Direct-test PCR for detection of meningococcal DNA and its serogroup characterization: standardization and adaptation for use in a public health laboratory

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A direct PCR test (DT-PCR) was established to detect *Neisseria meningitidis* DNA in clinical samples from patients with suspected bacterial meningitis. Specific primers for the 16S rDNA of *N. meningitidis* were designed to amplify a 600 bp DNA fragment. One hundred and ninety-three clinical samples were analysed, corresponding to 114 samples from patients diagnosed as positive and 79 as negative for infection by *N. meningitidis* using conventional methods (culture, latex agglutination and counterimmunoelectrophoresis). These samples were submitted to PCR by two different clinical sample preparation approaches (with and without DNA extraction and purification) and submitted to different PCR protocols to improve the results. In agarose gel detection, the sensitivity value for DT-PCR was 88.5 % and, using dot-blot DNA detection, the sensitivity increased to 96.4 %. The detection limit for meningococcus in cerebrospinal fluid was 2×10^2 c.f.u. ml⁻¹. Serogroup prediction was done using a multiplex PCR protocol and the sensitivity was 83 % for agarose gel DNA detection and 96.4 % using dot-blot DNA detection.

Received 24 January 2003

Accepted 8 May 2003

INTRODUCTION

Meningococcal meningitis caused by *Neisseria meningitidis* results from the ability of this bacterium to reach the central nervous system, causing inflammation of the meninges. Diagnosis is by detection of Gram-negative diplococcal bacteria or antigens in blood or cerebrospinal fluid (CSF) samples. Conventional methods are not specific and show low sensitivity, particularly in patients undergoing treatment (Ballard *et al.*, 1987; Coovadia *et al.*, 1989; Nato *et al.*, 1991; Gray & Fedorko, 1992).

Serogroup classification of meningococcus strains is often useful for investigating outbreaks, for case-contact management and for monitoring meningococcal vaccine effective-

ness. Conventional serological classification by a simple bacterial agglutination test can occasionally be difficult because of specificity problems associated with grouping antisera, downregulation of *N. meningitidis* capsule expression, the propensity of certain strains to autoagglutinate or the propensity of these bacteria to exchange DNA by transformation (Yakubu *et al.*, 1999; Tzanakaki *et al.*, 2001). Ultrasound-enhanced latex agglutination (LA), standard PCR and fluorescent PCR methods have been used to confirm the presence of meningococcal antigens or DNA in clinical samples (Jenkins *et al.*, 1997; Guiver *et al.*, 2000; Diggle *et al.*, 2001; Pollard *et al.*, 2002; Sobanski *et al.*, 2002).

The detection of meningococcal DNA in clinical samples by PCR has been performed with success by many groups using primers based on the gene encoding 16S rRNA (Greisen *et al.*, 1994; Rådström *et al.*, 1994; Hall *et al.*, 1995; Saruta *et al.*, 1997; Ley *et al.*, 1998; Bäckman *et al.*, 1999; Atobe *et al.*, 2000). For serogroup prediction, PCR can be

Abbreviations: CIE, counterimmunoelectrophoresis; CSF, cerebrospinal fluid; DT-PCR, direct-test PCR; GM-PCR, glass-matrix PCR; LA, latex agglutination.

applied using primers designed for genes that are specific for each serogroup (Orvelid *et al.*, 1999; Probert *et al.*, 2002), specific primers that characterize two different serogroups (Borrow *et al.*, 1997) or multiplex PCR that can distinguish all five of the most common meningococcal serogroups (Taha, 2000; Pollard *et al.*, 2002). Fluorescence-based PCR methods were developed to confirm meningococcal disease, improving results from gel-based methods, but they are still not able to distinguish all meningococcal serogroups (Guiver *et al.*, 2000; Corless *et al.*, 2001; Diggle *et al.*, 2001).

Meningococcal meningitis is a serious infection that can sometimes be fatal or lead to lifelong debility. For this reason, rapid and effective diagnosis and confirmation of serogroup type are very important. It is still important to simplify the technical steps involved in the existing PCR-based protocols without decreasing sensitivity and specificity. With this aim, we have compared two different clinical sample preparation protocols with PCR amplification of the meningococcal DNA. The chosen protocol was adapted to increase its sensitivity. The same clinical sample preparation methodology was used to determine meningococcus serogroup by PCR. In this paper, we describe a simple and rapid procedure for detection of the infectious agent that causes meningococcal meningitis and its serogroup characterization, without the need of further treatment of the clinical sample.

METHODS

Bacterial strains and culture methods. *N. meningitidis* serogroup B strain ATCC 13090 used in the PCR standardization procedures was cultured on chocolate-agar plates, supplemented with ram blood and growth factor VX, at 37 °C and 5% CO₂ for 24 h. Bacterial DNA isolated from culture was purified according to the method of van Soolingen *et al.* (1994) and stored at -20 °C. The following strains were used for PCR specificity test: *Streptococcus pneumoniae* IAL 1741, *Haemophilus influenzae* NCTC 419, *Escherichia coli* ATCC 15221, *Streptococcus agalactiae* ATCC 13813^T, *Shigella flexneri* ATCC 12022, *Listeria monocytogenes* ATCC 19111 and *Mycobacterium tuberculosis* H37Rv. For serogroup prediction using PCR, the following *N. meningitidis* strains were used: serogroup A (ATCC 13077), serogroup B (ATCC 13090), serogroup C and serogroup W₁₃₅ (isolated from a clinical sample; serogrouping performed with rabbit polyclonal antisera provided by the Adolfo Lutz Institute – National Reference Center for Meningitis, Brazil).

Clinical samples. One hundred and ninety-three CSF samples were obtained from the clinical samples collection of the Department of Bacteriology from the Biological Research Institute Central Laboratory of Rio Grande do Sul State (IPB-LACEN/RS), collected in the period 1995–2002. These samples were tested by counterimmunoelectrophoresis (CIE) and LA and maintained in 1 ml aliquots at -20 °C. Culture of the samples was performed at the hospitals and they were sent to IPB-LACEN/RS for characterization. Not all cultures could be included because some were not viable when they arrived at IPB-LACEN/RS. Suspicion of meningococcal infection was defined as invasive disease with purpura fulminans or petechiae or meningitis plus a high clinical suspicion of a meningococcal infection; laboratory-confirmed meningococcal infection was defined as isolation of *N. meningitidis* from a normally sterile site or demonstration of meningococcal antigen in the clinical sample.

The samples were submitted to two different analyses. The first, using 91

samples (group 1), was based on comparison of two strategies to prepare clinical specimens before PCR amplification. This group was divided into three sample categories on the basis of laboratory findings. In category I (*n*=26), samples had positive results in culture and antigen-detection methods. In category II (*n*=34), samples had positive results in antigen-detection methods, and, in category III (*n*=31), samples had negative results in all methods for meningococcal diagnosis. In this last category, patients were finally diagnosed for disease other than infection by *N. meningitidis* (unrelated bacterial or viral meningitis).

In order to compare different PCR strategies, we used the other 102 samples (group 2) from the same IPB-LACEN/RS collection. This group was also divided in three sample categories on the same basis as group 1, giving categories I (*n*=50), II (*n*=4) and III (*n*=48). Serogroup prediction by PCR was applied to samples from this group that presented positive results for the presence of meningococcal DNA by PCR.

Antigen-detection methods. The LA test was done with 50 µl of each biological sample by using a commercial kit (Pastorex Meningitis 61718; Sanofi Diagnostics Pasteur) according to the manufacturer's instructions. For CIE, 10 µl of each specimen was tested by the method described by Takeda *et al.* (1979).

Strategies to prepare DNA from clinical specimens. Ninety-one DNA samples were prepared from 500 µl CSF or serum using a glass matrix (Sephaglas; Pharmacia Biotech) and eluted in a final volume of 30 µl, as described by Rossetti *et al.* (1997). Aliquots (10 µl) of the extracted and purified DNA were submitted to PCR amplification. We call this procedure GM-PCR. Another 10 µl of the clinical samples was submitted to three cycles at 96 °C and 55 °C for 3 min (Raskin *et al.*, 1992) in a thermocycler (MiniCycler Hot Bonnet PTC-150; MJ Research) to disrupt the cell wall and expose the DNA and then placed directly into the PCR. This procedure was called direct-test PCR (DT-PCR).

The 102 clinical samples of group 2 were analysed by DT-PCR only, but we prepared the samples in two different ways. In the first method, the sample was added to the PCR mixture as described before. In the second method, we concentrated 500 µl of the CSF samples by using a centrifugation step for 5 min at 13 000 g and resuspended the pellet in 100 µl distilled water. An aliquot (10 µl) was then submitted to three cycles in a MiniCycler thermocycler.

PCR amplification and detection of fragments. To amplify *N. meningitidis* DNA, we designed primers based on the 16S rDNA sequence (Rådström *et al.*, 1994). The primers used were Men1 (5'-TGGGCAACCTGATTGCTT-3') and Men2 (5'-TTCTGGTATCCCC CACTCC-3') and the amplified DNA fragment was 600 bp in length.

GM-PCR and DT-PCR based on these primers contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 200 µM each dNTP, 2.5 U Taq DNA polymerase (Cenbiot; UFRGS), 50 pmol of each primer and 10 µl template in a total reaction volume of 50 µl. Thirty cycles of 94 °C for 2 min, 64 °C for 1 min and 72 °C for 2 min were followed by an elongation step at 72 °C for 5 min in a MiniCycler thermocycler.

Aliquots (13 µl) of all PCR products were separated by electrophoresis in 1.5% agarose gel (Gibco BRL) stained with ethidium bromide and visualized by UV. For the GM-PCR strategy, we transferred the DNA to nylon membranes (Hybond-N⁺; Amersham) for Southern blotting hybridization. The membrane was probed with the 600 bp fragment obtained by PCR that had been purified with a MicroSpin 300 column (Pharmacia). The detection system was the Amersham ECL kit.

Serogroup prediction by PCR. In order to assign the *N. meningitidis* serogroup, we used primers published by Taha (2000) that targetted *orf-2*, a gene cassette required for the biosynthesis of the capsule of

serogroup A, and a *siaD* gene for serogroups B, C, W₁₃₅ and Y. The expected sizes of the amplicons from this multiplex PCR are 450 bp (serogroup B), 400 bp (serogroup A), 250 bp (serogroup C) and 120 bp (serogroups Y and W₁₃₅). A further PCR for each serogroup was performed to confirm the result and to discriminate serogroups W₁₃₅ and Y. In each assay, the final 50 µl reaction mixture contained 15 µl of each sample, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 200 µM each dNTP, 2.5 U *Taq* DNA polymerase and 50 pmol of each serogroup primer.

PCRs were performed in a MiniCycler thermocycler with the following parameters: a first cycle of denaturation at 94 °C for 3 min, annealing at 55 °C for 30 s and polymerization at 72 °C for 20 s, followed by 35 cycles of 92 °C for 40 s, annealing at 55 °C for 30 s and polymerization at 72 °C for 20 s and a final cycle of polymerization at 72 °C for 10 min. Amplicons were analysed by electrophoresis on a standard 2 % agarose gel.

Samples presenting negative results in agarose gel were spotted onto Hybond-N⁺ by dot-blot and the DNA was fixed by exposure to UV light for 10 min. The membrane was probed with the 450 bp fragment obtained by PCR that corresponds to serogroup B meningococcus, purified as described above, with detection as described above.

Improvements to the DT-PCR protocol. Besides concentrating the samples, we tried to improve DT-PCR results by increasing the number of amplification cycles from 30 to 45. Also, samples with negative results in agarose gel were dot-blotted on Hybond-N⁺ and probed with the 600 bp PCR fragment as described above.

Determination of detection limit and specificity of the DT-PCR.

For the determination of sensitivity, a culture of *N. meningitidis* was diluted 10-fold into an *N. meningitidis*-free ('clean') CSF sample according to the McFarland scale. One-hundred microlitres of each dilution was cultured on chocolate-agar plates and grown for 24 h to determine the number of c.f.u. An aliquot (10 µl) of each dilution was amplified by DT-PCR and the amplicons were visualized in 1.5 % agarose gel.

The detection limit of DNA extracted from *N. meningitidis* was determined by spiking clean CSF with 100 ng to 500 fg DNA. An aliquot (10 µl) of each dilution was amplified by DT-PCR and the amplicons were visualized in 1.5 % agarose gel.

The specificity of the DT-PCR for *N. meningitidis* was analysed by submitting 100 ng DNA from *Streptococcus pneumoniae*, *H. influenzae*, *E. coli*, *Streptococcus agalactiae*, *Shigella flexneri*, *L. monocytogenes* and *M. tuberculosis* to the same PCR conditions used for *N. meningitidis* DNA.

Sequencing of the 16S rDNA product. The 600 bp product amplified by DT-PCR from the 16S rDNA was sequenced with a ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham). Sequencing reactions contained 10 ng DNA amplified by DT-PCR and purified with MicroSpin columns (Pharmacia Biotech) and 2.5 pmol Men1/Men2 primers. The sequence obtained was identical to the sequence deposited in GenBank (accession no. Z22776).

RESULTS

Conventional methods

Ninety-one specimens from 90 patients formed group 1. Of these, 60 were laboratory-confirmed meningococcal infections and 31 presented negative diagnosis. Fifty-five of 60 positive samples presented positive results for antigen-detection methods and the other five samples presented positive results only in culture (Table 1). For group 2, 102 clinical samples from 102 different patients were analysed. Fifty-four samples presented positive results in culture and/or meningococcal antigen detection and 48 were negative for meningococcal infection by conventional methods. Seventeen of these samples presented positive results for the

Table 1. Amplification of *N. meningitidis* 16S rDNA from clinical samples by GM-PCR and DT-PCR

Ninety-one samples from patients with or without meningococcal meningitis (group 1) were analysed. Samples were divided into three categories and GM-PCR and DT-PCR were carried out as described in Methods. DT-PCR results were determined from agarose gels only. Positivity values are numbers (%) positive for each group of samples analysed by different protocols. Total concordance represents the number of concordant results between conventional and molecular methods.

Criterion	GM-PCR		DT-PCR
	Agarose gel	Hybridization	
Positivity for sample groups			
I. Culture-confirmed cases (<i>n</i> = 26)	11 (42.3)	19 (73)	23 (88.4)
II. Antigen-detection-confirmed cases (<i>n</i> = 34)	10 (29.4)	26 (76.4)	29 (85.2)
III. Negative (<i>n</i> = 31)	0 (0)	0 (0)	0 (0)
Total concordance (<i>n</i> = 91)	52	76	83
Performance criteria			
Sensitivity (%)	60.6	80	88.2
Specificity (%)	100	100	100
Negative predictive value (%)	100	100	100
Positive predictive value (%)	44.2	67.3	79.4

presence of the antigen by CIE (61.6 % sensitivity) and 24 presented positive results by LA (75.4 % sensitivity) (Table 2).

Amplification of *N. meningitidis* DNA from clinical samples by GM-PCR and DT-PCR

In order to compare results between the GM-PCR and DT-PCR protocols, we analysed the 91 clinical samples of group 1. Table 1 shows the results of PCR tests compared with clinical diagnosis. From 26 clinical samples of category I, 11 (42.3 %) were positive for *N. meningitidis* when the DNA was extracted and purified by glass matrix and detected in agarose gels. When these samples were hybridized, the number of positives increased to 19 (73 %). However, when CSF samples were processed by DT-PCR, 23 (88.4 %) showed positive results directly in agarose gels. From 34 clinical samples of category II, 10 (29.4 %) were positive for *N. meningitidis* when the DNA was extracted and purified by glass matrix and detected in agarose gel. When these samples were hybridized, the number of positives increased to 26 (76.4 %). However, when CSF samples were processed by DT-PCR, 29 (85.2 %) showed positive results directly in agarose gels. From the 31 negative samples (category III), all presented negative results by all PCR methodologies. The sensitivity, specificity, predictive positive and predictive negative values for GM-PCR detected in agarose gel were respectively 60.6, 100, 100 and 44.2 %. When these samples were hybridized, the sensitivity, specificity, predictive positive and predictive negative values were respectively 80, 100, 100 and 67.3 %. For DT-PCR, the sensitivity and specificity were 88.2 and 100 % and the positive and negative predictive values were 100 and 79.4 %.

Improvement of the DT-PCR protocol

In order to improve the DT-PCR procedure, 102 CSF samples (group 2), both laboratory-confirmed for meningococcal disease and negative, were tested in three further ways and the results were compared with those of the DT-PCR protocol without these additional steps (protocol I) (Table 2). In protocol II, the samples were concentrated and the cell wall was disrupted in a thermocycler before addition to the PCR. In protocol III, the sample was prepared as in the first protocol except for increasing the number of cycles to 45. In protocol IV, samples were amplified for 45 cycles and the amplicons were detected on a nylon membrane by a dot-blot protocol. Of 54 laboratory-confirmed cases, 26 (68.3 %) were positive by DT-PCR. When samples were concentrated by the centrifugation step, the number of positive samples increased to 39 (83 %), and when the additional cycles were included in the procedure, the number of positive samples increased to 43 (88.5 %). When detection was made on a nylon membrane, 48 samples gave positive results (96.4 %).

Detection limit and specificity of DT-PCR

To determine the detection limit of the DT-PCR, we spiked CSF with *N. meningitidis* cells and with DNA extracted from *N. meningitidis*. Aliquots (10 µl) from each dilution were amplified by DT-PCR. The detection limit was 2 c.f.u. per reaction, or 2×10^2 c.f.u. ml⁻¹, when we used bacteria dilution and 1 pg per reaction when CSF spiked with bacterial DNA was used (Fig. 1).

The specificity of the amplification was determined by submitting DNA from other bacterial specimens that also cause meningitis to the same PCR conditions used for

Table 2. Diagnostic performance of DT-PCR protocols compared with culture and antigen-detection methods

One hundred and two CSF samples from patients with and without meningococcal meningitis (group 2) were studied. Positivity gives numbers (%) testing positive for *N. meningitidis* by different methods. Total concordance represents the number of concordant results between conventional and molecular methods. Ninety-three samples were analysed by CIE and 50 samples were analysed by LA. NA, Not applicable.

Criterion	CIE	LA	DT-PCR			
			I	II	III	IV
DT-PCR conditions						
Amplification cycles (<i>n</i>)	NA	NA	30	30	45	45
Sample concentration	NA	NA	No	Yes	Yes	Yes
Detection method	NA	NA	Agarose	Agarose	Agarose	Dot-blot
Positivity for sample groups						
I. Culture-confirmed cases (<i>n</i> = 50)	17 (34)	24 (48)	26 (52)	39 (78)	43 (86)	48 (96)
II. Antigen-detection-confirmed cases (<i>n</i> = 4)	0 (0)	4 (100)	3 (75)	4 (100)	4 (100)	4 (100)
III. Negative (<i>n</i> = 48)	0 (0)	0 (0)	2 (4.1)	4 (8.3)	4 (8.3)	8 (16.6)
Total concordance (<i>n</i> = 102)	65	37	75	87	91	92
Performance criteria						
Sensitivity (%)	61.6	75.4	68.3	83	88.5	96.4
Specificity (%)	100	100	96	92	92	85.7
Negative predictive value (%)	63.1	43.4	65.7	81.3	87.2	96
Positive predictive value (%)	100	100	96.4	93.1	93.1	87

Fig. 1. Detection limit of DT-PCR. Dilution series of *N. meningitidis* bacteria (a) and extracted DNA (b) in 'clean' CSF were amplified by DT-PCR (giving a 600 bp amplicon) and analysed in 1.5% agarose gel. Lanes: M, 1 kb ladder (Gibco BRL); PC, positive control; 1–10, dilutions of *N. meningitidis*; NC, negative control. In (a), lane 5 corresponds to 2 c.f.u. per reaction; in (b), lane 9 corresponds to 1 pg extracted *N. meningitidis* DNA.

amplification of *N. meningitidis* DNA by DT-PCR with 30 and 45 cycles of amplification in agarose and dot-blot DNA detection. The expected amplification product of 600 bp was only obtained with *N. meningitidis* DNA. No amplification products were observed in the other samples (results not shown).

Serogroup prediction by PCR

We characterized the meningococcus serogroup for samples from group 2 that presented positive results by DT-PCR using the protocol described by Taha (2000). The results are presented in Table 3 and Fig. 2. By conventional methods, 45 samples were characterized as serogroup B, four as serogroup C, one as non-agglutinant, one as bi-agglutinant for serogroup B and C and three as poly-agglutinant. When the DT-PCR amplicons were analysed in agarose gel, 35 of 45 samples presented positive results for serogroup B and four confirmed positive results for serogroup C.

Table 3. Serogroup prediction by PCR compared with conventional methods

Results are numbers of samples giving each serogroup.

Serogroup	Conventional	PCR
B	45	43*
C	4	4
B and C	1	1†
Non-agglutinant	1	1‡
Poly-agglutinant	3	3
Total	54	52

*Thirty-five of 45 samples were positive in agarose gels and a further eight were positive only in the dot-blot protocol.

†PCR amplification gave 250 bp fragment corresponding to serogroup C; no amplification observed for serogroup B.

‡PCR amplification gave 120 bp fragment corresponding to serogroup W₁₃₅.

Fig. 2. Serogroup prediction by PCR. Fragments amplified by PCR and separated in 2% agarose gel for serogroups A (400 bp), B (450 bp), C (250 bp) and W₁₃₅ (120 bp) of *N. meningitidis* are shown. Lane M, 1 kb ladder (Gibco BRL); CNR, negative control.

In the case of the sample characterized as bi-agglutinant by conventional methods, the PCR presented a positive result only for serogroup C and the non-agglutinant sample was characterized as belonging to serogroup W₁₃₅. The three samples characterized as poly-agglutinant were characterized by PCR as belonging to serogroup B. The sensitivity was 83%. Samples that presented negative results in agarose gel were analysed by the dot-blot protocol, and eight more samples presented positive results for serogroup B, reaching 96.4% sensitivity.

DISCUSSION

In this paper, we report the development and standardization of a direct-test PCR for detection of *N. meningitidis* DNA. Two PCR methods were compared to analyse which would present better results for diagnosis of meningococcal disease. Direct submission of the biological sample to amplification by PCR was shown to be better than a protocol that used DNA extraction and purification by glass matrix. We chose the GM-PCR protocol because our group already uses this method to detect *M. tuberculosis* DNA and it showed the best results with CSF samples of patients with tuberculous meningitis (Rossetti *et al.*, 1997). It is possible that some samples presented low concentrations of bacteria, that DNA losses could have occurred during the wash steps when the purification procedure with the glass matrix was applied or that some *Taq* DNA polymerase inhibitor(s) remained in the preparation. We have tried other commercial products such as DNazol and Glasmax (both from Gibco-BRL) to extract and purify the DNA, but the results were very similar to those found with the glass matrix used here (results not shown). The results showed that direct amplification of *N. meningitidis* DNA from clinical samples is possible by the use of three initial cycles in the PCR to disrupt the bacterial cell wall and release the DNA. This procedure was first proposed by Raskin *et al.* (1992) to analyse blood samples by PCR. Comparative analysis of GM-PCR and DT-PCR using clinical samples from group 1 showed that DT-PCR was

more sensitive. Eight of 60 clinical specimens presented negative results when tested by DT-PCR. One possible explanation for this result is the source of the samples tested. These samples belong to a sample collection that is frequently manipulated to perform routine tests, which could have caused DNA degradation. Possibly, in those eight samples that presented positive clinical diagnosis, the DNA was degraded by manipulation or by freeze-thawing or by the long storage time, or there may have been small amounts of DNA (Yamamoto, 2002). Another possibility is that the criteria used by physicians to give a positive clinical diagnosis for six patients were the positive result in CIE or LA. Some groups have demonstrated that the antigens used in those tests can present false-positive results that correspond to a cross-reaction between *E. coli* or *Haemophilus parainfluenzae* antigens (McGowan, 1992; Perkins *et al.*, 1995).

To improve the DT-PCR protocol, we changed the process of biological sample preparation before submitting the sample to amplification. Using samples from group 2, we observed that 25 of 54 culture- and/or antigen-detection-positive samples presented negative results by DT-PCR. When we increased the number of amplification cycles from 30 to 45, the number of negative samples was reduced to seven and the sensitivity increased to 88.5 %. It is possible that the seven samples that presented negative results in DT-PCR contained too few bacteria to be detected by this protocol. To test this possibility, we applied the dot-blot DNA detection protocol, and we observed that five samples presented positive hybridization, increasing the sensitivity to 96.4 %. The use of the dot-blot protocol gave 87.2 % specificity compared with 92 % obtained in DNA agarose gel detection. It is possible that unspecific fragments were generated in some samples that could not be visualized in agarose gels. These fragments hybridized weakly to the probe, and may be considered as false-positive results. This suspicion was confirmed by using Southern blot DNA transfer from the agarose gel; two samples were shown not to contain the 600 bp DNA fragment that corresponds to amplification of *N. meningitidis* DNA. The dot-blot protocol should be used only in cases of strong clinical suspicion of meningococcal disease with negative results by DT-PCR with agarose gel DNA detection.

Other methods for non-culture-based diagnosis of *N. meningitidis* have been reported, based on amplification of the dihydropteroate synthase gene (Kristiansen *et al.*, 1991), insertion sequence IS1106 (Ni *et al.*, 1992), 16S rDNA or the 16S–23S rDNA spacer region (Greisen *et al.*, 1994; Rådström *et al.*, 1994; Saruta *et al.*, 1997; Kotilainen *et al.*, 1998; Bäckman *et al.*, 1999; Margall *et al.*, 1999; Seward & Towner, 2000). The sensitivity and specificity of these PCR assays for the diagnosis of meningococcal meningitis range from 80 to 95 % and the limit of detection in agarose gels ranges from 30 to 3 c.f.u. per reaction tube. With DT-PCR, we have been able to detect 2 c.f.u. or 1 pg of *N. meningitidis* DNA, with 88.5 % sensitivity and 92 % specificity, using DNA detection on agarose gel. For this type of diagnosis, the detection limit and high specificity are important because of the need for an

accurate, rapid and specific result to apply the correct therapeutic procedure without the use of commercial kits for DNA extraction and purification. Therefore, it would be adequate for detection of bacteria in CSF samples, since 85 % of CSF samples with bacterial infection have been reported to contain more than 10^3 c.f.u. ml⁻¹ (La Scolea & Dryja, 1984).

Protocols based on real-time PCR have been used for simultaneous detection of *N. meningitidis*, *H. influenzae* and *Streptococcus pneumoniae* using *ctrA*, *bexA* and *ply* gene targets, respectively (Corless *et al.*, 2001). This methodology promises to be the ultimate PCR diagnosis, but it is still too expensive to be applied in a public health laboratory and the sensitivity for meningococcal disease is 88.4 %.

Our results showed that the DT-PCR method presents several advantages over other protocols. The complete procedure takes about 6 h and is simple, specific and has low costs for implementation in the routine of a public health laboratory. The addition of an internal control for the DT-PCR amplifications and the establishment of an improved protocol to collect clinical samples to avoid the problems of DNA degradation or contamination will be important to improve the method. The inclusion of the dot-blot protocol for all negative samples in agarose gels showed good results and is faster and cheaper than the Southern blot method.

The use of serogroup prediction by PCR was a reliable way to confirm the positive results obtained by DT-PCR and also to assign the meningococcus serogroup. As in the DT-PCR protocol, it is possible that the 11 samples that showed negative results contained too few bacteria to be detected by this protocol. Dot-blot DNA detection increased the sensitivity to 96.4 %. However, we could confirm 83 % of cases that sometimes showed positive results simply by antigen-detection methods. Even the results of detection in agarose gel can be very helpful. In terms of epidemiology, this tool will be very useful for the application of preventative measures and to assess vaccine effectiveness.

DT-PCR of biological samples combined with serogroup prediction is a rapid protocol that may be used for confirmation of meningococcal meningitis diagnosis and will be particularly useful in situations where culture is difficult because of previous treatment with drugs.

ACKNOWLEDGEMENTS

We would like to thank Nelson Pinheiro Machado and Fernando Kappcke from the Section of Bacteriology of IPB-LACEN/RS for help with conventional methods of bacterial meningitis. Special thanks are due to Patricia Izquierdo Cafrune and Dr Marilene Vainstein for critical reading of the manuscript. L. F. B. was supported by scholarships from CNPq and CAPES. FAPERGS, CNPq and CDCT/FEPPS supported this work.

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