very susceptible to contamination in most laboratories.⁹ We also were confronted with contamination, leading, after 40 amplification cycles, to amplified DNA of the correct size that hybridised with our probes in 5% of *H. influenzae* culture-negative samples. By reducing the number of cycles to 35 and strengthening adherence to a strict set of protocols, problems caused by contamination in the CSF samples were subsequently avoided.

Studies on isolated DNA and bacterial cells, both revealed a reproducible sensitivity of about 10–50 *H. influenzae* cells (or its DNA equivalent), primer set 1 consistently being somewhat less sensitive than primer set 2. These sensitivities were achieved with agarose gel 2% electrophoresis and were not increased appreciably by Southern blot analysis with the ³²P-labelled probes HI–III and HI–VI. Slot blot or dot blot analysis may increase the sensitivity as has been described, e.g., for *L. pneumophila*.⁵

The two primer sets used in PCR for the detection of *H. influenzae* had different specificities. Primer set 1 was derived from a capsulation-associated gene¹² and reacted with *H. influenzae* serotype b strains and with all other serotypes, although to a

variable extent, but not with non-capsulate *H. influenzae* strains (table I). This was not unexpected as genes, similar to the Bex A gene of serotype b, have been described in the other serotypes of *H. influenzae*.¹² Primer set 2, derived from the gene for outer-membrane lipoprotein P6,^{10,11} reacted with all *H. influenzae* strains and also with such closely related species as *H. haemolyticus* and *H. aegyptius*. This is in agreement with previous results obtained with monoclonal antibodies, showing that these three species have the same outer-membrane protein P6 epitope.¹⁹ Somewhat unexpected was the reaction of this primer set with two of the nine *H. parainfluenzae* strains, but it was known that the same monoclonal antibodies also react with a minority of *H. parainfluenzae* strains.¹⁹ This probably indicates that some strains of *H. parainfluenzae* have a protein related to the lipoprotein P6 of *H. influenzae*.

PCR DNA amplification showed a sensitivity for the detection of *H. influenzae* in CSF from patients with bacterial meningitis similar to culture. This sensitivity was superior to that of antigen detection in CSF by immuno-electrophoresis or latex-agglutination:²⁰ the two CSF samples with noncapsulated *H. influenzae* strains and nine of the 38 *H. influenzae* serotype b culture-positive CSF samples gave negative reactions in these antigen detection tests. For PCR, mostly supernates from CSF were used, whereas sediments were used for culture. In the few samples for which cerebrospinal fluids *in toto*, were used for both techniques, PCR was positive even in the presence of only a few cfu as determined by culture. Furthermore, PCR was positive with two CSF samples from patients with *H. influenzae* meningitis who had been treated for several days with antibiotics and for which culture results had become negative (data not shown).

We conclude that the PCR DNA amplification is equivalent to culture in respect of the sensitivity of detection of *H. influenzae* in CSF. It appears to be a matter of preference and secondary benefits (e.g., antibiotic sensitivity tests) whether to choose to detect *H. influenzae* in this infectious disease by culture or PCR DNA amplification. Moreover the PCR method is valuable for identification of *H. influenzae* if cultures are negative because of antibiotic treatment before sampling; the two different primer sets make distinction between capsulate and noncapsulate *H. influenzae* possible.

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