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A broad range assay for rapid detection and etiologic characterization of bacterial meningitis: performance testing in samples from sub-Saharan^{☆,☆☆,★}

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Abstract

This study aimed to conduct a pilot evaluation of broad-based multiprobe polymerase chain reaction (PCR) in clinical cerebrospinal fluid (CSF) samples compared to local conventional PCR/culture methods used for bacterial meningitis surveillance. A previously described PCR consisting of initial broad-based detection of *Eubacteriales* by a universal probe, followed by Gram typing, and pathogen-specific probes was designed targeting variable regions of the 16S rRNA gene. The diagnostic performance of the 16S rRNA assay in “127 CSF samples was evaluated in samples from patients from Togo, Africa, by comparison to conventional PCR/culture methods. Our probes detected *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. Uniprobe sensitivity and specificity versus conventional PCR were 100% and 54.6%, respectively. Sensitivity and specificity of uniprobe versus culture methods were 96.5% and 52.5%, respectively. Gram-typing probes correctly typed 98.8% (82/83) and pathogen-specific probes identified 96.4% (80/83) of the positives. This broad-based PCR algorithm successfully detected and provided species level information for multiple bacterial meningitis agents in clinical samples.

Keywords

Bacterial meningitis; Clinical samples; Meningitis belt; Real-time PCR; Disease surveillance

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1. Introduction

Acute bacterial meningitis is a significant cause of morbidity and mortality throughout the world, particularly in developing countries. The highest burden of bacterial meningitis occurs in the “meningitis belt” of sub-Saharan Africa, which experienced a record outbreak in 1996 with 250,000 cases and 25,000 registered deaths (<http://www.who.int/mediacentre/factsheets/fs141/en/>, World Health Organization, 2009). Historically, 3 main pathogens, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, have accounted for 75–80% of cases globally, with the majority of others accounted for by *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Rafi et al., 2010). The particular pathogens causing meningitis are constantly evolving, as evidenced by the emergence of *Neisseria* serogroups other than serogroup A and the change in the relative occurrence of pneumococcal meningitis in sub-Saharan Africa.

In the developing world, the capacity of the microbiologic laboratory to interface with the clinical setting for patients with suspected bacterial meningitis has historically been highly variable, with regard to both individual patient care and surveillance (Chanteau et al., 2007). Many remote health care settings have relied on macroscopic evaluation of the cerebrospinal fluid (CSF) only, with additional testing such as latex agglutination and bacteriologic evaluation, occurring only in more advanced districts or urban hospital settings (Chanteau et al., 2007; Chatelet et al., 2005). Molecular testing, which has recently shown capable performance, offers the promise of an alternative approach to both diagnosis and surveillance, and has recently been successfully integrated into routine surveillance in some developing countries (Chanteau et al., 2006, 2007; Chatelet et al., 2005; Sidikou et al., 2003).

Relative to culture, polymerase chain reaction (PCR) diagnostic assays for bacterial meningitis offer speed, as well as high sensitivity and specificity (Chanteau et al., 2006; Chatelet et al., 2005; Robbins et al., 2005; Saravolatz et al., 2003; Sidikou et al., 2003). Accordingly, many experts now consider either PCR or culture to be an acceptable gold standard recognizing that, in many resource-limited settings, definitive microbiologic evaluation is not feasible (Chanteau et al., 2007; Robbins et al., 2005; Rose et al., 2010).

While promising, the current approach to PCR-based assessment of CSF in developing country settings involves the use of multiple individual PCR tests that focus on the top 3 bacterial meningitis-causing organisms. Furthermore, in the largest field surveillance study published to date, PCR was found to have relatively low sensitivity for detection of pneumococcus and *H. influenzae* type b (Chatelet et al., 2005). We recently designed and pilot tested a broad-based multiprobe PCR assay for the identification of common bacterial pathogens and further evaluated it in a small clinical study in the USA (Rothman et al., 2010). Here, we further evaluated our rapid broad-based multiprobe PCR assay by conducting a performance study, relative to both current local conventional PCR methods and traditional microbiologic culture testing for bacterial meningitis, with samples from sub-Saharan Africa.

2. Methods

We evaluated a previously developed probe-based PCR method (Rothman et al., 2010; Yang et al., 2002) with regard to diagnostic performance in detecting the etiology of suspected bacterial meningitis from a repository of surveillance samples from Togo, Africa, using field-based multiplex PCR and classical microbiology as the reference standard. The study was approved by the Institutional Review Board of The Johns Hopkins University and was conducted with authorization of the Togolese Ministry of Health.

2.1. Sample collection and handling of clinical specimens

For the pilot clinical evaluation, we used CSF samples previously collected during enhanced surveillance activities in Togo conducted by the Togolese Ministry of Health in cooperation with the Agence de Médecine Préventive (Paris, France) (Traore et al., 2004). CSF samples were collected from January 2005 to December 2007 at sentinel surveillance sites in Togo for any patient treated for suspected acute bacterial meningitis (Traore et al., 2004). All visibly cloudy samples were tested by latex agglutination. Only samples which reached the reference laboratory within 3 h after lumbar puncture underwent traditional microbiologic testing including Gram-staining microscopy, white blood cell count, and culture.

Primary PCR testing on all CSF samples was performed at the Centre Muraz, Bobo-Dioulasso, Burkina Faso, using a previously described conventional PCR method, which involved amplification of multiple genes specific for *N. meningitidis*, *H. influenzae*, and *Streptococcus pneumoniae*, followed by a multiplex PCR for *N. meningitidis* serogroup identification (Chatelet et al., 2005). As part of the surveillance program, excess CSF was also stored for advanced diagnostic testing and research at -80°C . Clinical and laboratory data were entered in a standardized surveillance database.

2.2. Sample selection for testing at the Johns Hopkins University International Research Laboratory

Deidentified CSF samples ($n=127$) were selected with a positive- to-negative case ratio of approximately 2:1 in Togo and were shipped on dry ice to the Johns Hopkins University International Research Laboratory (JHU IRL). Testing was performed utilizing our probe-based PCR assay (Rothman et al., 2010; Yang et al., 2002). Samples were processed as previously described by laboratory technicians (Rothman et al., 2010; Yang et al., 2002) who were blinded to the microbiologic results. After the probe-based PCR assays were performed, results were compared with the traditional testing (culture, Gram stain, latex agglutination, and PCR) results.

2.3. Laboratory methodology

We designed a PCR assay for bacterial meningitis, building on a multiprobe PCR platform which was designed at JHU IRL; assay design, analytic, and limit of detection (LOD) testing have been previously described (Rothman et al., 2010; Yang et al., 2002).

2.4. PCR assay design

Our assay employs universal primers (p891F: 5'-TGGAGCATGTGGTTTAATTCGA-3'; p1033R: 5'-TGCGGGACTTAACCCAACA3') and a probe (uniprobe: 5'-VIC-CACGAGCTGACGACARCCATGCA3'-MGB) which target conserved regions of the 16S rRNA gene (Rothman et al., 2010; Yang et al., 2002), as well as Gram typing (Gram positive: 5'-FAM-AGGTGGTGCATGGTTGTCGTCAGC3'-MGB; Gram negative: 5'-VIC-ACAGGTGCTGCATGGCTGTGTCGTCAGCT3'-MGB) and pathogen-specific probes targeting the hypervariable V6 region within the same primer region (Yang et al., 2008). Probes for the 6 most common causes of clinically relevant bacterial meningitis were designed by sequence analysis using GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and ClustalW (<http://www.genome.jp/tools/clustalw/>) to determine sequence variability and confirmed for specificity utilizing BLAST (<http://blast.ncbi.nlm.nih.gov/>). Sequences for the previously published 6 probes are as follows: *N. meningitidis*: 5'-VIC-TCCGTCTCCGGAGGATTCCGTAC 3'-MGB; *Streptococcus pneumoniae*: 5'-TETTCACCTCTGTCCCGAAGGAAACTCTATCTCTAGA3'-MGB; *H. influenzae*: 5'-FAM-AAGGCACAAGCTCATCTCTGAGCTCTTCTTAGG3'-MGB; *L. monocytogenes*: 5'-TETAAGGGAAAGCTCTGTCTCCAGAGTGGTCAA3'-MGB;

Staphylococcus aureus: 5'FAM-CCTTTGACAACTCTAGAGATAGAGCCTTCCC3'-MGB, and *E. coli*: 5'FAM-ACATTCTCATCTCTGAAAACCTCCGTGGATGTC3'-MGB (Rothman et al., 2010).

2.5. Sample preparation for PCR

A 500-μL aliquot of the CSF sample was treated with 50 μL of lysis buffer (Magna PureLC Kit-I, Roche Diagnostics, Indianapolis, IN, USA) and incubated at room temperature (25 °C) for 20 min. The specimen was centrifuged at 3200 × *g* for 10 min, and the pellet was resuspended in 50 μL of molecular-grade water. The full nucleic acid extraction protocol was performed as previously described (Rothman et al., 2010; Yang et al., 2002).

2.6. PCR amplification and detection

As the amount of CSF received was limited, it was necessary to incorporate a preamplification step to increase the amount of template DNA in the clinical sample. The preamplification step was analytically validated for use with CSF specimens of low volume (<1 mL) utilizing multiple organisms including *N. meningitides*, *Streptococcus pneumoniae*, and *H. influenzae* at various concentrations (data not shown). The preamplification step consisted of a short 15-cycle PCR using the universal primers p891F and p1033R. A mixture containing 25 μL of 2× Taqman Universal PCR Mix (PE Applied Biosystems, Foster City, CA, USA) and 1.5 μL of each primer, p891F and p1033R, at 67 μmol/L underwent an ultrafiltration step using a Microcon YM-100 centrifugal filter device (Millipore, Bedford, MA, USA) at 3200 × *g* for 10 min, followed by the addition of 1 μL of 2.5 U of low exogenous DNA *Taq*-DNA polymerase (Amplitaq Gold LD, PE Applied Biosystems) to the filtered reaction mixture. The reaction mixture containing 30 μL of PCR mixture and 20 μL of sample DNA was preamplified under the following thermal cycling conditions: pre-incubation at 50 °C for 2 min, denaturation at 95 °C for 10 min, 15 cycles at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Post preamplification, steps were taken to prevent contaminating the second amplification reactions as much as possible. The technicians responsible for performing these reactions changed gloves and laboratory coats, and cleaned pipettes with 10% bleach and 70% ethanol, and the preamplification samples were opened in a separate room from where the second amplification samples were being prepared.

The second amplification consisted of amplifying the first PCR product with the same set of primers and detection accomplished with the addition of either the uniprobe or type-specific probes. PCR was performed with a 50-μL total reaction mixture composed of filtered reaction mixture of 25 μL of 2× Taqman PCR mix (PE Applied Biosystems), 1.5 μL of primers p891F and p1033R, 1 μL of 10 μmol/L probe, 1 μL of 2.5 U *Taq* DNA polymerase (Amplitaq Gold LD), and a 1:10 diluted 20-μL sample of DNA, from the preamplification step. PCR was performed under the following conditions: preincubation at 50 °C for 2 min, denaturation at 95 °C for 10 min, 50 cycles at 95 °C for 15 s, and annealing/extension temperature at 60 °C for 60 s.

Amplification, data collection, and analysis were performed using the ABI 7900 HT System (PE Applied Biosystems). Our detection algorithm consisted of initial broad-based screening of *Eubacteriales* using uniprobe PCR. Those samples that were positive by uniprobe PCR were further analyzed for specific pathogen identification with parallel PCRs consisting of both a Gram-typing (Gram-positive [GP] and Gram-negative [GN] probe) PCR reaction and our pathogen-specific (*N. meningitidis* [NM], *Streptococcus pneumoniae* [SP], *H. influenzae* [HI], *L. monocytogenes* [LM], *Staphylococcus aureus* [SA], and *E. coli* [EC] probe) PCR

reactions. Discrepant samples were further analyzed by standard Sanger sequencing methodology.

Analytic testing, LOD determination, and assay specificity were performed and determined as previously described. Briefly, single colonies of *N. meningitidis* ATCC 6250, *H. influenzae* ATCC 49247, *Streptococcus pneumoniae* ATCC 49619, *L. monocytogenes* ATCC 7648, *E. coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 were grown overnight at 37 °C in tryptic soy broth (Becton Dickinson, Spark, MD, USA) and serially diluted in culture-negative CSF. Nucleic acid was extracted from each dilution as described above, and PCR was performed on the extract (Rothman et al., 2010; Yang et al., 2002).

Negative controls were standardized by pooling multiple culture-negative CSF samples together and processing them as a single negative. Each standardized negative control was tested in triplicate. Additionally, positive and negative controls were utilized for nucleic acid extraction as well as for PCR purposes. Positive controls had to amplify in order for a given run to be considered valid and the results usable. The average and SD of the C_T for the triplicate negative controls from each run were calculated and utilized to determine a cutoff providing positive and negative definitions for each sample. A positive definition was defined as a sample with a C_T value more than 3 SDs below the calculated cutoff; a negative definition was defined as a sample with a C_T value above the calculated cutoff (Rothman et al., 2010; Yang et al., 2008).

The 127 CSF samples were tested using the PCR assay algorithm described above. After laboratory results were recorded, findings were compared with the original conventional PCR findings and traditional microbiologic results. Accuracy of the uniprobe PCR was determined by the observed clinical sensitivity and specificity as compared to conventional PCR results and conventional microbiologic testing with 95% confidence intervals calculated using Stata (StataCorp, College Station, TX, USA).

3. Results

3.1. Comparison of our PCR assay with the conventional PCR results

Of the 127 samples collected from patients with suspected bacterial meningitis, 83 were conventional PCR positive and 44 were conventional PCR negative. When comparing our PCR assay with the field-based PCR, all (83/83) conventional PCR-positive samples tested positive by our uniprobe PCR (sensitivity 100%; 95% CI 95.8–100%), while 24 of 44 conventional PCR-negative samples tested negative by our uniprobe PCR (specificity 54.6%; 95% CI 40.1–68.3%) (Table 1A). Among the 83 positives, conventional PCR results were as follows: 67 *NM* (39 *NMA*, 10 *NMW135*, 15 *NMX*, and 3 *NM* of indeterminate serogroup), 9 *Streptococcus pneumoniae*, 4 *H. influenzae*, and 3 mixed etiologies. Detailed characterization of the 83 conventional PCR-positive and uniprobe-positive samples by our Gram-typing and probe-based assay is shown in Table 2. Gram-typing probes correctly typed 82/83 samples according to their respective bacterial Gram type (10 GP [1 false positive], 73 GN). Pathogen-specific probes provided correct species level information for 80/83 samples according to their respective culture identification (64 *NM* [3 false negatives], 9 *SP*, 4 *HI*, 2 dual-positive *NM* and *HI*, 1 triple-positive *NM*, *HI*, and *EC*).

Further characterization of the 20 samples that were conventional PCR negative and uniprobe positive is shown in Table 3. Seventeen were positive by a Gram-typing probe and 6 of these were positive by a pathogen-specific probe, of which one was a dual positive. Three of the 20 samples were sequenced as *Corynebacterium* and *Micrococcus luteus*, which are known to be common laboratory contaminants (Allothman et al., 2003; Chattopadhyay and Thomas, 1978). Only one of these samples that were positive by uniprobe, GP probe,

and *SP* probe was confirmed as *Streptococcus pneumoniae* by sequencing. The 16 other discordant samples were either “quantity not sufficient” for sequencing or produced sequence data that could not be evaluated.

3.2. Comparison of our PCR assay with the traditional microbiologic results

Comparing our PCR assay with microbiologic testing, of the 127 samples collected, 126 of 127 had at least one of the standard microbiologic tests performed. There were 86 positive by at least one of the 3 standard microbiologic testing methods and 40 were negative. As shown in Table 1B, of the 86 samples positive by traditional microbiologic testing, 83 tested positive by uniprobe PCR (sensitivity 96.5%; 95% CI 90.2–98.8%) and of the 40 samples negative by conventional PCR testing, 21 tested negative (specificity 52.5%; 95% CI 37.5–67.1%). Among the 86 samples that were positive by microbiologic testing, 67 were positive by conventional culture, 85 were positive by Gram staining, and 57 were positive by latex agglutination.

4. Discussion

Currently available nonmolecular diagnostic assays for bacterial meningitis have intrinsic limitations which restrict utility for both expeditious clinical decision making and surveillance in developing countries. CSF Gram stain and cell counts, while relatively quick and low cost, have low sensitivity and are nonspecific (Aronin and Quagliarello, 2009; Feigin et al., 1992). Latex agglutination provides meningococcal serogroup identification for some serogroups including serogroup A, making it useful in the African meningitis belt, but the assay does not detect the currently emerging serogroup X and may underestimate positivity by up to 11% (Chatelet et al., 2005). CSF culture, considered the gold standard in developed countries, typically takes at least 24 h to report, has diminished sensitivity for those already on antibiotics, and may be impractical in resource poor settings (e.g., African meningitis belt) due principally to difficulty in transporting specimens from field sites to reference laboratories which are capable of performing traditional culture and microbiologic testing (Chatelet et al., 2005; Robbins et al., 2005; Saravolatz et al., 2003).

As a result, for purposes of bacterial meningitis surveillance in developing countries, a diagnostic strategy has been advocated which includes routine molecular testing (most commonly PCR) for every sample in combination with microbiologic testing when feasible (Chanteau et al., 2007; Traore et al., 2004). Multiple platforms have recently been advanced for molecular testing (Boisier et al., 2005; Ceyhan et al., 2008; Chakrabarti et al., 2009; Chanteau et al., 2006; Chatelet et al., 2005; Chen et al., 2009; Corless et al., 2001; Duan et al., 2009; Falla et al., 1994; Lafourcade et al., 2010; Saravolatz et al., 2003; Taha et al., 2005) and evaluated for surveillance purposes for bacterial meningitis in developing countries (Boisier et al., 2005; Chatelet et al., 2005; Chanteau et al., 2006; Lafourcade et al., 2010; Sidikou et al., 2003; Robbins et al., 2005). Although many have shown promising performance characteristics with clinical samples, many prior methods developed have been restricted to detecting a limited number of pathogens, based on pathogen-specific gene targeting approach in their PCR design (Boisier et al., 2005; Chatelet et al., 2005; Chanteau et al., 2006; Lafourcade et al., 2010; Sidikou et al., 2003). The findings reported here in this pilot study demonstrate that our multiprobe-based PCR assay showed excellent performance characteristics relative to both a conventional PCR currently in use and traditional microbiologic testing methods (i.e., Gram stain, latex agglutination, and/or culture). Whether the technique is useful as an adjunctive diagnostic for bacterial meningitis surveillance in resource-deficient settings requires further evaluation with regard to feasibility, costs, robustness, and sustainability.

Our broad-based uniprobe PCR had comparable if not better sensitivity than other recent published broad-based PCR assays (Chakrabarti et al., 2009; Chen et al., 2009; Duan et al., 2009; Rafi et al., 2010; Saravolatz et al., 2003) with 100% sensitivity (83/83) for presence of *Eubacteriales* in clinical samples for patients with suspected bacterial meningitis. Of these, our pathogen-specific probes correctly identified 96% (80/83), which is acceptable for most surveillance purposes. Of the 3 of 83 (all *N. meningitidis* by conventional multiplex PCR) that were missed by our pathogen-specific probes, 2 were correctly Gram typed by our method. Sequence analysis for these 3 probe-specific misses failed to confirm original culture findings with 1 yielding 'no quality' results and 2 yielding *Pseudomonas*. Further clinical resolution of these specific cases could not be performed here, due to the identity-unlinked nature of our study.

Recent PCR-based surveillance studies have reported relatively low sensitivity for detection of *Streptococcus pneumoniae* and *H. influenzae* type b, due to low DNA yield on extraction (Chatelet et al., 2005). Our PCR design (in-house extraction and 2-step PCR) and the findings reported here (low LODs, high sensitivity) offer the advantage of identifying missed cases of *Streptococcus pneumoniae* meningitis and *H. influenzae* type b meningitis. Specifically, as shown in Table 3, our assay identified an additional 6 cases positive for *Streptococcus pneumoniae*, *H. influenzae*, or *E. coli*. This is also evidenced by the ability of the assay to detect multiple pathogens present in CSF samples as indicated by the 2 samples that were positive for both *N. meningitidis* and *H. influenzae*, as well as the sample that was positive for *N. meningitidis*, *H. influenzae*, and *E. coli*.

The assay utilized 2 amplification steps, a preamplification step and a secondary amplification step. No evaluation was performed to determine whether the preamplification step contributed to the high sensitivity of the assay, although similar methodologies have been employed previously in nested and semi-nested PCR to elevate PCR sensitivities. It should be noted that any assay utilizing preamplification and secondary amplification steps can be prone to contamination from preamplification, particularly if unidirectional workflow is not followed. Any laboratory attempting methodologies involving more than 1 amplification step should utilize measures to limit the potential for contamination resulting from using more than 1 amplification step.

Despite its high sensitivity, our uniprobe assay had a specificity of only 54.5% when compared to conventional PCR and 52.5% relative to traditional reference microbiology. Although the comparison between a conventional PCR and a real-time PCR is not ideal, as real-time-based PCR methods are more sensitive and specific than conventional PCR methods, this comparison is useful because it compares one amplification-based assay to another amplification-based assay. One possible explanation for the 20 uniprobe PCR-positive but conventional PCR-negative findings could be the improved extraction methods which are incorporated into our protocol. Potential explanation for the 19 uniprobe PCR-positive but reference microbiology-negative findings could include potential antibiotic pretreatment prior to specimen collection (our de-identified protocol does not allow us to assess this) or problems with sample transportation, storage, and processing (Chakrabarti et al., 2009; Rafi et al., 2010; Tunkel et al., 2004; von Gottberg et al., 2008). The fact that further analysis of several of these samples in our laboratory by sequencing yielded common laboratory contaminants is supportive of this later explanation, at least for a subset of samples. An alternative explanation for our relatively poor specificity is that these cases truly represent false-positive findings, a phenomenon which has been well described in prior studies using broad-based 16S rRNA eubacterial PCR assays (Yang et al., 2002). These assays are inherently more prone to false-positive findings, due to sequence homology with exogenous bacterial DNA. Lastly, as this methodology involved 2 amplification steps, a preamplification step and a secondary amplification step, it is possible that contamination

was introduced into these samples during transfer of the preamplification reaction to the secondary amplification reaction.

Future studies that include additional clinical data will help to adjudicate discrepant findings and provide a more reliable estimate of the specificity of our assay. Other broad-based PCR studies that reported higher specificity to that described here incorporated clinical data to better resolve discrepant findings (Corless et al., 2001; Duan et al., 2009; Rafi et al., 2010; Saravolatz et al., 2003). Use of a third “tie-breaker” test (e.g., PCR targeting an alternative pathogen-specific gene) could also assist in further discrimination of discordant samples in future studies, but was not possible here due to sample volume.

The CSF samples studied here came from Togo, Africa, and included a wide range of serogroups for *N. meningitidis* (Table 2). Reliable identification of each of these meningococci (A, X, W135) by our uniprobe, Gram-typing, and pathogen-specific probes gives us confidence that our assay is robust for detecting a broad range of serogroups. For example, our *N. meningitidis* probe detected *N. meningitidis* serogroups W135 and X that have caused major epidemics during the last decade in the meningitis belt (Mueller et al., 2006; von Gottberg et al., 2008). Other PCR-based meningitis surveillance assays that have been used to track serogroup patterns of meningitis are constrained by the use of specific primer targets (i.e., each specific for an individual serogroup) (Chanteau et al., 2006; Chatelet et al., 2005). Our assay circumvents this limitation since the target of our type-specific probe is specific for *N. meningitidis* but is sufficiently degenerate to detect a broad range of serogroups. While advantageous for sensitive detection of multiple serogroups (relevant for treatment), our assay is, however, limited because it cannot differentiate individual serogroups for meningococcal and pneumococcal meningitis, which is a limitation for informing vaccine strategy. For the latter purpose, further refinement of primer design using other 16S targets could, in theory, be designed in a similar fashion to what our group has demonstrated recently (i.e., for *Salmonella* serovar differentiation) (Gaydos et al., 2010). It still has value for meningitis surveillance because of its demonstrated capability to detect and identify organisms other than *N. meningitidis* responsible for bacterial meningitis, such as *Streptococcus pneumoniae* and *H. influenzae*, as well as the capability to detect and identify organisms that are responsible for atypical bacterial meningitis, such as *Staphylococcus aureus* and *E. coli*.

Although our assay is relatively rapid, another limitation is that technical aspects still limit time to detection and identification to approximately 6 h. The probe-based PCR platform described here is relatively cumbersome due to the multistep process, technical limitations of the multiprobe design (i.e., limited number of fluorophores that can be used in a single reaction), and inability to identify unanticipated pathogens without further sequence analysis. Incorporating automated sample processing and alternative detection methods, such as high-resolution melt analysis, hold promise to further reduce time to detection (Gaydos et al., 2010; Yang et al., 2009). Additional limitations to the methodology described here are the level of technical complexity, involving 2 separate amplification steps; filtration of the master mix utilized; reliance on a robotic platform for nucleic acid extraction; and the use of a floating negative control cutoff that requires data interpretation. Additionally, as this method involves a preamplification step that generates additional template for amplification, it can be prone to contamination that could yield false-positive results. In its current format, the methodology utilized here would not be suited for a primary field use diagnostic or a primary diagnostic for resource poor settings. However, despite the limitations, this methodology could be very useful as a confirmation diagnostic, or adjunctive diagnostic to field, or traditional gold standard testing.

In summary, the broad-based PCR algorithm utilized in this study provided highly sensitive detection of bacterial species responsible for the majority of cases of meningitis seen in areas of high bacterial meningitis incidence. Additionally, the broad-based PCR algorithm coupled to Gram-typing and species-specific probes provided species level identification of the causative bacterial pathogens observed in this study for bacterial meningitis.

References

- Allothman A, Jelani A, Althaqafi A, Rich M, Williams E. Contamination of patient hospital charts by bacteria. *J Hosp Infect.* 2003; 55:304–5. [PubMed: 14629976]
- Aronin, SI.; Quagliarello, VJ. Bacterial meningitis: diagnostic pearls. Medscape Web site. 2009. Available at: http://www.medscape.com/viewarticle/451596_2
- Boisier P, Djibo S, Sidikou F, et al. Epidemiological patterns of meningococcal meningitis in Niger in 2003 and 2004: under the threat of *N. meningitidis* serogroup W135. *Trop Med Int Health.* 2005; 10:435–43. [PubMed: 15860090]
- Ceyhan M, Yildirim I, Balmer P, et al. A prospective study of etiology of childhood acute bacterial meningitis, Turkey. *Emerg Infect Dis.* 2008; 14:1089–96. [PubMed: 18598630]
- Chakrabarti P, Das BK, Kapil A. Application of 16S rDNA based seminested PCR for diagnosis of acute bacterial meningitis. *Indian J Med Res.* 2009; 29:182–8. [PubMed: 19293446]
- Chanteau S, Rose AMC, Djibo S, Nato F, Boisier P. Biological diagnosis of meningococcal meningitis in the African meningitis belt: current epidemic strategy and new perspectives. *Vaccine.* 2007; 25S:A30–6. [PubMed: 17517452]
- Chanteau S, Sidikou F, Djibo S, Moussa A, Mindadou H, Boisier P. Scaling up of PCR- based surveillance of bacterial meningitis in the African meningitis belt: indisputable benefits of multiplex PCR assay in Niger. *Trans R Soc Trop Med Hyg.* 2006; 100:677–80. [PubMed: 16359713]
- Chatelet I, Traore Y, Gessner B, Antignac A, Njanpop-Lafourcade B, Ouedraogo M, et al. Bacterial meningitis in Burkina Faso: surveillance using field-based polymerase chain reaction testing. *Clin Infect Dis.* 2005; 40:17–25. [PubMed: 15614687]
- Chattopadhyay B, Thomas E. Bacterial contamination of laboratory forms. *J Clin Pathol.* 1978; 31:1004–5. [PubMed: 711900]
- Chen L, Duan Q, Cai M, Wu Y, Shang S. Rapid diagnosis of sepsis and bacterial meningitis in children with real-time fluorescent quantitative polymerase chain reaction amplification in the bacterial 16S rRNA gene. *Clin Pediatr.* 2009; 48:641–7.
- Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarek 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; 39:1553–8. [PubMed: 11283086]
- Duan Q, Shang S, Wu Y. Rapid diagnosis of bacterial meningitis in children with fluorescence quantitative polymerase chain reaction amplification in the bacterial 16S rRNA gene. *Eur J Pediatr.* 2009; 168:211–6. [PubMed: 18548276]
- Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of *Haemophilus influenzae*. *J Clin Microbiol.* 1994; 32:2382–6. [PubMed: 7814470]
- Feigin RD, McCracken GH Jr, Klein JO. Diagnosis and management of meningitis. *Pediatr Infect Dis J.* 1992; 11:785–814. [PubMed: 1448332]
- Gaydos, CA.; Won, H.; Yang, S.; Hsieh, YH.; Kecojevic, A.; Tennant, S., et al. Program and Abstracts Book of the International Conference on Emerging Infectious Diseases (Atlanta). Vol. 61. American Society for Microbiology; 2010. Rapid identification of typhoidal and non-typhoidal salmonella species using a broad based PCR assay coupled with high-resolution melt analysis [abstract 40].
- Lafourcade BN, Sanou O, Linden M, Levina N, Karanfil M, Yaro S, et al. Serotyping pneumococcal meningitis cases in the African meningitis belt by use of multiplex PCR with cerebrospinal fluid. *J Clin Microbiol.* 2010; 48:612–4. [PubMed: 20007384]
- Meningococcal meningitis. World Health Organization Web site. 2009. Available at: <http://www.who.int/mediacentre/factsheets/fs141/en/>

- Mueller JE, Borrow R, Gessner BD. Meningococcal serogroup W135 in the African meningitis belt: epidemiology, immunity and vaccines. *Expert Rev Vaccines*. 2006; 5(3)
- Rafi W, Chandramuki A, Mani R, Satishchandra P, Krishna S. Rapid diagnosis of acute bacterial meningitis: role of broad range 16S rRNA PCR. *J Emerg Med*. 2010; 38:225–30. [PubMed: 18790588]
- Robbins J, Schneerson R, Gotschlich E. Surveillance for bacterial meningitis by means of polymerase chain reaction. *Clin Infect Dis*. 2005; 40:26–7. [PubMed: 15614688]
- Rose AMC, Mueller JE, Gerstl S, Njanpop-Lafourcade BM, Page A, Nicolas P, et al. Meningitis dipstick rapid test: evaluating diagnostic performance during an urban *Neisseria meningitidis* serogroup A outbreak, Burkina Faso, 2007. *PLoS One*. 2010; 5(6):e11086. [PubMed: 20552035]
- Rothman R, Ramachandran P, Yang S, Hardick A, Won H, Kecojevic A, et al. Use of quantitative broad-based polymerase chain reaction for detection and identification of common bacterial pathogens in cerebrospinal fluid. *Acad Emerg Med*. 2010; 17:741–7. [PubMed: 20653589]
- Saravolatz L, Manzor O, VenderVelde N, Pawlak J, Belian B. Broad-range bacterial polymerase chain reaction for early detection of bacterial meningitis. *Clin Infect Dis*. 2003; 36:40–5. [PubMed: 12491200]
- Sidikou F, Djibo S, Taha MK, Alonso JM, Djibo A, Kairo KK, et al. Polymerase chain reaction assay and bacterial meningitis surveillance in remote areas, Niger. *Emerg Infect Dis*. 2003; 9:1486–8. [PubMed: 14718100]
- Taha MK, Alonso JM, Cafferkey M, et al. Interlaboratory comparison of PCR-based identification and genotyping of *Neisseria meningitidis*. *J Clin Microbiol*. 2005; 43:144–9. [PubMed: 15634963]
- Traore Y, Tameklo TA, Njanpop-Lafourcade B, Lourd M, Yaro S, Niamba D, et al. Incidence, seasonality, age distribution, and mortality of pneumococcal meningitis in Burkina Faso and Togo. *Clin Infect Dis*. 2004; 48:S181–9. [PubMed: 19191614]
- Tunkel A, Hartman B, Kaplan S, Kaufman B, Roos K, Scheld W, et al. Practice guidelines for the management of bacterial meningitis. *Clin Infect Dis*. 2004; 39:1267–84. [PubMed: 15494903]
- Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CG, et al. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *J Clin Microbiol*. 2002; 40:3449–54. [PubMed: 12202592]
- Yang S, Ramachandran P, Hardick A, Hsieh Y, Quianzon C, Kuroki M, et al. Rapid PCR-based diagnosis of septic arthritis by early Gram-type classification and pathogen identification. *J Clin Microbiol*. 2008; 46:1386–90. [PubMed: 18305128]
- Yang S, Ramachandran P, Rothman RE, Hsieh YH, Hardick A, Won H, et al. Rapid identification of biothreat and other clinically relevant bacterial species by use of universal PCR coupled with high-resolution melting analysis. *J Clin Microbiol*. 2009; 47:2252–5. [PubMed: 19458181]
- von Gottberg A, du Plessis M, Cohen C, Prentice E, Schrag S, de Gouveia L, et al. Emergence of endemic serogroup W135 meningococcal disease associated with a high mortality rate in South Africa. *Clin Infect Dis*. 2008; 46:377–86. [PubMed: 18181736]

Table 1A

Conventional PCR results versus uniprobe PCR results.

	Conventional PCR +	Conventional PCR –	Total
Uniprobe PCR (+)	83 ^a	20 ^b	103
Uniprobe PCR (–)	0	24	24
Total	83	44	127

^aFor details see Table 1B.

^bFor details, see Table 2.

Table 1B

Traditional microbiologic testing (culture, latex agglutination, and Gram-stain results) versus uniprobe PCR results.

	Microbiologic testing +		Microbiologic testing –	Total
Uniprobe PCR (+)	83	19		102
Uniprobe PCR (–)	3	21		24
Total	86	40		126 ^a

^aOne specimen did not have standard microbiologic test results.

Detailed results for conventional PCR–positive, uniprobe-positive samples: uniprobe, Gram-typing probe, and pathogen-specific probe PCR results.

Table 2

Conventional PCR results	n	n'	Uniprobe	Gram typing	Pathogen-specific results
<i>Neisseria meningitidis</i> (serotypes: A, n = 39; W135, n = 10; X, n = 15; indeterminate, n = 3)	67	64	+	GN+	NM+
		2	+		–
<i>Streptococcus pneumoniae</i>		1	+	GP+	–
<i>Haemophilus influenzae</i> (type b)	9	9	+	GP+	SP+
<i>N. meningitidis</i> (A) + <i>H. influenzae</i> (b)	4	4	+	GN+	HI+
	3	3	+	GN+	NM+ HI+ EC+
Total	83	83	83/83	82/83 ^a	80/83 ^b

n = Total no. of conventional PCR positives; n' = further delineated sample numbers; Uniprobe = universal probe PCR result; NM = *N. meningitidis* probe PCR result; SP = *Streptococcus pneumoniae* probe PCR result; HI = *H. influenzae* probe PCR result; LM = *L. monocytogenes* probe PCR result; EC = *E. coli* probe PCR result; SA = *Staphylococcus aureus* probe PCR result; GN = Gram-negative probe PCR positive; GP = Gram-positive probe PCR positive.

^aEighty-two of 83 positive by respective Gram typing; 1 NM+ sample was GP+.

^bEighty of 83 positive by respective pathogen-specific probe; 3 NM+ missed by NM probe; 2 sequenced as *Pseudomonas*, 1 no quality sequencing data; EC detected in 1 of 3 NM+ HI+ samples.

Table 3

Detailed results for 20 conventional PCR–negative, uniprobe–positive samples.

Conventional PCR results	n'	Uniprobe	Standard microbiologic testing results	Gram-typing PCR	Pathogen-specific PCR	Sequencing results
<i>Negative (n = 20)</i>						
	1	+	+ Latex agglutination	GP+	SP+	Sequencing confirmed <i>Streptococcus pneumoniae</i>
	3		+ Gram stain/culture	GN+	HI+	QNS for sequencing
	1		–	GN/GP+	SP+ HI+	QNS for sequencing
	1		–	GN+	EC+	
	2		–	GN+	–	
	1		+ Gram Stain	GP+	–	Sequenced as <i>Corynebacterium</i>
	2		–	GP+	–	Sequenced as <i>Micrococcus luteus</i>
	1		–	GP+	–	Sequencing showed no quality data
	2		+ Gram Stain	GN+	–	
	3		–	GN+ GP+	–	
	3		–	QNS	QNS	QNS for further testing
Total	20	20/20	7/20 ^a	17/20 ^b	7/20 ^c	4/20 ^d

Uniprobe = Universal probe PCR result; GN = Gram-negative probe PCR positive; GP = Gram-positive probe PCR positive; SP = *Streptococcus pneumoniae* probe PCR result; HI = *H. influenzae* probe PCR result; EC = *E. coli* probe PCR result; QNS = Quantity not sufficient.

'–' = Indicates a negative result.

^aSeven of 20 conventional PCR–negative samples were positive by 1 of 3 reference methods (latex agglutination, Gram stain, culture).

^bSeventeen of 20 conventional PCR–negative samples were positive by Gram-typing PCR.

^cSeven of 20 conventional PCR–negative samples were positive by pathogen-specific PCR.

^dFour of 20 conventional PCR–negative samples were positive by sequencing; 3 were sequenced as common laboratory contaminants.