Detection of *Neisseria meningitidis* DNA in blood samples using direct-PCR test

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Background
Meningococcal disease caused by *Neisseria meningitidis* is a widely distributed complex human disease affecting all age categories. Successful and effective treatment of patients with this disease depends on precise and early diagnosis of the disease.

Objective
The aim of this study was to evaluate the possible use of direct PCR (D-PCR) for the detection and amplification of *N. meningitidis* DNA in blood samples compared with the conventional PCR (C-PCR) method, which needs bacterial culture and DNA extraction.

Materials and methods
Specific primers on the basis of the 16S rDNA of *N. meningitidis* were used for the amplification of 600 bp DNA fragment. Two strategies were followed: D-PCR, which relies on amplification of DNA directly in blood without DNA extraction using the KAPA Blood PCR Kit, and the C-PCR, which relies on the extraction of bacterial DNA using the Qiagen QiAmp DNA Mini Kit. The following blood samples were included in each strategy: A blood sample with bacterial cerebrospinal meningitis confirmed by blood culture, a normal blood sample seeded with *N. meningitidis* ATCC (13090) reference strain as positive control for the standardization of the PCR procedure, a normal blood sample as a negative control, and an internal negative control test sample (H_2O).

Results
Both D-PCR and C-PCR tests gave the expected amplified DNA fragment of 600 bp on agarose gel electrophoresis of both patients' blood sample and *N. meningitidis* seeded normal blood sample, whereas no amplified products were detected when both tests were performed on normal blood sample or the internal negative H_2O control.

Conclusion
Direct blood PCR assay could be a possible easy, rapid, nonexpensive, and specific method for the detection of meningococci in blood samples, particularly in situations in which culture is difficult because of previous treatment, and also could facilitate the large-scale screening of various medical conditions.

Keywords:
blood sample, conventional PCR, direct PCR, *Neisseria meningitidis*

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Introduction

Meningococcal meningitis is an infection caused by the bacteria *N. meningitidis*, which results in inflammation of the membranes covering the brain and the spinal cord. Twelve serogroups of *N. meningitidis* have been identified, six of which (A, B, C, W135, X, and Y) can cause epidemics [1,2]. Meningococcal meningitis occurs sporadically throughout the world and accounts for a major cause of mortality in all societies. Successful and effective treatment of patients depends on precise and early diagnosis [3,4].

The initial diagnosis of this disease is usually on the basis of suggestive clinical symptoms and signs of invasive meningococcal disease followed by the conventional methods for isolation and identification of the Gram-negative bacteria, which include culture on selective media, detection of specific antigens in blood, or cerebrospinal fluid (CSF) [5,6].

However, in patients with invasive meningococcal disease, antibiotics are prescribed routinely before the collection of samples as a part of the prehospital management of bacterial meningitis. Consequently, this may result in difficulty to culture the organisms [7]. Besides, culturing requires a minimum of 8–12 h of incubation time before performing the biochemical and/or immunological tests to identify the bacterium, which is time consuming [8,9]. It has been shown that the time required to obtain a positive culture result can be even longer in patients infected with slow-growing organisms or with low bacterial counts. In addition, conventional serological identification using simple bacterial agglutination test can be difficult because of the 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 [10,11]. Unfortunately, the conventional methods also show low specificity and sensitivity.

Therefore, the development and introduction of nonculture-based diagnostic methods such as the PCR into clinical practice is necessary [12,13]. These assays overcome the drawbacks of the conventional methods and improve the sensitivity and specificity [14].

Application of PCR to clinical specimens has many potential pitfalls because of the susceptibility of PCR to inhibitors, contamination, and experimental conditions. The nature of the content and amount available of the clinical specimens such as urine, blood, sputum, and CSF are different; therefore, careful design of the PCR assay is very important [14].

PCR using DNA from blood samples is a valuable tool in the field of medical diagnostics. However, DNA extraction from blood is a laborious and sample-consuming step and hampers the automation of PCR for large-scale studies. Many researchers have attempted to develop direct-PCR (D-PCR) protocols that do not require DNA extraction for time saving, convenience, avoidance of infection in sample handlers, prevention of loss of trace samples in the DNA purification step, and potential automation for large-scale diagnosis [15]. In addition, attempts to perform D-PCR from blood without DNA extraction have been difficult to achieve, as numerous endogenous and exogenous blood constituents may inhibit PCR.

Few data were published on D-PCR detection of *N. meningitidis* in clinical specimens [14,16]. It is still necessary to facilitate the technical steps involved in the existing PCR-based methods without decreasing the sensitivity and specificity [9]. This study was performed with the aim to evaluate the use of D-PCR as a possible simple and rapid assay for direct detection and amplification of meningococcal DNA in blood samples without the need of pretreatment of the clinical sample compared with the conventional PCR (C-PCR) method, which needs bacterial culturing and DNA extraction.

Materials and methods

Strategies for *N. meningitidis* detection using a PCR

Detection and amplification of *N. meningitidis* DNA in the blood samples were carried out by two strategies.

The first strategy was the D-PCR using the KAPA Blood PCR Mix B Kit (#KK7003; Kapa Biosystems, Woburn, MA, USA), which is designed for the amplification of DNA fragments directly from whole blood without DNA extraction. It involves a second-generation polymerase enzyme derived through a process of molecular evolution and the first DNA polymerase engineered specifically for the amplification of DNA directly from whole blood. It eliminates the need for DNA extraction. The PCR mix contains all components required for PCR, except primers and blood template. DNA fragments may be amplified directly from the reactions containing 1–20% (v/v) whole blood, without pretreatment of the blood samples or DNA extraction.

The second strategy was the C-PCR, which relies on the isolation and purification of bacterial DNA using QIAamp DNA Mini Kit (#51304; Qiagen, Hilden, Germany). This kit provides QIAamp Mini Spin Columns with silica membrane-based nucleic acid isolation and purification, QIAGEN Proteinase K, reagents, buffers, and collection tubes. DNA binds specifically to the QIAamp silica-gel membrane, whereas contaminants pass through. PCR inhibitors are completely removed by wash steps, and pure nucleic acid is eluted with water. This procedure reduces hands-on preparation time to 20 min, as no phenol–chloroform extraction is required.

Samples

The following samples were included in each strategy.

A clinical sample: One milliliter of blood sample was collected in a tube containing EDTA from a patient diagnosed clinically as having bacterial cerebrospinal meningitis and confirmed by the blood culture method for *N. meningitidis*.

A positive control sample: One milliliter of blood sample was collected in a tube containing EDTA from a normal volunteer and then was seeded with *N. meningitidis* serogroup B ATCC (13090) reference strain for the standardization of PCR procedure.

A normal negative control sample: One milliliter of blood sample was collected in a tube containing EDTA from a healthy normal volunteer.

An internal negative control test sample (H_2O without DNA).

Methods

Bacterial reference strain and culture method

N. meningitidis serogroup B strain ATCC (13090), provided from Naval Medical Research Unit 3 (NAMRU-3; Cairo, Egypt) was used in the PCR standardization procedures. Bacteria was cultured

on 5% blood agar supplemented with 0.5% glucose, 0.01% glutamine, 0.5% yeast extract, and 5% CO_2 at 37°C for 24 h [17].

Preparation of the seeded/spiked blood sample with the bacterial reference strain

To determine the specificity of the test, positive blood sample control was prepared using *N. meningitidis* serogroup B strain ATCC (13090). Briefly, 1 ml blood sample collected in tubes containing EDTA from a normal volunteer was seeded with a single colony of *N. meningitidis*. The seeded blood sample was boiled at 95°C for 10 min, and the cell debris was removed by centrifugation at 13 000*g* for 5 min. The supernatant was preserved for use in each D-PCR and C-PCR [1].

Direct-PCR amplification on blood

D-PCR was performed using KAPA Blood PCR Mix B (Kapa Biosystems). A typical reaction was set up according to the manufacturer's instructions by mixing the components in the order (50 µl PCR grade water, 2.5 µl of 5 µmol/l forward primer, 2.5 µl of 5 µmol/l reverse primer, 2.5 µl of 2% Tween-20, 25 µl of 2´ KAPA Blood PCR Mix B, and 5 µl of EDTA blood). Tubes were spun to collect all components at the bottom and then vortexed to mix before reactions were placed in the thermocycler. Amplification of *N. meningitidis* DNA was performed using the primers on the basis of the 16S rDNA sequence [18]. The primers used were Men1 (5′-TGGGCAACCTGATTGCTT-3′) and Men2 (5′-TTCTGGTATCCCCCACTCC-3′), and the amplified DNA fragment was 600 bp in length. The PCR conditions for DNA amplification were initial denaturation for 5 min at 95°C followed by 40 cycles (95°C, 30 sec; 55°C, 30 s; 72°C, 1 s) and a 5-min delay at 72°C in a Mini Cycler thermocycler (FPR0G02Y Techne Progene, Stone, Staffordshire, UK).

Conventional PCR on whole blood

1. Isolation and purification of DNA were carried out using QIAamp DNA mini Kit according to the manufacturer's instructions. A volume of 200 µl of patient's whole blood and 200 µl of blood seeded with bacterial cells were loaded to the column. The optimized buffers stabilize nucleic acids and enhance selective DNA adsorption to the QIAamp membrane. Alcohol is added and lysates are loaded onto the QIAamp spin column. Wash buffers are used to remove impurities. Pure and ready to use DNA is then eluted with water.
2. Amplification of DNA was performed according to the manufacturer's instructions, with 5 × buffer A (Kapa Biosystems), 3 mmol/l $MgCl_2$, 0.2 mmol/l each dNTP, 5 U/µl KAPA2G Fast DNA Polymerase (Kapa Biosystems), 50 pmol of each primer, and 10 µl *N. meningitidis* of the extracted DNA samples in a total reaction volume of 50 µl. The PCR conditions for DNA amplification were initial denaturation for 1 min at 95°C followed by 40 cycles (95°C, 10 s; 55°C, 10 s; 72°C, 1 s) and a 5-min delay at 72°C in a Mini Cycler thermocycler (FPR0G02Y Techne Progene).

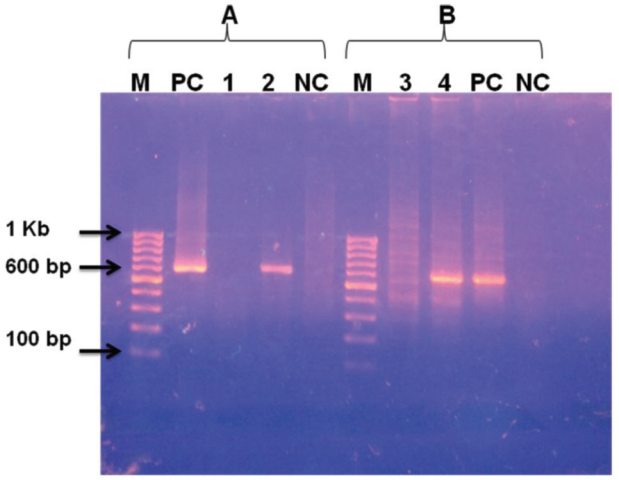
Electrophoresis detection

All PCR products were separated by electrophoresis in 1·5% agarose gel (Gibco BRL, San Francisco, CA, USA), stained with ethidium bromide, and visualized using UV [19]. A 100 bp molecular weight ladder marker was included (Gibco BRL).

Results

D-PCR technique performed on either patient's blood infected with *N. meningitidis* or normal blood spiked with *N. meningitidis* serogroup B strain ATCC (13090) resulted in amplified product of 600 bp size and the fragment was analyzed in 1.5% agarose gel, which confirmed the specificity of the test. The same result was obtained when the assay was carried out on extracted *N. meningitidis* DNA from the same blood samples and analyzed using C-PCR (Fig. 1a and b). No amplified products were detected when either D-PCR or C-PCR was performed on normal blood sample or negative internal control (H_2O), which confirmed the validity of the test.

Figure 1



Amplification of *Neisseria meningitidis* 16S rDNA in blood samples analyzed using (a) direct PCR without DNA extraction and (b) conventional PCR with DNA extraction shown in 1·5% agarose gel stained with ethidium bromide, giving a 600 bp amplicon. Lanes: M: 100 bp molecular weight ladder; PC, Positive control *N. meningitidis* ATCC (13090) seeded blood; NC, Negative internal control (H_2O), 1 and 3: normal blood control, 2 and 4: infected patient's blood sample.

Discussion

N. meningitidis is a heterotrophic Gram-negative diplococcal bacterium best known for morbidity and mortality during the childhood in industrialized countries and is responsible for epidemics in Africa and Asia. Effective and successful treatment of patient infected with *N. meningitidis* relies on the rapid diagnosis of the disease [9,20].

The gold standard for diagnosis is the culturing of *N. meningitidis* from sterile body fluid such as blood and CSF. However, culture-based methods for the identification of fastidious organism such as *N. meningitidis* are difficult and in some cases can lead to failure [5,6,21,22].

Besides, the incubation time required for culturing before performing the biochemical test to identify the bacterium is time consuming [8,17]. Furthermore, serological classification can be challenging because of capsule expression, downregulation of *N. meningitidis,* and the tendency of certain strains to autoagglutinate [10,11].

Several studies were published on different PCR-based methods as a powerful, rapid, and reliable method for laboratory confirmation of meningococcal infections and epidemiological surveillance, especially in individuals who received previous treatment with antibiotic showing difficulty in the culture method [14,23–25].

This study is a preliminary study for the development and standardization of D-PCR test to detect *N. meningitidis* DNA in blood samples compared with the C-PCR after extraction of DNA. Our study successfully showed that D-PCR technique performed on either patient's blood infected with *N. meningitidis* or normal blood spiked with *N. meningitidis* serogroup B strain ATCC (13090) resulted in amplified product of 600 bp size and the fragment was analyzed in 1.5% agarose gel, which confirmed the specificity of the test. The same result was obtained when the assay was carried out on the same blood samples and analyzed using C-PCR, which needs DNA extraction. No amplified products were detected when either D-PCR or C-PCR was performed on normal blood sample or negative internal control (H_2O), which confirmed the validity of the test.

PCR-based clinical and forensic tests often have low sensitivity or even false-negative results caused by potent PCR inhibitors found in blood and soil. It is widely accepted that purification of target DNA before PCR is necessary for successful amplification. Direct submission of the biological sample for amplification by the D-PCR protocol used in this study overcome the problems that were reported by Yamamoto [14] on the C-PCR protocols, such as DNA losses during the wash steps, DNA degradation by manipulation, freeze-thawing, or long storage time, or small amounts of DNA. Taha *et al.* [26] added that the variable results obtained for some samples were because of the differences in sample handling and DNA preparation, showing the impact of DNA extraction procedure on the PCR performance.

Several attempts were made to obtain target amplicons by direct amplification in clinical samples, such as the simple salting out procedure and pretreatment of whole blood with heat, microwave, or formamide. However, those methods failed to generate successful results compared with PCR using extracted DNA [27].

KAPA2G Fast DNA Polymerase (Kapa Biosystems) used in the present study significantly shortens the complete procedure time to 2 h compared with 6 and 3 h in other studies and lowers the enzyme inhibition risk compared with the wild-type *Taq* polymerase, which was used in the study by Baethgen *et al.* [1] and Qurbanalizadegan *et al.* [9], respectively.

Our results are in accordance with the D-PCR protocols for the detection of *N. meningitidis* from CSF sample that were reported in the study by Baethgen *et al.* [1] and Qurbanalizadegan *et al.* [9]. They were able to detect 1 pg of *N. meningitidis* DNA, with 88.5% sensitivity and 92% specificity, using DNA detection on agarose gel. In addition, the detection of meningococcal DNA in clinical samples using PCR has been performed successfully with primers on the basis of the gene encoding 16S rDNA [28–31]. In addition, for serogroup prediction PCR can be used with primers designed for the genes that are specific for each serogroup [32].

Real-time PCR assay have been used for the detection of *N. meningitidis*, *Haemophilus influenza*, and *Streptococcus pneumonia* [33]. However, this methodology is still too expensive and complex for a public health laboratory. In an attempt to overcome PCR inhibition, enhance PCR amplification, and simplify the PCR protocol, *Zhang et al.* [27] demonstrated improved PCR-enhancing cocktails containing nonionic detergent, L-carnitine, D-(+)-trehalose, and heparin. These cocktails, in combination with two inhibitor-resistant Taq mutants – OmniTaq and Omni Klentaq – enabled efficient amplification of exogenous, endogenous, and high-GC content DNA targets directly from the crude samples containing human plasma, serum, and whole blood without DNA purification. These enhancer cocktails also improved the performance of the novel

Taq mutants in real-time PCR amplification using crude samples, both in SYBR Green fluorescence detection and TaqMan assays.

Conclusion

Direct blood PCR assay could be a possible easy, rapid, cheap, and specific method for the detection of meningococci in blood samples, particularly in situations in which culture is difficult because of previous treatment, and also could facilitate the large-scale screening of various medical conditions.

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Conflicts of interest

There are no conflicts of interest.

References

1 Baethgen LF, Moraes C, Weidlich L, Rios S, Kmetzsch CÍ, Silva MSN, *et al.* Direct-test PCR for detection of meningococcal DNA and its serogroup characterization: Standardization and adaptation for use in a public health laboratory. J Med Microbiol 2003; 52:793–799.

2 Lemos APS, Brandão AP, Gorla MCO, Paiva MV, Simonsen V, Melles, CEA. Phenotypic characterization of *Neisseria meningitidis* strains isolated from invasive disease in Brazil from 1990 to 2001. J Med Microbiol 2006; 55:751–757.

3 Rosenstein NE, Perkins BA, Stephens DS, Popovic T, Hughes JM. Meningococcal disease. N Engl J Med 2001; 344:1378–1388.

4 Swartz MN. Bacterial meningitis – a view of the past 90 years. N Engl J Med 2004; 351:1826–1828.

5 Gray SJ, Sobanski MA, Kaczmarski EB, Guiver M, Marsh WJ, Borrow R, *et al.* Ultrasound-enhanced latex immunoagglutination and PCR as complementary methods for non-culture-based confirmation of meningococcal disease. J Clin Microbiol 1999; 37:1797–1801.

6 Porritt RJ, Mercer JL, Munro R. Ultrasound-enhanced latex immunoagglutination test (USELAT) for detection of capsular polysaccharide antigen of *Neisseria meningitidis* from CSF and plasma. Pathology 2003; 35:61–64.

7 Wood AL, Gill MJ. PCR and the investigation of meningococcal infection. Epidemiol Infect 2001; 127:269–274.

8 Bingen E, Lambert-Zechovsky N, Mariani-Kurkdjian P, Doit C, Aujard Y, Fournerie F, Mathieu H. Bacterial counts in cerebrospinal fluid of children with meningitis. Eur J Clin Microbiol Infect Dis 1990; 9:278–281.

9 Qurbanalizadegan M, Ranjbar R, Ataee RA, Hajia M, Goodarzi Z, Farshad S, *et al.* Specific PCR assay for rapid and direct detection of *Neisseria meningitidis* in cerebrospinal fluid specimens. Iran J Public Health 2010; 39:45–50.

10 Yakubu DE, Abadi FJR, Pennington TH. Molecular typing methods for Neisseria meningitidis. J Med Microbiol 1999; 48:1055–1064.

11 Tzanakaki G, Urwin R, Musilek M, Kriz P, Kremastinou J, Pangalis A, *et al.* Phenotypic and genotypic approaches to characterization of isolates of *Neisseria meningitidis* from patients and their close family contacts. J Clin Microbiol 2001; 39:1235–1240.

12 Borrow R, Claus H, Chaudhry U, Guiver M, Kaczmarski EB, Frosch M, Fox AJ. siaD PCR ELISA for confirmation and identification of serogroup Y and W135 meningococcal infections. FEMS Microbiol Lett 1998; 159:209–214.

13 Guiver M, Borrow R, Marsh J, Gray SJ, Kaczmarski EB, Howells D, *et al.* Evaluation of the Applied Biosystems automated Taqman polymerase chain reaction system for the detection of meningococcal DNA. FEMS Immunol Med Microbiol 2000; 28:173–179.

14 Yamamoto Y. PCR in diagnosis of infection: Detection of bacteria in cerebrospinal fluids. Clin Diagn Lab Immunol 2002; 9:508–514.

15 Yang YG, Kim JY, Song Y-H, Kim D-S. A novel buffer system, AnyDirect, can improve polymerase chain reaction from whole blood without DNA isolation. Clin Chim Acta 2007; 380:112–117.

16 Taha M-K, Fox A. Quality assessed nonculture techniques for detection and typing of meningococci. FEMS Microbiol Rev 2007; 31:37–42.

17 La Scolea Jr LJ, Dryja D. Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance. J Clin Microbiol 1984; 19:187–190.

18 Radstrom P, Backman A, Qian N, Kragsbjerg P, Pahlson C, Olcen 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.

19 Raskin S, Phillips 3rd JA, Kaplan G, McClure M, Vnencak-Jones C. Cystic fibrosis genotyping by direct PCR analysis of Guthrie blood spots. PCR Methods Appl 1992; 2:154–156.

20 Smith K, Diggle MA, Clarke SC. Automation of a fluorescence-based multiplex PCR for the laboratory confirmation of common bacterial pathogens. J Med Microbiol 2004; 53:115–117.

21 Ballard TL, Roe MH, Wheeler RC, Todd JK, Glode MP. Comparison of three latex agglutination kits and counter immunoelectrophoresis for the detection of bacterial antigens in a pediatric population. Pediatr Infect Dis J 1987; 6:630–634.

22 Nato F, Mazie JC, Fournier JM, Slizewicz B, Sagot N, Guibourdenche M, *et al.* Production of polyclonal and monoclonal antibodies against group A, B, and C capsular polysaccharides of *Neisseria meningitidis* and preparation of latex reagents. J Clin Microbiol 1991; 29:1447–1452.

23 Sadler F, Borrow R, Dawson MM, Kaczmarski EB, Cartwright K, Fox AJ. Improved methods of detection of meningococcal DNA from oropharyngeal swabs from cases and contacts of meningococcal disease. Epidemiol Infect 2000; 125:277–283.

24 Taha M-K, Olcén P. Molecular genetic methods in diagnosis and direct characterization of acute bacterial central nervous system infections. APMIS 2004; 112:753–770.

25 Rebelo MC, Boente RF, Matos JDA, Hofer CB, Barroso DE. Assessment of a two-step nucleic acid amplification assay for detection of *Neisseria meningitidis* followed by capsular genogrouping. Mem Inst Oswaldo Cruz 2006; 101:809–813.

26 Taha M-K, Alonso J-M, Cafferkey M, Caugant DA, Clarke SC, Diggle MA, *et al.* Interlaboratory comparison of PCR-based identification and genogrouping of *Neisseria meningitidis*. J Clin Microbiol 2005; 43:144–149.

27 Zhang Z, Kermekchiev MB, Barnes WM. Direct DNA amplification from crude clinical samples using a PCR enhancer cocktail and novel mutants of Taq. J Mol Diagn 2010; 12:152–161.

28 Greisen K, Loeffelholz M, Purohit A, Leong D. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 1994; 32:335–351.

29 Hall LMC, Duke B, Urwin G. An approach to the identification of the pathogens of bacterial meningitis by the polymerase chain reaction. Eur J Clin Microbiol Infect Dis 1995; 14:1090–1094.

30 Bäckman A, Lantz P-G, Rådström P, Olcén P. Evaluation 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.

31 Atobe JH, Hirata MH, Hoshino-Shimizu S, Schmal MR, Mamizuka EM. One-step heminested PCR for amplification of *Neisseria meningitidis* DNA in cerebrospinal fluid. J Clin Lab Anal 2000; 14:193–199.

32 Probert WS, Bystrom SL, Khashe S, Schrader KN, Wong JD. 5′ Exonuclease assay for detection of serogroup Y *Neisseria meningitidis*. J Clin Microbiol 2002; 40:4325–4328.

33 Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 2001; 39:1553–1558.