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Full Length Research Paper

Detection of *Neisseria meningitidis* and unknown *Gammaproteobacteria* in cerebrospinal fluid using the two-step universal method

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Bacterial meningitis is insufficiently diagnosed based on microscopic, cultural, and multiplex-polymerase chain reaction (M-PCR). The use of already established universal method (UM) offers the ultimate solution to the detection and potential identification of bacteria in cerebrospinal fluid (CSF) samples. We have applied the UM together with a newly established Anchored Multiplex PCR (AMD4; *Streptococcus pneumoniae*, *Haemophilus influenzae, Neisseria meningitidis,* and *Listeria monocytogenes*) to screen 130 CSF samples obtained from suspected meningitis cases for any bacterium. This two-stage UM approach was able to show that three of the samples contained bacterial DNA, only one of the three samples (K1) was shown to contain *N. meningitidis* whereas the other two samples (A35 and H1) were negative with AMD4. Nucleotide sequencing and BLAST analyses of 16S amplicons obtained by the UM from samples A35 and H1 showed no significant homology (<90%) to any available 16S sequence, yet indicated both bacteria (A35 and H1) to share 94.2% similarity. Both bacteria belonged to *Gammaproteobacteria*. The bacterium from sample K1 was isolated by culture and identified as *N. meningitidis*. The other two samples were negative by culture according to the clinical laboratories at both hospitals; A35 was from a patient who had received empirical antimicrobial therapy prior to sample collection. The remaining 127 samples were shown by the UM to be negative in accordance with clinical and laboratory findings. The UM can contribute significantly to the identification of bacterial meningitis cases to initiate empirical antimicrobial therapy within 3 h of sample collection. Simultaneously, bacterial meningitis can be ruled out from samples producing negative UM results. AMD4 application will detect and identify the major pathogens of bacterial meningitis whereas the UM will detect any bacterium, UM can potentially identify any bacterium as long as it is represented in the nucleotide databases; if not represented, it is labeled as unknown. We recommend the utilization of the UM in clinical testing; we also recommend culturing, characterization and identification of these unknown bacterial agents of meningitis as well as others.

Key words: Meningitis, cerebrospinal fluid (CSF), cerebrospinal fluid, the universal method, 16S rDNA, bacterial detection, bacterial identification.

INTRODUCTION

Despite improvements in health care system, acute bacterial meningitis remains a life threatening infectious emergency (Chakrabarti et al., 2009). While most infections occur in infants, they are found in healthy children and adolescents as well. Successful treatment requires rapid detection then identification of the bacterium (Saravolatz et al., 2003). Bacterial meningitis may lead to permanent neurological sequelae such as hearing loss, mental retardation, and seizures. Behavioral changes may occur in up to 50% of survivors, especially when diagnosis and treatment are delayed (Saravolatz et al., 2003; Dubos et al., 2008; Welinder-Olsson et al., 2007). Potential long-term neurological sequelae include cranial nerve paralysis, hemiparesis, hydrocephalus, seizures as well as visual and hearing impairment which can have a profound impact on the quality of life of the

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Table 1. Bacterial pathogens known to cause meningitis.

Pathogen	Source /References
Acinetobacter baumannii	Siegman-Igra et al.,1993; Kim et al., 2009
Bacillus anthracis	Ebrahimi et al., 2011
Chryseobacterium meningosepticum	Ozkalay et al., 2006
Citrobacter farmer	Tan et al., 2010
Enterococcus spp.	Poppert et al., 2005
Escherichia coli	Brouwer et al., 2010; Porter, 2011
Gemella haemolysans	Anil et al., 2007
Globicatella sanguinis	Héry-Arnaud et al., 2010
Gram negative bacilli	Kim et al., 2009; Porter, 2011
Haemophilus influenzae	Failace et al., 2005; Porter, 2011
Lactobacillus rhamnosus	Robin et al., 2010
Listeria monocytogenes	Brouwer et al. , 2010; Porter, 2011
Mycobacterium tuberculosis	Thwaites et al., 2011
Neisseria meningitidis	Porter, 2011
Proteus vulgaris	Tebruegge et al., 2008
Salmonella spp.	Tebruegge et al., 2008
Staphylococcus epidermidis	Kim et al., 2009
Staphylococcus spp., *Staphylococcus aureus*	Brouwer et al., 2010
Streptococcus spp., *Streptococcus agalactiae*	Failace et al., 2005; Marchandin et al., 2005; Geiβdörfer et al., 2008; Domínguez-Punaro et al., 2010
Streptococcus pneumoniae	Marchandin et al., 2005; Porter, 2011
Streptococcus spp. Group B	Porter, 2011
Streptococcus suis	Domínguez-Punaro et al., 2010
Ureaplasma urealyticum	Geiβdörfer et al., 2008

survivors. Death rate may reach 25% (Kim, 2003; Tebruegge and Curtis, 2008; Gray and Fedorko, 1992; Failace et al., 2005). Meningitis cases are usually attributed to a single agent; however, mixed infections are possible (Marchandin et al., 2005). Table 1 summarizes the bacterial agents that have been reported to cause meningitis.

Here, we propose a modified method for bacterial detection and identification. Previously, it has been demonstrated that 34 species distributed among 24 bacterial genera could be detected and identified using primer pairs obtained from 16S rDNA sequence information (Barghouthi, 2011), in addition, previous method (Failace, 2005) used for the detection of *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* was modified to detect *Listeria monocytogenes* as well as other bacteria such as *Staphylococcus epidermidis*. Thus, AMD4, in combination with UM should be sufficient to rule out or detect 4 deadly meningitis causing bacteria and still simultaneously detect other bacterial DNA present in the sample. This two step Universal Method was applied for the detection of three bacteria from unrelated bacterial meningitis cases caused by different unknown *Gammaproteobacteria* that were reported as “no growth” by two clinical laboratories (from two samples) and one by *N. meningitidis* that was reported as “growth of *N. menigitidis*”. This work illustrates the validity of the two-step UM in the direct detection and identification of bacteria from cerebrospinal fluid (CSF). Further efforts to isolate and characterize such bacteria are amply justified in the pursuit of understanding bacterial meningitis; antibiotic susceptibility, patient care, and epidemiology.

MATERIALS AND METHODS

In the present study, a robust method for detection and identification of bacterial meningitis was applied. The method is a polymerase chain reaction (PCR) based system that amplifies segments of 16S rDNA bacterial gene allowing both detection and identification of CSF bacterial pathogens. Two methods are applied simultaneously to each CSF sample, the Universal Method golden primer mixture G7 which is a multiplex of 16S Alquds University General Primers: (QUGP-Fn3: 5’-CAGGATTAGATACCCTGGTAGTCC-3’; QUGP-F4: 5’-CCGCCTGGGGAGTACG-3’;QUGP-Fn5: 5’-ACTCCTACGGGAGGCAGCAG-3’; QUGP-Fn6: 5’-CCAGCAGCCGCGGTAATAC-3’; QUGP-Rn1: 5’-GGCTACCTTGTTACGACTTC-3’;QUGP-Rn2: 5’-TGACGGGCGGTGTGTACAAG-3’;QUGP-Rn3: 5’-GGCGTGGACTACCAGGGTATC-3’) and the anchored species specific PCR multiplex (AMD4; Table 2) were used for simultaneous

Table 2. The sequences of the species specific forward primers and their respective amplicon sizes are indicated. The sequences for QUGPs are given in the methods section.

Specific forward primer	***Species specific PCR and sequencing primers (5'→3')***	***Tm*** °C [§]	***Amplicon Size with anchor primer QUGP-Rn2 / QUGP-Rn3***
Haemophilus influenzae	TGAGAGGCCGCATGCCATAGGATGA Φ	*64*°C	1201/ 600bp
Klebsiella pneumoniae Escherichia coli, Mycobacterium, and others¶	ATTGCACAATGGGCGCAAGCCTGAT	65°C	1044 /442bp
*Listeria monocytogenes Digestion of (TCCGGA)**	AAGTGTGGCGCATGCCACGCTT Φ	65°C	1231 / 631 bp (381+250)
Neisseria meningitidis	TTTGTCAGGGAAGAAAAGGCTGTTGC Φ	*61*°C	969 /369 bp
Staphylococcus epidermidis and others[Ω]	GCCTAATACATGCAAGTCGAGCGAACAG	62°C	1373/768 bp
*Streptococcus pneumonia Digestion of (TCCGGA)**	GTGTGAGAGTGGAAAGTTCACACTG Φ	58°C	952/351 bp (102+249bp)
S. agalactia	GCAAGTAGAACGCTGATGTTTGGTGTTTAC	61°C	1359/756 bp

*Restriction enzymes that may cut the sequence TCCGGA are *Bsp13I, Kpn2I,* or *BseAI* producing the digestion products shown, Φ AMD4 mix was made of indicated primers mixed with QUGP-Rn2 or QUGP-Rn3, sequences are given in the materials and methods section, **§** calculated T_m accounting for base-stacking, ¶ Include: *Gulosibacter sp., Micrococcus luteus, K. oxytoca, K. variicola, Pseudoalteromonas sp., Pantoea agglomerans, P. eucalypti, P. ananatis, P. dispersa, Enterobacter cowanii, Serratia marcescens*, among others, Ω Others include other *Staphylococcus* spp*., Bacillus* spp., *Aerococcus* spp. among others.

detection and identification of bacteria in CSF samples. The protocol used for the detection and identification of bacterial pathogens in CSF samples is illustrated (Figure 1).

Patients

Clinical CSF samples were collected from 130 suspected meningitis patients by lumber puncture, (neonates and internal word). All samples were tested by clinical laboratories using conventional methods (culture). For this study, a duplicate sample was obtained from Al Ahli Hospital (H samples), Alia Hospital (A samples), and Caritas Hospital (K samples) with appropriate permissions between January 2009 and August 2010. Duplicate CSF samples were collected in DNA free screw cap sterile microfuge tubes and stored at –65°C.

DNA extraction from pure cultured reference species

The most common CSF bacteria (*N. meningitides, Haemophilus influenzae, Streptococcus pneumoniae,* and *Listeria monocytogenes*) were obtained from Al-Quds University Bacterial Collection in the form of pure cultures (Barghouthi, 2011).

To achieve a uniform DNA extraction procedure that is applicable to Gram positive and Gram negative bacteria, the following protocol was performed: Phosphate buffered saline PBS-2-mercaptoethanl mixture (PBS; NaCl 80 g, KCl 2.0 g, Na_2HPO_4 14.4 g, KH_2PO_4 2.4 g, pH 7.4, final volume 1 L H_2O), 2-mercaptoehanol (2ME; Sigma Chemicals) was added to the autoclaved PBS; 0.5 µl/ml. As a reducing agent, 2ME may inhibit or inactivate bacterial enzymes such as proteases and nucleases that may interfere with the quality of DNA or the efficiency of the PCR reaction. A 1 µl sterile plastic loop was used to transfer a loop-full of bacteria colonies to 400 µl of PBS-2ME. After 5 min of incubation at ambient room temperature, the bacterial cells were collected by centrifugation at 13,000 rpm for 1 min (Hettich microcentrifuge, Tuttlingen, Germany), supernatant was aseptically discarded. To the remaining pellet 50 µl of lysis mixture (25 µl of 0.5N NaOH and 25 µl of 1% Sodium Dodecyl Sulfate; SDS) were added, mixed, and steamed over a boiling water bath for 10 min. The lysate was diluted with 150 µl of sterile pure water, mixed, and centrifuged for 3 min at 13,000 rpm, the supernatant was then used in PCR reactions. The remaining supernatant was transferred to a fresh sterile screw cap microfuge tube and stored at -65°C for further use.

Bacterial DNA extraction from CSF samples

One hundred microlitre of CSF sample were placed in a labeled sterile screw cap microfuge tube, 0.5 µl of 2-mercaptoethanol (2ME) was added, after incubation at 37°C for 5 min, centrifuged for 2 min at 13,000 rpm, supernatant was removed and appropriately discarded. The remaining steps for DNA extraction were exactly as described above.

Universal method bacterial detection and anchored-multiplex PCR

The UM was applied as described by Barghouthi (2011). Briefly, the golden mixture multiplex (G7) which amplifies any of the ten target sequences distributed among bacterial 16S rDNA genes. G7 takes advantage of the probability of any one of those 10 sequence of appearing in any bacterium, G5 supplies another 3 target sequences that can be applied as well. UM detects all bacteria and identifies many bacterial species (Barghouthi, 2011). One of the produced PCR products is then sequenced for bacterial identification through BLAST analysis of available DNA sequences in gene data bases (Altschul et al., 1990; BLAST, 2001).

The anchored-multiplex (AM) method is a modified multiplex that was developed for this study. The two general reverse primers (QUGP-Rn2 or QUGP-Rn3; Table 2) that represents most bacteria; particularly those causing meningitis: *N. meningitidis*, *S. pneumoniae*, *H. influenzae, L. monocytogenes, S. aureus,* enterobacteria and others were QUGP-Rn2 and QUGP-Rn3 (Barghouthi, 2011).

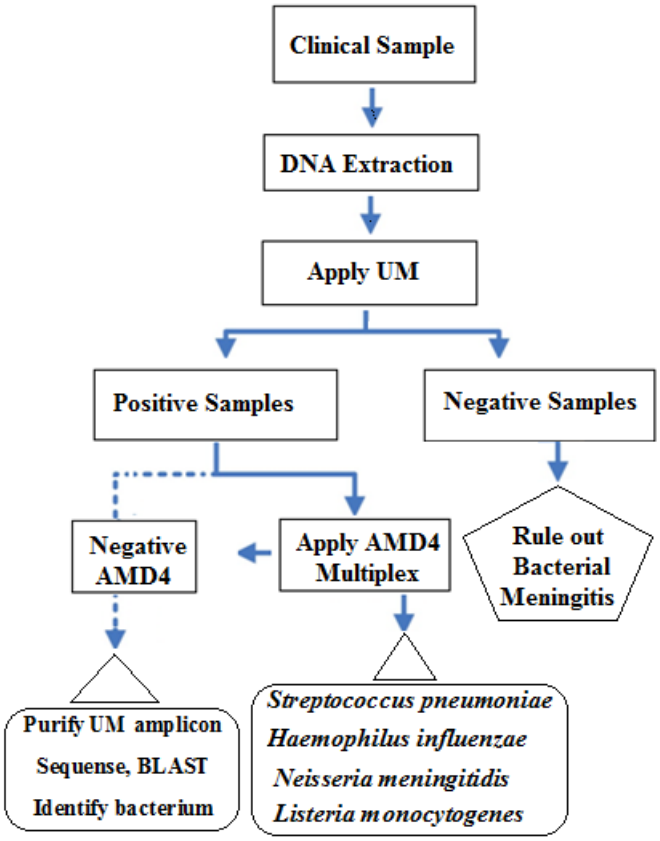


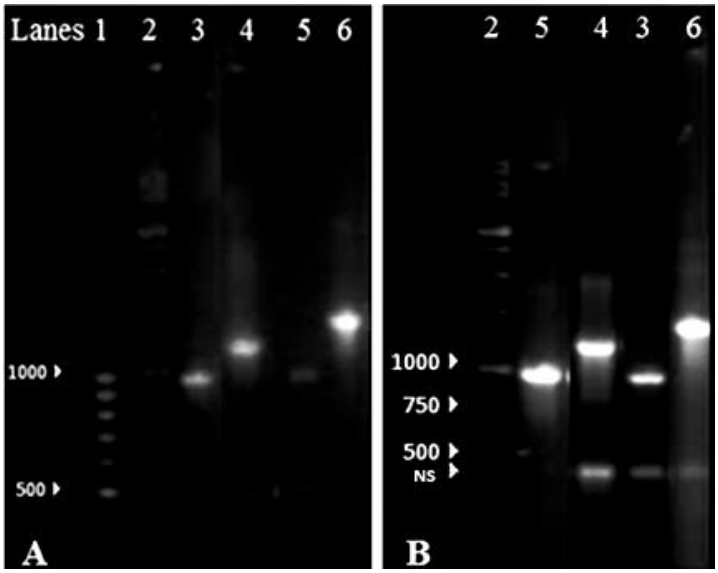
Figure 1. Bacterial detection and identification working plan.

QUGP-Rn2 produces longer PCR amplicon with the forward species specific primers than does QUGP-Rn3. Table 2 shows the expected PCR amplicons with different bacteria when QUGP-Rn2 or QUGP-Rn3 are used. All primers were designed to be functional at a fixed annealing temperature of > 58°C as determined by the online *Tm* calculator (www.promega.com). The primers used in this study to generate Anchored-Multiplex D4 (AMD4) and others are shown in Table 2.

AMD4 is a mixture containing the four species specific forward primers and the QUGP-Rn3 or QUGP-Rn2, the multiplex was named AMD4, since the QUGP-Rn3 or Rn2 served as the reference point to determine the PCR product size. Species specific primers were also tested individually with one of the general QUGPs primers. The mixture was directed against *N. meningitidis*, *S. pneumoniae*, *H. influenzae,* and *L. monocytogenes.* The concept of the anchored –multiplex takes advantage of the fact that the two reactions (G7 and AM) can be applied simultaneously or sequentially to reduce the requirement for amplicon sequencing and to facilitate bacterial identification. The following procedure was performed: One hundred pmol (10 µl) of each specific forward primer was mixed with 40 µl (400 pmol) of one of the reverse primers (QUGP-Rn2 or Rn3) and 20 µl of sterile, DNA-free water. This produced 10 pmol/ µl concentration of each primer, except for the reverse primer which was used at 40 pmol to prevent its depletion in case of the presence of more than one target sequence and to balance the forward primers. PCR reactions were carried out as 25 µl-reactions in thin walled tubes; primers and DNA were added at 0.5 µl each (Ready MasterMix, Promega Biochemical company). Short amplicons obtained with QUGP-Rn3 produced better band resolution than the long amplicons seen with QUGP-Rn2 (Table 2).

RESULTS AND DISCUSSION

AMD4 was prepared by mixing the general reverse primer QUGP-Rn2 or -Rn3 with the species specific primers. When AMD4 was applied specifically to each one of the reference bacterial species, specific PCR amplicons were generated (Figure 2). AMD4 (QUGP-Rn2) produced the appropriate PCR amplicons; a 952 bp amplicon with *S. pneumoniae*; a 1201 bp amplicon with *H. influenzae*; a 969 bp amplicon with *N. meningitidis;* and 1231 bp amplicon with *L. monocytogenes*. Other potential bacterial pathogens (*Klebsiella pneumoniae, Escherichia coli,* and *Staphylococcus epidermidis*) were tested individually; using their specific forward primers anchored to one of the reverse primers QUGP-Rn2 or QUGP-Rn3 they produced the predicted PCR amplicons (Table 2). The results (Figure 2A) illustrate that AMD4 produced amplicons identical to those obtained when each specific primer was applied separately (Figure 2B). The results also showed the lack of cross reactivity between primers and other non-target inter-or intra-species DNA, emphasizing the specificity of the AMD4 mixture. Amplicons obtained with AMD4 were in complete



Fiugure 2. (A) Amplicons produced with Anchored Multiplex D4 (AMD4) using the reverse primer QUGP-Rn2 for each of four bacterial pathogens. Lanes: 1, 100-bp molecular markers; 2, 1Kbp Markers; 3, *Streptococcus pneumoniae* 952bp amplicon; 4, *Haemophilus influenzae* 1201 bp; 5, *Neisseria meningitidis* 969bp*;* 6, *Listeria monocytogenes* 1231bp**. (B)** Amplicons produced without multiplex from direct application of each specific forward primer with QUGP-Rn2, lanes are as in panel A, non-specific bands ~300bp (NS) were only observed in panel B and absent from panel A . Lanes with the same number contain the same bacterial amplicon (lanes 3 and 5 are switched in panel B).

agreement with the theoretically predicted PCR amplicons (Figure 2 and Table 2).

Sensitivity of the AMD4 multiplex

The ability of AMD4 to detect target bacteria in CSF samples was tested and established. Reference bacterial colony was serially diluted in human CSF that was judged to be bacteria-free by its negative reaction to UM-G7. AMD4-PCR was then applied to DNA extracted from each dilution. The results showed that AMD4 was able to specifically detect bacteria diluted in CSF (Figure 3A). AMD4 was able to generate shorter amplicons when QUGP-Rn2 was replaced by QUGP-Rn3 (Figure 3B).

Clinical CSF samples

Molecular detection of bacteria in CSF from 130 clinical samples collected from three different hospitals in Southern West Bank, Palestine, has successfully shown 127 samples to have no bacterial DNA indicating the absence of bacterial meningitis. Typical PCR negative samples tested with UM-G7 are shown (Figure 4).

The remaining three samples (A35, H1, and K1) were positive by UM-G7. When these three samples were tested with AMD4, only sample K1 produced a unique PCR product (969 bp) indicative of *N. meningitidis.* The result was in accordance with the hospital clinical laboratory culture results where *N. meningitidis* was isolated.

The two unknown amplicons obtained with G7 (QUGP-Fn6 and QUGP-Rn3) from clinical sample A35 and H1 (Figure 4) were subjected to nucleotide sequencing followed by BLAST analysis. The results (Figure 5A and B) indicated that A35 and H1belonged to *Gammaproteobacteria.* To show that the two bacteria were different, amplicon sequence of H1 was aligned against that of A35. Similarity was found to be 94.2% indicating non-identity (Figure 6). The results were consistent with patient clinical findings but disagreed with clinical laboratory findings which reported the two cases as “no growth”.

Aseptic CSF sample processing is critical; collection of CSF sample in DNA-free tubes, DNA extraction from 100 µl-samples (larger CSF volumes can be used), treatment of samples with 2-mercaptoethanol, and washing steps which ensured successful PCR-based detection and identification, were established. One of the aims was to identify CSF samples that were free of bacteria, especially since the UM-G7 detection is sensitive to any

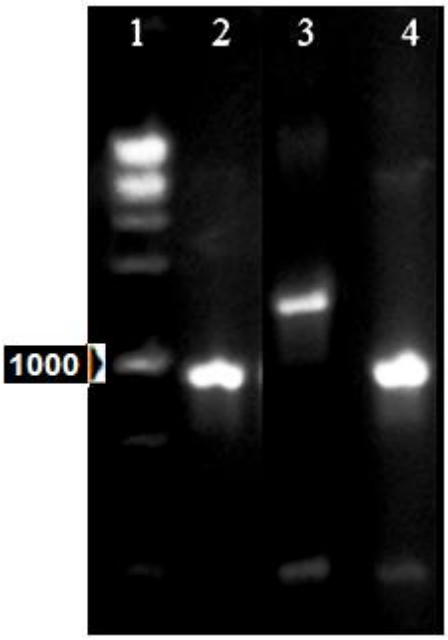


Figure 3A. Sensitivity of bacterial detection with AMD4 (QUGP-Rn2). Bacterial colony was serially diluted in bacteria-free CSF. Each PCR reaction received DNA equivalent to 2.5 CFU. *Neisseria meningitidis* 969bp, lane2; *Haemophilus influenzae* 1201bp, lane 3; *Streptococcus pneumoniae* 952bp, lane 4. Dilutions were made in 100 µl aliquots of bacteria-free human CSF. DNA was then extracted from each dilution into 200 µl. Amplification was carried out on 0.5 µl of extracted DNA.

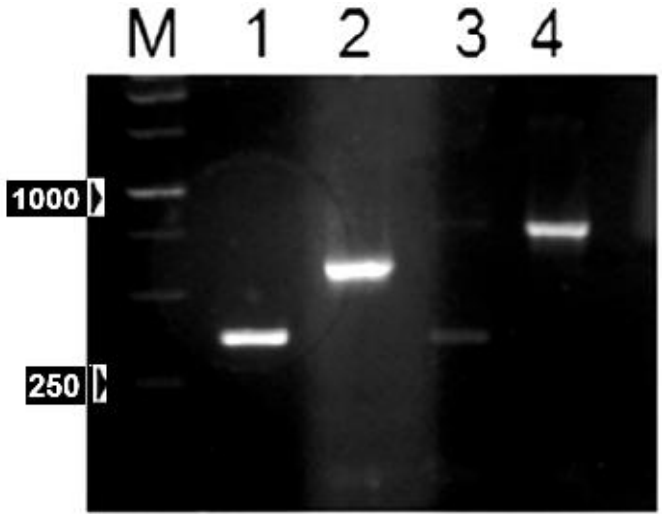


Figure 3B. AMD4 (QUGP-Rn3) generated smaller PCR products that improved resolution of PCR bands; lane l *N. meningitidis* 370bp; lane 2 *H. influenzae* 600bp; lane 3 *S. pneumoniae* 351bp; and lane 4 *L.monocytogenes* 631 bp. To discriminate *S. pneumoniae*, its amplicon can be digested with *Bsp13I* that does not digest *N. meningitidis* amplicon (Table 2).

bacterial DNA contamination and may result in increased false positive results. The 127 UM-negative samples were consistent with clinical laboratory findings; CSF samples that were collected in containers and the empty containers themselves (other than the screw cap sterile microfuge tubes) had produced positive results with UM-PCR indicating that contaminated containers sterilized by radiation or gas application most likely will produce positive UM-G7 amplification, probably due to the presence of preserved bacterial DNA. This problem was solved by direct collection of CSF samples into sterile screw-cap microfuge tubes.

The AMD4 was designed to quickly identify the major bacterial species associated with meningitis. The concept of Anchored Multiplex addressed two important issues; firstly, one reverse primer shared by all target bacteria will act as a reference point (anchor) to all amplicons whereas the species specific forward primer will dictate the size of amplicon and makes it distinguishable from other bacterial species. Secondly, the utility of anchoring primer (QUGP Rn2 or Rn3) substituted for the use of the species specific reverse primers and reduced the number of primers within the multiplex AMD4 (AMD4 contained five primers; four forward and one reverse instead of 8 primers). Such strategy, allows for the incorporation of additional 16S species specific forward primers. Although a non-specific band (~300 bp) was observed (Figure 2B), the band was absent or less evident when AMD4 was utilized for amplification, (Figure 2A).

Another criterion that was standardized in this study was the amplification reaction annealing temperature; all primers were designed to function at Ca ~60°C. Accordingly, both AMD4 and UM were carried out using the same PCR-amplification parameters. The choice of 60°C as the annealing temperature had several advantages over other annealing temperatures; prevention of inter-and intra-primer pairing and hairpin formation especially with GC rich primer regions and the provision of an extended free 3´-end. Bacterial specific primers needed to be 16-28 nucleotide long based on their sequences and GC content, 60°C was suitable for all primers. This feature allowed simultaneous testing of CSF samples using AMD4 and UM under the same amplification conditions. If a CSF sample was negative with both methods, it was scored as free of bacterial DNA, if a sample was positive, as in sample K1, with both methods, it was simultaneously identified as *N. meningitidis* based on amplicon size. However, when only UM-G7 was positive as with samples H1 and A35 (negative reactions with AMD4.), the Universal Method identification was utilized in bacterial identification. H1 and A35 were missed by cultural and microscopic methods conducted by hospital clinical laboratory. Clinically, bacterial meningitis was indicated by fever,

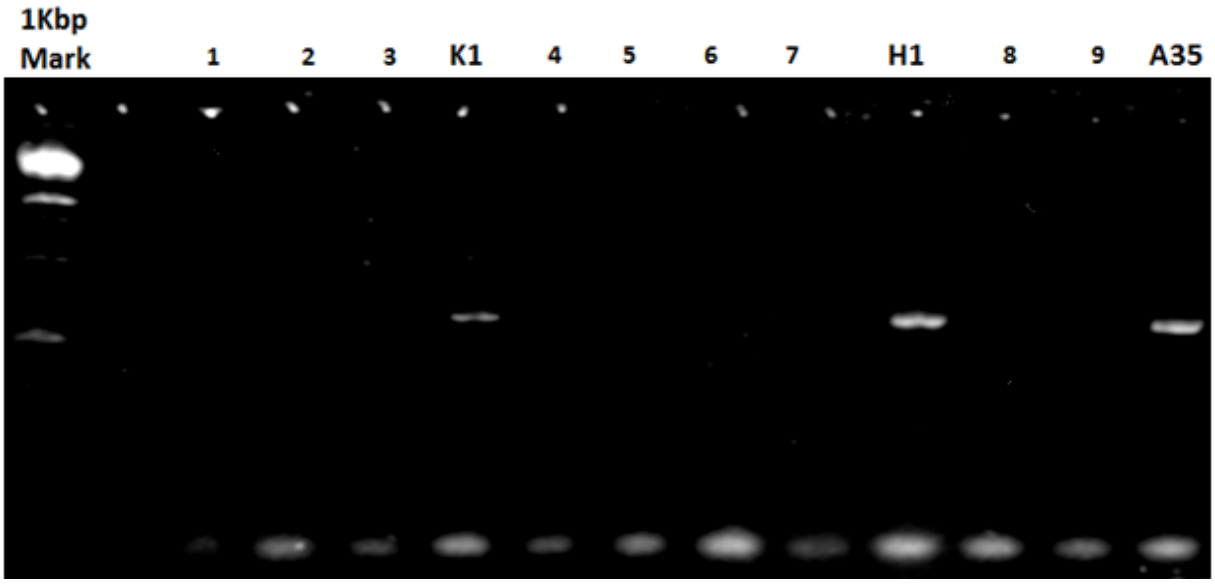


Figure 4. Positive and negative CSF samples with UM-G7, only positive samples are indicated the remaining 9 samples (1-9) were negative. The 290bp amplicons produced by the action of QUGP Fn6 and QUGP Rn3 were detected in samples K1, H1, and A35 when subjected to UM-G7. K1, H1, and A35 were from three different hospitals.

5'-TTCCTGTTTGCCTACCGCTTTCGCGCCTGCAGCGTCAGTAATGGGAAGGCAGGGAGCCGC
TTGCCACTGGTGTTCCTCCCGATATTTACGCATTTCACCGCTACACCGGGAATTCCACTCCCCTCTGCCGTA
CTCTAGCCAGCCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACTAACCGC
CTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGA-3'

A

Figure 5A. 273-nucleotide DNA sequence obtained for sample A35 with QUGP-Rn3 primer. Notice that the underlined 3'-end sequence is complementary to the sequence of QUGP- Fn6 used in the amplification process. The 3'-end adenine (bold) may have been added by Taq DNA polymerase in a template-independent fashion (Geiβdörfer et al., 2008).

5'-ACCTGTTTGCTTACAGCTTTCGCGCCTGAGCGTCAGTAACTGGGACCAGGGAGCC
GCCTCCGCCACTGGTGTTCCTCCCGATATCTACGCATTTCACCGCTACACCGGGAATTCCACTCCCCTCTGC
CGTACTCTAGCCAGCCAGTATCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACTAA
CCGCCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGG-
3'

B

Figure 5B. 271-nucleotides DNA sequence obtained for sample H1 with QUGP-Rn3, the underlined sequence is complementary to QUGP-Fn6.

CSF physical properties, leukocyte counts, and CSF-glucose level. Sample A35 was from a 4-year old patient who presented with fever, the culture results and gram stain were negative, possibly due to antimicrobial treatment that had been commenced prior to lumbar puncture/CSF sample collection; bacterial meningitis was evident from high white blood cell counts (WBC) and low CSF-glucose level. The UM-G7 successfully detected the bacterial presence in this sample (A35) even though antibiotic was administered prior to sample collection. The 290 bp UM-G7 amplicon (generated by QUGP-Fn6●QUGP-Rn3) was sequenced in both directions. BLAST alignment failed to detect identical bacterium, but indicated that the bacterium was most closely related to *Gammaproteobacteria* (92%; Figure 4; http://www.bacterio.cict.fr/g/gammaproteobacteria.html). Gamma (2011).

The second UM-positive sample (H1); was from a 3

Figure 6. Emboss conducted alignment of A35 amplicon against H1 amplicon showed 94.2% similarity which indicated two different *Gammaprotobacteria* (BLAST results are not shown).

growth". UM was able to detect bacterial presence, sequencing and BLAST analysis indicated that bacterium was related to *Gammaproteobacteria* but different from A35 isolate (Figure 6).

Two important issues remain to be critically addressed; the possible contamination of the CSF sample with exogenous bacteria and the antibiotic susceptibility of the identified bacterial pathogen. As emphasized above, aseptic CSF collection in DNA-free containers, processing the minimum amount of CSF sample, and sequencing of amplicons may be sufficient to rule out contamination for the following reasons. Assuming low level contamination (<1000 cells/ml CSF) will escape PCR sensitivity levels producing negative results if less than 0.25 bacterial chromosome is introduced into the PCR reaction (since most bacteria harbor 4 copies of the 16S gene per chromosome, some species show more than one chromosome; usually 2), this can be achieved when processing 100 µl of CSF are extracted into >200 µl and only 0.5 µl of the extract is applied to the PCR reaction, most probably will result in a negative PCR reaction. Simultaneously, another assumption has to be made; clinical bacterial meningitis cases must show bacterial counts of >1000 cells/ml of CSF to be detectable by the same calculations. Higher levels of contamination (> 1000 cells/ml of CSF) will produce positive UM-G7 amplicons, such contaminations may be caused by a single species or a mixture of bacteria and will produce a false positive result when the case is a true negative case of bacterial meningitis. On the other hand, contamination will confuse true positive meningitis cases. In other words, a CSF sample that is UM-G7 positive should be treated as a true positive sample since bacterial meningitis cannot be ruled out. The case of bacterial meningitis can be further complicated by mixed infections and sample contamination which complicate diagnosis. In cases of spine or brain (CNS) neurosurgeries prophylactic and post-surgical antibiotic therapy are administered regardless of infection (Marchandin et al., 2005).

Samples H1 and A35 are unlikely to be the result of contamination since 97.7% of all the samples (127/130) were UM-G7-negative indicating excellent aseptic techniques. In addition, high-level contamination (>1000 cells/ml of CSF) is required to be detectable by PCR; high level contamination most likely is a "mixed"

contamination and will prevent useful sequencing and alignment (BLAST, 2011).

Samples H1 and A35 most probably contained a single type of bacterium that only produced a pure amplicon which in turn produced clean sequences. The identified bacteria were related to the Gram negative *Gammaproteobacteria* which is consistent with Gram negative rods/bacilli reported to cause meningitis (Table 1).

The second problem is the determination of antibiotic susceptibility of bacterial pathogens; this problem is inherent to all molecular techniques; unlike culture, disc diffusion, and E-test. Molecular techniques do not allow complete antibiotic profiling of the pathogen, although attempts have been successful in showing certain markers of antibiotic resistance, yet they fall short of providing a practical and reliable antibiotic susceptibility profiling for therapeutic decision making.

In conclusion, the UM potential to rule out bacterial meningitis was illustrated; all UM negative samples (127 CSF samples) were in agreement with clinical and laboratory findings in ruling out bacterial meningitis. The UM-G7 showed unprecedented potential for its capacity to detect and identify bacterial pathogens. The UM only limitation for identifying bacterial pathogens was its reliance on the availability of DNA sequences for BLAST analysis, but data bases are growing as new sequences are added. A cultured spiral bacterium (QUBC 70) was not identified after sequencing and BLAST due to the absence of identical sequences from available nucleotide bases (Barghouthi, 2011). Later, BLAST showed QUBC 70 to share 99% of its amplicon sequence with *Alphaproteobacteria*. The number of available complete genome sequences was 1860 in March, 2011 and 2001 genomes in February 2012. Additional sequences are available through Nucleotide bases, yet none of the data bases are complete (BLAST; nucleotide BLAST, 2011). New pathogens may be identified such as those detected in samples H1 and A35, they were Gram negative, *Gammaprotobacteria* which encompasses several known pathogenic bacteria (Gamma). We hope that UM and UM-G7 be adopted by most clinical laboratories for the diagnosis of bacterial meningitis and bacteremia.

This work emphasizes the need to re-focus attention at bacterial agents that may be involved in bacterial meningitis. It calls for the full investigation, culturing, identification, and characterization of bacterial pathogens involved in meningitis. The under reporting of bacterial meningitis may be simply due to inadequate detection and culturing methods applied by clinical laboratories.

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