ORIGINAL ARTICLE 10.1111/j.1469-0691.2005.01327.x

Dynamics of PCR-based diagnosis in patients with invasive meningococcal disease

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ABSTRACT

Invasive meningococcal disease continues to be a life-threatening condition and rapid diagnosis is important for the administration of appropriate treatment. This study focused on the use of PCR for the diagnosis of meningococcal aetiology and the dynamics of PCR-based diagnosis over time in various biological samples. Sixty cerebrospinal fluid (CSF) and 144 serum samples collected during the first week of hospitalisation from 37 patients with laboratory-confirmed invasive meningococcal disease were investigated. Overall, 91.9% of CSF samples and 45.9% of serum samples were PCR-positive, while culture of CSF and blood was positive for only 35% and 39% samples, respectively. Positive PCR results were obtained until day 7 with CSF and until day 5 with serum. It is therefore recommended that samples for molecular diagnosis should be collected early in the course of suspected invasive meningococcal disease.

Keywords Diagnosis, meningococcal disease, molecular diagnostics, *Neisseria meningitidis*, PCR

Original Submission: 1 April 2005; **Revised Submission:** 18 June 2005; **Accepted:** 21 July 2005

Clin Microbiol Infect 2006; 12: 137–141

INTRODUCTION

Invasive disease (sepsis and/or meningitis) caused by *Neisseria meningitidis* is a potentially life-threatening condition that can also lead to life-long disfigurement. Clinical suspicion of this infection is usually based on the presence of haemorrhagic skin rash associated with symptoms of meningitis or acute sepsis. Rapid and accurate diagnosis is essential both for optimal management of patients and for timely antibiotic prophylaxis for contacts. Confirmation of the aetiological diagnosis allows physicians to use narrow-spectrum antibiotics, to shorten the duration of treatment and to provide prognostic information.

Conventional microscopy, as well as culture methods, may fail to allow the diagnosis of invasive meningococcal disease (IMD) because of early administration of antibiotics. Blood cultures are positive in *c.* 50% of untreated patients with meningococcal sepsis, but this positivity rate is reduced to 5% after the first dose of antibiotic. Similarly, microscopy or culture is positive with cerebrospinal fluid (CSF) samples in 90% of untreated cases of meningococcal meningitis, but there is a dramatic reduction if antibiotics have been administered previously [1]. PCR detects small quantities of bacterial DNA and does not require the presence of viable bacteria. Therefore, it can confirm the aetiological agent even after antibiotic administration. Moreover, PCR can determine the serogroup of meningococci involved. Serogroup classification of meningococcal isolates is useful for epidemiological surveillance, for case/contact management, and for monitoring the effectiveness of meningococcal vaccines. Detection of meningococcal DNA in biological samples has been achieved with primers based on the 16S rRNA gene [2,3], and more specific primers can be used for serogroup identification (*siaD* gene for serogroups B, C, Y and W135, and *mynB/sacC* gene for serogroup A) [4,5]. Fluorescence- and

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gel-based methods have been developed for visualisation and identification of PCR products [6–8].

In the Czech Republic, an active surveillance programme registers *c.* 100 cases of IMD annually, with a reported case fatality rate of *c.* 10% [9]. Since 2001, PCR has been used increasingly for laboratory confirmation and epidemiological surveillance of IMD in the Czech Republic. In 2003, the aetiology of 41.6% of all registered cases of IMD was confirmed by PCR. Moreover, PCR was the only method that enabled the aetiology to be confirmed for 17.8% of cases [10]. Since 2003, guidelines for pre-hospital management of suspected cases of IMD in the Czech Republic have recommended intravenous administration of the first dose of antibiotic and corticosteroid before transport to the hospital, with blood for culture and PCR being taken before treatment is started [11]. Conflicting findings have been published concerning the dynamics of bacterial DNA stability in various biological samples. This information could contribute to a better understanding of the pathophysiology of IMD, as well as indicating the most appropriate time for taking samples. The aim of the present study was to determine the dynamics of PCR-based diagnosis in blood and CSF samples, and to compare the efficacies of different PCR primer pairs.

MATERIALS AND METHODS

Patients

The individuals included in this prospective study were patients with positive PCR results from CSF and/or blood, hospitalised at the Department of Infectious Diseases of the University Hospital, Prague, between January 2002 and March 2004, who fulfilled one of the following criteria: (1) culture isolation of *N. meningitidis* from a normally sterile site (n = 20); or (2) typical clinical symptoms and positive latex agglutination (LA) test or direct microscopy (n = 5); or (3) typical clinical symptoms and no other aetiological agent confirmed (n = 12). Typical clinical symptoms were defined as: haemorrhagic skin rash (petechiae and ecchymoses) and presence of symptoms of sepsis/septic shock and/or meningitis. According to these criteria, 37 patients (20 males, 17 females; mean age 23.1 years; range 6 months to 58 years) were included in the study. Nine (24%) patients were diagnosed with sepsis and 28 (76%) with meningitis.

Collection and handling of specimens

For PCR, serum or whole blood samples were taken on the day of admission and during the first week of hospitalisation. CSF was collected on the day of admission and usually on day 5 after admission, or earlier if the patient's condition was not improving as expected. Sixty CSF samples and 144 blood samples were collected from 37 patients. Blood samples were stored at 4°C and CSF samples at −20°C. On the day of admission, biological material was sampled for direct microscopy, culture and LA tests. Antibiotic therapy was started on the day of admission or before hospitalisation. The day when therapy was started was recorded as day 0. Following admission, all patients were treated under standard regimens with cefotaxime or benzylpenicillin. Clinical data were obtained from medical reports and the physicians involved in treatment. Informed consent was received from the parents or guardians of all patients. The research was carried out in accordance with the Helsinki Declaration.

PCR

DNA was extracted from blood or serum (200 μL) and the sediment was obtained following centrifugation of *c.* 1 mL of CSF with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Primers used for PCR are listed in Table 1; *crgA* and nested 16S rRNA primers were used to detect *N. meningitidis*, and *siaD* primers were used to identify serogroups B and C. Reaction mixtures (50 μL) consisted of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, Triton X-100 0.1% v/v, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Biogen, Prague, Czech Republic), 0.5 μM primers, 0.5 U of recombinant *Taq* DNA polymerase (Top-Bio, Prague, Czech Republic) and 25 μL of DNA extract. Amplification was performed in an Amplitron II Thermocycler (Thermolyne, Dubuque, IA, USA) as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C

Table 1. Oligonucleotide primers used for PCR

PCR assay	Oligonucleotides	Gene	Sequences	Amplicon (bp)	Species
One step	98-6	*crgA*	5′-GCTGGCGCCGCTGGCAACAAATTC-3′	230	*Neisseria meningitidis*
	98-10		5′-CTTCTGCAGATTGCGGCGTGCCTG-3′		
	98-19	*siaD*	5′-GGATCATTTCAGTGTTTTCCACCA-3′	450	*N. meningitidis* B
	98-20		5′-GCATGCTGGAGGAATAAGCATTAA-3′		
	98-17	*siaD*	5′-TCAAATGAGTTTGCGAATAGAAGGT-3′	250	*N. meningitidis* C
	98-18		5′-CAATCACGATTTGCCCAATTGAC-3′		
Semi-nested					
Step I	U3	16S rRNA	5′-AACT(C/A)CGTGCCAGCAGCCGCGGTAA-3′	1031	Bacteria
	ru8	16S rRNA	5′-AAGGAGGTGATCCA(G/A)CCGCA(G/C)(G/C)TTC-3′		
Step II	NM	16S rRNA	5′-TGTTGGGCAACCTGATTG-3′	710	*N. meningitidis*
	ru8	16S rRNA	5′-AAGGAGGTGATCCA(G/A)CCGCA(G/C)(G/C)TTC-3′		

for 1 min and 72°C for 1 min, followed by 72°C for 2 min. This first-step reaction was then diluted 1:500 in sterile, DNA-free water, and 5 μL was added to a second reaction, which consisted of 20 cycles. Each PCR run included negative (water) and positive controls. The PCR products were visualised under UV light following electrophoresis on agarose 2% w/v gels and staining with ethidium bromide. Amplicon sizes are listed in Table 1.

Conventional identification

CSF and blood samples from patients with suspected IMD were sent to the microbiological laboratory for direct microscopy and culture. *N. meningitidis* strains were cultured on blood agar plates at 37°C in CO_2 5% v/v for 48 h. Commercially available LA kits were used for direct testing of CSF (Slides méningite-Kit 5; bioMérieux, Marcy l'Etoile, France) and blood (Pastorex meningitis kit; Bio-Rad, Marnes-la-Coquette, France) with one drop (30–40 μL) of biological sample according to the manufacturers' instructions.

Statistical analysis

Results of diagnostic methods were expressed as percentages. Results of tests performed before and after the onset of antibiotic treatment were compared using Fisher's exact test and GraphPad Prism v. 4.00 (Graph Pad Software, San Diego, CA, USA). The level of statistical significance was defined as $p \leq 0.05$.

RESULTS

Positive PCR results were obtained for 17 (45.9%) blood samples and 34 (91.9%) CSF samples. In 15 (40%) cases, positive results were obtained with both sample types. Based on the PCR results, the infecting organisms belonged to serogroup B (n = 17; 46%), serogroup C (n = 15; 41%) or serogroup Y (n = 1; 3%), or else the serogroup was not determined (n = 4; 10%). Twenty (54%) patients had received antibiotics before collection of blood for PCR, and 16 (43%) patients received antibiotics before collection of CSF samples.

In contrast to PCR, there were 14 (39%) positive blood cultures and 13 (35%) positive CSF cultures. Ten (28%) patients received antibiotics before collection of blood for culture, and 14 (38%) received antibiotics before collection of CSF for culture. In the group of pretreated patients, there was a significant reduction in the frequency of culture-positive results (p 0.01) and a non-significant reduction in the frequency of LA-positive results. The frequency of positive PCR results was not changed significantly. The results obtained are summarised in Table 2.

The aetiology of meningococcal disease was confirmed by PCR, culture and LA for 14 (38%) patients, by PCR and culture for nine (24%) patients, and by PCR and LA for two (5%) patients. For 12 (32%) patients, PCR was the only method that yielded a positive result. Table 3 summarises the relationship between the test results and the form of meningococcal disease (i.e., sepsis or meningitis).

Figs 1 and 2 illustrate the period following initiation of antibiotic treatment after which positive PCR results were obtained with CSF and blood samples. The last positive CSF sample was detected on day 7 (Fig. 1), while the last positive blood sample was detected on day 5 (Fig. 2). Meningococcal DNA was detected by the group-specific primers until day 7 in CSF, and until day 2 in blood. With semi-nested PCR, positive results were obtained until day 6 in CSF, and until day 5 in blood. The low number of samples did not permit statistical evaluation of these results.

Table 2. Influence of antibiotic therapy on the result by diagnostic method in blood and cerebrospinal fluid (CSF)

	Test[a]	Tests done	Positive (%)	p value
Collection of CSF samples				
Before onset	PCR	21	21 (100)	
of antibiotic treatment	LA	15	9 (60)	
	Culture	23	12 (52)	
After onset	PCR	16	13 (81)	0.07
of antibiotic treatment	LA	9	2 (22)	0.10
	Culture	14	1 (7)	0.01
Collection of blood samples				
Before onset	PCR	17	8 (47)	
of antibiotic treatment	LA	12	5 (42)	
	Culture	26	12 (46)	
After onset	PCR	20	9 (45)	1.00
of antibiotic treatment	LA	14	4 (29)	0.68
	Culture	10	2 (20)	0.25

LA, latex agglutination test.
[a]For CSF, only the first samples collected on the day of admission were included. A combination of one-step and semi-nested PCR was used.

DISCUSSION

A PCR method for detection of *N. meningitidis* serogroups B and C [4] was introduced in 2000 by the National Reference Laboratory for Meningo-

Table 3. Positive results of PCR, latex agglutination (LA) and culture for cerebrospinal fluid (CSF) and blood samples, grouped in relation to the clinical form of invasive meningococcal disease

	PCR-positive[a] *n* (%)		LA-positive[a] *n* (%)		Culture-positive[a] *n* (%)	
Clinical presentation	CSF	Blood	CSF	Blood	CSF	Blood
Sepsis	7 (78)	7 (88)	0	0	1 (11)	3 (33)
Meningitis	27 (100)	11 (40)	12 (57)	9 (43)	12 (43)	11 (41)

[a]All positive results, independent of timing of collection, are included.

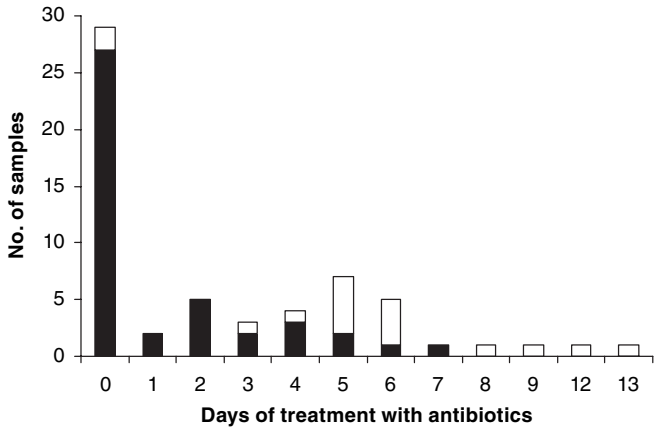

Fig. 1. Period after the initiation of antimicrobial therapy during which PCR-positive results were obtained with cerebrospinal fluid samples (white, negative results; black, positive results).

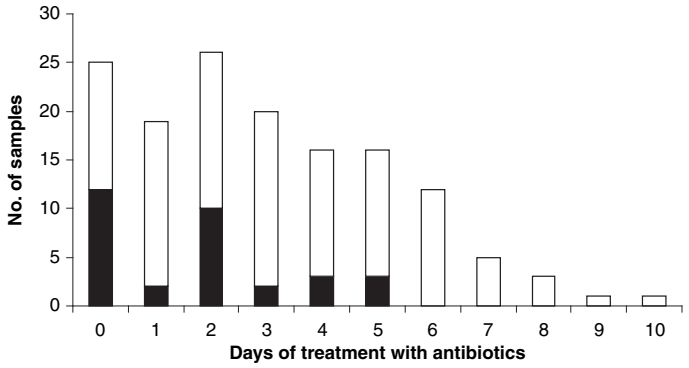

Fig. 2. Period after the initiation of antimicrobial therapy during which PCR-positive results were obtained with blood samples (white, negative results; black, positive results).

coccal Infections, Prague, and provides high sensitivity (85%) and specificity (95%) [12]. The original method was modified as described by Zambardi *et al.* [13] and makes use of the primers described by Frosh *et al.* [14]. Subsequently, the method has been upgraded to detect simultaneously *N. meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* [2,15]. In 2003, the National Reference Laboratory participated in an inter-laboratory comparison of PCR-based identification of *N. meningitidis*, which achieved a mean sensitivity and specificity of 90% and 93%, respectively [16].

The combined PCR method used in the present study was more reliable than culture and LA for the diagnosis of IMD. In 12 (32%) cases, the aetiology was identified only by PCR. Three cases were confirmed subsequently by multilocus sequence typing direct from CSF [17]. The other nine cases displayed the typical clinical course of the disease and no other agent was detected. The present study confirms the importance of PCR for identifying the aetiology in cases of IMD, with a significant increase in the number of laboratory-confirmed cases of meningococcal disease. Almost half the patients in the present study received antibiotics before hospital admission, and PCR was particularly useful with such patients.

Previous studies have reported variable findings on the duration of detection by PCR of meningococcal DNA after antibiotic treatment. Ragunathan *et al.* [18] reported that blood and CSF were PCR-positive for 24 h and 72 h, respectively, after the start of antibiotic therapy, while Bryant *et al.* [19] obtained positive nested and real-time PCR results with blood samples up to day 9 after the start of intravenous antibiotics. The present study detected meningococcal DNA up to day 7 in CSF, and up to day 5 in blood after the start of antibiotic therapy. The results reflect the fact that DNA persists after bacteria have been killed, and that clearance of meningococcal DNA from CSF is slower than from blood. Differences in results among these studies may be explained by several factors, including: (1) the number of bacteria in CSF at the beginning of treatment; (2) the susceptibility of bacteria to antibiotics; (3) the

number and killing activity of leukocytes in CSF (leukocytes destroy bacterial DNA after phagocytosis and bacterial lysis); and (4) the permeability of the blood–brain barrier, which influences penetration of antibiotics and the immune reaction components, as well as clearance of bacterial residues. As the overall detection rate for PCR decreases during the course of disease, it is recommended that biological samples (especially CSF) be taken for PCR investigation as soon as possible. Nevertheless, despite the availability and usefulness of PCR for identifying the aetiology in cases of IMD, culture of CSF and blood remains indispensable for antibiotic sensitivity testing and for further detailed research into strains of meningococci.

ACKNOWLEDGEMENTS

We thank physicians from the Department of Infectious Diseases, University Hospital Bulovka, Prague for their help in collecting the clinical samples, and R. Pospisilova and V. Pavlikova from the National Reference Laboratory for Meningococcal Infections for technical support. The data from this study were presented, in part, at the 13th European Congress of Clinical Microbiology and Infectious Diseases (Glasgow, 2003) and the 14th European Congress of Clinical Microbiology and Infectious Diseases (Prague, 2004).

REFERENCES

1. Cartwright K, Reilly S, White D, Stuart J. Early treatment with parenteral penicillin in meningococcal disease. *BMJ* 1992; **305**: 143–147.
2. Rådström P, Bäckman A, Qian N, Kragsbjerg P, Pahlson C, Olcén P. Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and streptococci using a seminested PCR strategy. *J Clin Microbiol* 1994; **32**: 2738–2744.
3. Bäckman A, Lantz PG, Rådström P, Olcén P. Evolution of an extended diagnostic PCR assay for detection and verification of the common causes of bacterial meningitis in CSF and other biological samples. *Mol Cell Probes* 1999; **13**: 49–60.
4. Taha M.-K. Simultaneous approach for non-culture PCR based identification and serogroup prediction of *Neisseria meningitidis*. *J Clin Microbiol* 2000; **38**: 855–857.
5. Orvelid P, Backman A, Olcen P. PCR identification of the group A *Neisseria meningtidis* in cerebrospinal fluid. *Scand J Infect Dis* 1999; **31**: 481–483.
6. Sambrook I, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
7. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridisation. *PCR Methods Appl* 1995; **4**: 357–362.
8. Diggle MA, Edwards GF, Clarke SC. Automation of fluorescence-based PCR for confirmation of meningococcal disease. *J Clin Microbiol* 2001; **39**: 4518–4519.
9. Kriz P. Surveillance of invasive meningococcal disease in the Czech Republic. *Euro Surveill* 2004; **9**: 11–12.
10. Krizova P, Kalmusova J, Musilek M. Invazivni meningokokove onemocneni v Ceske republice v roce 2003. *Zpravy CEM* 2003; **13**: 78–84.
11. Roznovsky L, Gutvirth J, Benes J *et al*. Standard efektivni klinicke pece v prednemocnicni neodkladne peci: invazivni meningokokova onemoceni. *Urg Med* 2002; **3**: 18–20.
12. Kalmusova J, Pavlikova V, Krizova P *et al*. Prvni vysledky PCR diagnostiky invazivniho meningokokoveho onemocneni. *Klin Mikrobiol Infekc Lek* 2001; **7**: 15–19.
13. Zambardi G, Druetta A, Roure C *et al*. Rapid diagnosis of *Mycobacterium tuberculosis* infections by an ELISA-like detection of polymerase chain reaction products. *Mol Cell Probes* 1995; **9**: 91–99.
14. Frosh M, Edwards U, Bousset K, Krausse B, Weisberg C. Evidence for a common molecular origin of the capsule gene loci in Gram-negative bacteria expressing group II capsular polysaccharides. *Mol Microbiol* 1991; **5**: 1251–1263.
15. Kalmusova J, Bronska E, Krizova P. Diagnostika invazivniho meningokokoveho, hemofiloveho a pneumokokoveho onemocnení metodou PCR. *Klin Mikrobiol Infekc Lek* 2004; **10**: 130–133.
16. Taha MK, Alonso JM, Cafferkey M *et al*. Interlaboratory comparison of PCR-based identification and genogrouping of *Neisseria meningitidis*. *J Clin Microbiol* 2005; **43**: 144–149.
17. Kriz P, Kalmusova J, Felsberg J. Multilocus sequence typing of *Nesseria meningitidis* directly from cerebrospinal fluid. *Epidemiol Infect* 2002; **128**: 157–160.
18. Ragunathan L, Ramsay M, Borrow R, Guiver R, Gray S, Kaczmarski EB. Clinical features, laboratory findings and management of meningococcal meningitis in England and Wales: report of a 1997 survey. Meningococcal meningitis: 1997 survey report. *J Infect* 2000; **40**: 74–79.
19. Bryant PA, Li HY, Zaia A *et al*. Prospective study of a real-time PCR that is highly sensitive, specific, and clinically useful for diagnosis of meningococcal disease in children. *J Clin Microbiol* 2004; **42**: 2919–2925.