ORIGINAL ARTICLE 10.1111/j.1469-0691.2005.01109.x

Simultaneous single-tube PCR assay for the detection of *Neisseria meningitidis*, *Haemophilus influenzae* type b and *Streptococcus pneumoniae*

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ABSTRACT

Rapid, accurate and inexpensive diagnosis of bacterial meningitis is critical for patient management. This study describes the development and evaluation of a multiplex PCR assay for the detection of *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b, which globally account for 90% of cases of bacterial meningitis. The single-tube assay, based on the *ctrA*, *ply* and *bex* targets, respectively, enabled detection of 5–10 pg DNA. When the assay was tested with clinical samples (n = 425), its sensitivity for the three targets was 93.9%, 92.3% and 88%, respectively, while the overall specificity and positive predictive value of the assay was 100%. The negative predictive value was 99.1–99.5%. The methodology permits rapid and accurate detection of the three main pathogens that cause bacterial meningitis.

Keywords Diagnosis, *Haemophilus influenzae*, meningitis, *Neisseria meningitidis*, PCR, *Streptococcus pneumoniae*

Original Submission: 6 October 2004; **Revised Submission:** 9 December 2004; **Accepted:** 23 December 2004

Clin Microbiol Infect 2005; 11: 386–390

INTRODUCTION

Meningitis receives a high level of medical, public and media attention because of its rapid onset and high level of morbidity and mortality [1]. The rapid progression of symptoms and potentially devastating effects of this disease necessitate early recognition and immediate treatment [2]. *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b are the main causes of bacterial meningitis [3–5], globally accounting for 90% of reported cases of acute bacterial meningitis in infants and children aged >60 days [6].

Bacterial pathogens with increased virulence and transmissibility have highlighted the importance of effective methods for rapid identification and epidemiological surveillance. Rapid identification has important public health implications, particularly with regard to case contact management, detecting and evaluating clusters of cases, and intervening in outbreaks. Developments in meningococcal and pneumococcal polysaccharide–protein conjugate vaccines have increased the need for accurate laboratory confirmation of these infections in order to monitor the effects of vaccine implementation and their continuing efficacy [7]. Until the introduction of the meningococcal serogroup C vaccine, the incidence of confirmed cases of meningococcal disease continued to increase during a time of raised public and medical awareness and improved laboratory techniques [8,9]. For many years, epidemiological investigations of outbreaks of meningococcal disease have relied on the serological characterisation of isolates. This culminated in the development of a characterisation scheme that included the capsular polysaccharide (serogroup) and two of the major outer-membrane protein antigens, PorA and PorB [10].

More recently, PCR-based identification methods have been used by the National Meningococcal Reference Laboratory in Greece to confirm cases of *N. meningitidis*, especially those in which early antibiotic treatment prevents detection by culture [11]. Target genes investigated for identification of *N. meningitidis* in cultured microorganisms or clinical specimens from infected

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patients include IS*1106* [12], *porA* [13] and 16S rRNA [14]. PCR assays have also been developed for the conserved regulatory gene, *crgA* [15], and the *ctrA* gene [16], which is involved in transport of capsular polysaccharide [17]. Advances in real-time amplification technology have enabled selective amplification of multiple genes in one reaction by utilising spectrally distinct phosphoramidite dye-labelled probes [18]. Despite its high efficiency, this method is expensive and technically challenging; thus its routine use is restricted.

The present study describes the development and evaluation of a single-tube multiplex PCR for the simultaneous detection of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* type b in clinical samples as a tool for improved non-culture diagnosis of the three major causes of bacterial meningitis.

MATERIALS AND METHODS

Patient specimens

During a 2-year period (2002–2003), 425 samples from 336 patients were sent to the National Meningococcal Reference Laboratory, Athens, from different hospitals throughout Greece. These consisted of 227 blood samples, 194 cerebrospinal fluid (CSF) samples, three pleural samples and one bronchial sample. Details of the collection of clinical data and samples have been published previously [11].

Patient samples were classified as follows: (1) culture-confirmed cases with one of the three bacterial targets isolated from either blood and/or CSF (53 patients; 36 blood samples, 32 CSF samples, three pleural fluid samples and one bronchial fluid sample); (2) culture-negative cases, but with Gram-negative or -positive cocci visualised in a direct smear, or antigen detected in CSF (14 patients; nine blood samples, 12 CSF samples); (3) clinically suspected cases of bacterial meningitis, but with culture and other tests yielding negative results (54 patients; 41 blood samples, 33 CSF samples); (4) cases with clinically diagnosed bacterial meningitis caused by other bacteria (65 patients; 33 blood samples, 41 CSF samples), identified as *Brucella* ($n = 1$), *Listeria monocytogenes* ($n = 4$), *Staphylococcus aureus* ($n = 7$), *Staphylococcus epidermidis* ($n = 5$), group A streptococci ($n = 7$), group B streptococci ($n = 6$), *Cryptococcus* ($n = 2$), amoebae ($n = 1$), *Escherichia coli* ($n = 10$), *Klebsiella pneumoniae* ($n = 12$), *Enterococcus faecalis* ($n = 6$), *Citrobacter koseri* ($n = 3$), *Salmonella* Typhimurium ($n = 4$), *Acinetobacter baumannii* ($n = 2$) and *Enterobacter cloacae* ($n = 4$); and (5) cases with clinically diagnosed viral meningitis (150 patients; 108 blood samples, 76 CSF samples).

Bacteria

All bacterial isolates from patient samples were sent to the National Meningococcal Reference Laboratory by each referring hospital for further identification. Bacterial isolates from blood or CSF samples were cultured on chocolate and/or blood agar and grown at 37°C in CO_2 5% v/v. Latex agglutination kits (Pastorex; Sanofi Diagnostics, Marnes-la-Coquette, France; or Wellcogen Bacterial Antigen Kit; Abbott Murex, Dartford, UK) were used for detection of antigens in CSF.

DNA isolation

Bacterial colonies were suspended in 500 μL of sterile double-distilled H_2O, vortexed, boiled for 15 min, and centrifuged at 20 000 *g* for 12 min. The supernatant was retained and the DNA concentration was estimated spectrophotometrically.

DNA was extracted from whole blood samples with Nucleospin kits (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

CSF, pleural or bronchial fluid samples were processed as described by Zambarti *et al.* [19]. In brief, 500 μL of sample was centrifuged at 1700 *g* for 10 min. The supernatant was discarded to leave 150 μL, which was added to a mixture of 650 μL of sterile double-distilled H_2O and 150 μL of Chelex/Tween-80 buffer. The samples were then heated at 100°C for 30 min, and centrifuged at 10 000 *g* for 8 min.

PCR amplification

Amplification reactions (50 μL) contained 0.6 μM each oligonucleotide primer (Sigma-Aldrich, Seezle, Germany), 3 mM $MgCl_2$, 200 μM dNTPs, 1× PCR buffer, 1 U of *Taq* DNA polymerase (Abgene, Epsom, UK) and 20 μL of DNA sample. PCR conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 25 s, 57°C for 40 s and 72°C for 1 min in a Robocycler Gradient 96 Cycler (Stratagene, La Jolla, CA, USA). Amplicons were visualised under UV fluorescence following electrophoresis in agarose 3.5% w/v gels and staining with ethidium bromide. Positive controls of DNA from standard strains of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*, as well as negative controls, were included in each assay.

Specific primers for the *N. meningitidis ctrA* gene, the *H. influenzae bex* gene and the *S. pneumoniae ply* gene were based on those described previously [18], which gave amplicons of 110, 99 and 80 bp, respectively. However, in order to distinguish more readily between the amplicon sizes by electrophoresis, a new forward *bex* primer was designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi) [20] to give amplicon sizes of 181, 110 and 80 bp, respectively (Table 1).

Serial dilutions of spectrophotometrically quantified DNA (500–1 pg/50-μL reaction) from each organism were amplified with the individual and multiplex assays to determine the sensitivities of the assays. Specificities were tested with DNA extracted from *Pseudomonas aeruginosa*, β-haemolytic streptococci (groups A and B), *L. monocytogenes*, *E. cloacae*, *Staph. epidermidis*, *A. baumannii*, *Staph. aureus* and *Neisseria lactamica* ($n = 10$).

RESULTS

PCR amplification

Amplification of each target was unaffected by the presence of DNA (500–1 pg) from the other

Microorganism	Gene amplified	Sequence (5′ → 3′)	Amplicon size
Haemophilus influenzae type b	*bex*	Forward: TATCACACAAATAGCGGTTGG Reverse: GGCCAAGAGATACTCATAGAACGTT	181 bp
Neisseria meningitidis	*ctrA*	Forward: GCTGCGGTAGGTGGTTCAA Reverse: TTGTCGCGGATTTGCAACTA	110 bp
Streptococcus pneumoniae	*ply*	Forward: TGCAGAGCGTCCTTTGGTCTAT Reverse: CTCTTACTCGTGGTTTCCAACTTGA	80 bp

Table 1. Oligonucleotides used in multiplex PCRs to identify the three main microorganisms that cause bacterial meningitis

two organisms. In multiplex reactions, the detection limits for *H. influenzae*, *N. meningitidis* and *S. pneumoniae*, as determined in repeated experiments, were 5 pg, 5 pg and 10 pg, respectively.

The specificity of the assay was tested with DNA extracts from *N. lactamica* and eight organisms likely to be present in CSF and blood samples. Products of 310 bp were amplified from two isolates of *N. lactamica*, but these could be distinguished easily from the 110-bp *N. meningitidis* product.

Fig. 1 shows examples of the results obtained when the multiplex PCR was applied to clinical samples. In total, 425 samples from 336 patients were tested. Table 2 summarises the results obtained, grouped according to the organism involved and the patient category.

Sensitivity and specificity

The sensitivity of the multiplex PCR assay was evaluated with data from culture-confirmed and probable cases (categories 1 and 2, respectively) for each organism individually (Table 3). Sensitivity ranged from 88% to 93.9%, with positive predictive values and negative predictive values of 100% and >99%, respectively.

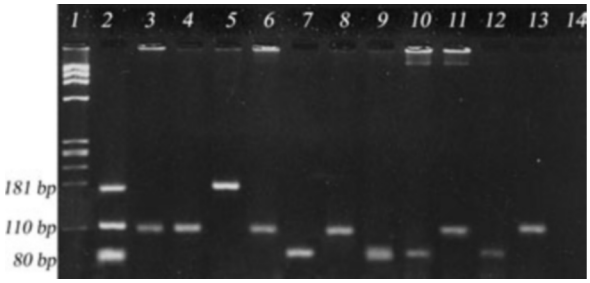

Fig. 1. Examples of the results obtained when the multiplex PCR assay was applied to clinical samples, showing detection of *Haemophilus influenzae* type b (181 bp), *Neisseria meningitidis* (110 bp) and *Streptococcus pneumoniae* (80 bp). Lanes: 1, φX174 *Hae*III digest; 2, positive control for all three targets; 3–13, clinical samples from blood (lanes 3, 6, 10, 11) or CSF (lanes 4, 5, 7, 8, 9, 12 and 13); 14, negative control.

The specificity of the assay was evaluated in relation to data from 215 patients classified as suffering from meningitis caused by viruses (n = 150) or other bacteria (n = 65). No amplicons were obtained with any of the samples from these patients, suggesting that the specificity of the assay was 100%.

DISCUSSION

The present study combined primers for the detection of the *ctrA* gene of *N. meningitidis*, the *ply* gene of *S. pneumoniae* and the *bexA* gene of *H. influenzae* in a multiplex PCR assay. These genes have been used previously for the clinical diagnosis of meningitis caused by these three bacteria [16,21,22], but the present study demonstrated that these three organisms could be detected simultaneously in a single tube assay. After optimisation and evaluation with DNA extracts from purified cultures, the multiplex assay was applied to clinical samples known to contain small amounts of bacterial DNA in the presence of large amounts of human genomic DNA, as well as inhibitors (e.g., haem in blood) [23,24]. *N. meningitidis* and *H. influenzae* type b DNA was amplified efficiently when present at 5 pg/reaction, while the detection limit for *S. pneumoniae* DNA was 10 pg/reaction; however this did not appear to affect the detection of *S. pneumoniae* in clinical samples.

The sensitivity of the *N. meningitidis* reaction was 93.9%, compared to figures of 88.4–91% reported previously [18,25]. This sensitivity was calculated from the results for clinical samples in categories (1) and (2). In category (1), the PCR-negative samples were collected 48 h following antibiotic treatment. In category (2), all the samples were PCR-positive.

The sensitivity of the *S. pneumoniae* reaction was 92.3%, compared to 91.8% in a previous

Table 2. Detection of bacterial DNA in clinical samples, grouped according to the organism involved and the patient category

	Culture-confirmed		Probable		Suspected	
Organism	**Positive PCR**	**Negative PCR**	**Positive PCR**	**Negative PCR**	**Positive PCR**	**Negative PCR**
Neisseria meningitidis						
Patients (n = 60)	25/27	2/27	6/6	0	18/27	9/27
Samples (n = 85)	(blood = 21; CSF = 15)	(blood = 2; CSF = 2)	(blood = 4; CSF = 5)		(blood = 13; CSF = 6)	(blood = 9; CSF = 8)
Streptococcus pneumoniae						
Patients (n = 48)	17/18	1/18	7/8	1/8	17/22	5/22
Samples (n = 66)	(blood = 11; CSF = 7;) pleural = 3; bronchial = 1)	(blood = 1; CSF = 1)	(blood = 1; CSF = 6)	(blood = 4; CSF = 1)	(blood = 9; CSF = 11)	(blood = 5; CSF = 5)
Haemophilus influenzae type b						
Patients (n = 13)	7/8	1/8	0	0	5/5	0
Samples (n = 16)	(blood = 1; CSF = 6)	(blood = 0; CSF = 1)			(blood = 5; CSF = 3)	
Other bacteria						
Patients (n = 65)	0	20/20	0	0	0	45/45
Samples (n = 74)		(blood = 9; CSF = 11)				(blood = 24; CSF = 30)
Virus						
Patients (n = 150)	0	2/2	0	148/148	0	0
Samples (n = 184)		(blood = 1; CSF = 2)		(blood = 107; CSF = 74)		

CSF, cerebrospinal fluid.

Table 3. Sensitivity of the multiplex PCR assay for detection of the three individual targets

	PCR-positive patients (samples)				
Organism	**Culture confirmed**	**Probable**	**Sensitivity**	**PPV**	**NPV**
Neisseria meningitidis	25/27 (36/40)	6/6 (9/9)	93.9%	100%	99.1%
	92.6% (90%)	100% (100%)	(95% CI 86.8–93.9)	(95% CI 92.4–100)	(95% CI 98–99.1)
Streptococcus pneumoniae	17/18 (22/24)	7/8 (7/12)	92.3%	100%	99.1%
	94.4% (91.7%)	87.5% (58.3%)	(95% CI 83.4–92.3)	(95% CI 90.3–100%)	(95% CI 98–99.1%)
Haemophilus influenzae type b	7/8 (7/8)	0 (0)	88%	100%	99.5%
	87.5% (87.5%)		(95% CI 63.9–88)	(95% CI 73–100)	(95% CI 98.7–99.5)

PPV, positive predictive value; NPV, negative predictive value.

study [18]. The sensitivity of the reaction did not depend on the specimen type, whereas the sensitivities were 100% and 69% for CSF and blood samples, respectively, in a previous study [26]. However, this gene target has been reported to be less specific than some commercially available kits [27] and may lead to overestimation of the number of positive samples.

The lowest sensitivity of the three reactions was recorded for the *H. influenzae* type b *bex* gene. Seven (87.5%) of eight culture-confirmed cases were PCR-positive, in contrast to a previous study, in which the sensitivity was 100% [18]. The clinical sample that was PCR-negative was collected 3 days after antibiotic treatment. There was a low number of *H. influenzae* type b cases because of use of the Hib vaccine in Greece since 1992. The overall specificity of the assay was calculated to be 100%. Since cross-reactions between *N. meningitidis* and several other species have been reported in antigen detection assays [28], and with *N. lactamica* isolates in molecular assays [16], the present study included ten *N. lactamica* isolates from carriers in the tests for specificity. Amplification products of 310 bp were amplified from two *N. lactamica* isolates, but these could be distinguished easily from the 110-bp *N. meningitidis* amplicon. The positive predictive value of the assay for all three targets was 100%, and the negative predictive value was 99.1–99.5%.

Overall, this single-tube PCR assay is a simple, reliable and easily implemented method for the confirmation of bacterial meningitis, and is a rapid and cost-effective way of analysing large numbers of samples. The development of the multiplex PCR assay for non-culture detection of meningococcal DNA will enable confirmation of cases, particularly when early treatment with antibiotics impairs detection by culture. This is an important step towards more accurate recognition and surveillance of bacterial meningitis infections.

ACKNOWLEDGEMENTS

The authors would like to thank all the microbiologists and clinicians who sent samples. The work was supported by a grant from the Hellenic Centre for Disease Control (KEEL).

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