Use of a new multiplex quantitative polymerase chain reaction based assay for simultaneous detection of *Neisseria meningitidis, Escherichia coli K₁, Streptococcus agalactiae*, and *Streptococcus pneumoniae*

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ABSTRACT

Background and Objectives: *Neisseria meningitidis, Escherichia coli K₁, Streptococcus agalactiae,* and *Streptococcus pneumoniae* cause 90% of bacterial meningitis. Almost all infected people die or have irreversible neurological complications. Therefore, it is essential to have a diagnostic kit with the ability to quickly detect these fatal infections.

Materials and Methods: The project involved 212 patients from whom cerebrospinal fluid samples were obtained. After total genome extraction and performing multiplex quantitative polymerase chain reaction (qPCR), the presence or absence of each infectious factor was determined by comparing with standard strains.

Results: The specificity, sensitivity, positive predictive value, and negative predictive value calculated were 100%, 92.9%, 50%, and 100%, respectively. So, due to the high specificity and sensitivity of the designed primers, they can be used instead of bacterial culture that takes at least 24 to 48 hours.

Conclusion: The remarkable benefit of this method is associated with the speed (up to 3 hours) at which the procedure could be completed. It is also worth noting that this method can reduce the personnel unintentional errors which may occur in the laboratory. On the other hand, as this method simultaneously identifies four common factors that cause bacterial meningitis, it could be used as an auxiliary method diagnostic technique in laboratories particularly in cases of emergency medicine.

Keywords: Cerebrospinal fluid; Meningitis; Quantitative polymerase chain reaction; Simultaneous detection; Diagnosis testing

INTRODUCTION

According to the Centers for Disease Control and

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Prevention (CDC), meningitis is called the inflammation of the meninges. Meningitis is considered as a potentially fatal disease that despite major medical advances over the past two decades is still the leading deadly symptom for the mortality caused by several infectious diseases, worldwide. Mortality rates vary by geographic area, age group, and type of pathogen (1).

Meningitis is caused by viruses, fungi, parasites, and most importantly bacteria. Bacterial meningitis

is an acute infectious disease that needs immediate confirmation and treatment. Once untreated, the consequences can be dangerous and irreversible. The most important microorganisms causing bacterial meningitis are *Neisseria meningitidis, Escherichia coli K₁, Streptococcus agalactiae,* and *Streptococcus pneumoniae* (2).

Symptoms include fever, headache, lethargy, vomiting, altered brain function, and light sensitivity. Bacterial meningitis is difficult to diagnose due to the similarity of clinical signs caused by other etiologic agents. Therefore, it is necessary to use a specific method to identify the agents involved in bacterial meningitis. To diagnose bacterial meningitis, cerebrospinal fluid (CSF) culture and analysis is the main method in identifying the causative agents of meningitis. Other diagnostic methods include CSF cell counts and immunological diagnosis such as latex agglutination. However, these diagnostic procedures are time consuming while the patients in critical clinical conditions (3).

Moreover,, the mentioned methods have some limitations such as low bacterial titers in CSF fluid, lengthy incubation period of bacterial cultures, cross-reactivity in immunological methods, and antibiotic use by patients prior to CSF sample collection which impairs the culture response and the identification of the pathogen causing bacterial meningitis (4).

Now, molecular methods, such as PCR, are more effective in detecting the possible presence of bacterial DNA in the CSF samples of patients with suspected bacterial meningitis (5). Standard PCR has the ability to detect possible bacterial DNA in CSF fluid even if the causative agent is not alive. However, this method is prone to possible contamination of samples during the processing stage which may result in false positive results (6). Therefore, the only reliable method is to use qPCR with high accuracy, speed, sensitivity and specificity, and minimum contamination (7). Detection of the bacteria causing meningitis leads to better understanding of the disease epidemiology and proper treatment.

The aim of this study was to evaluate the factors of *N. meningitidis, E. coli K₁, S. agalactiae,* and *S. pneumoniae* by multiplex qPCR with further comparison of results with those of standard methods (bacterial culture). This new detecting approach is of great importance as two of these organisms (*pneumococcus* and *meningococcus*) account for more than half of the bacterial meningitis with highest mortality rate.

MATERIALS AND METHODS

Culture strains, primer design & PCR set up. The Standard strains of *S. pneumoniae* (strain ATCC BAA-255 / R6), *N. meningitidis* serogroup B (strain MC58), *S. agalactiae* 2603V/R, and *E. coli K₁* JM101 strains were used as standard controls to determine the analytical sensitivity and specificity of the PCR assay. And, in this project we used *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 as negative control. Standard strains were cultured on Luria Bertani broth medium (Merck. Germany) and their genomes extracted using QIAamp® genomic DNA kits (Qiagen, Hilden, Germany). In the next step, using the alelleID 6 software, specific probes and primers (Table 1) were designed and commercially synthesized by Takapoozist Company. The target genes were UDP *N*-Acetylglucosamine 2-Epimerase *(neuC)* gene from *E. coli K₁* JM101, the extracellular pore-forming toxin (cAMP factor) gene *(cfb)* from *S. agalactiae*, the autolysin- encoding gene *(lytA)* from *S. pneumoniae*, and the capsule biosynthesis locus *(ctrA)* from *N. meningitides*. Finally, PCR gradients and multiplex-PCR set up were performed for all bacterial target genes. All PCR products of the genes from the standard strains were sequenced by Microsynth AG (Balgach, Switzerland).

PCR product cloning. The PCR products were purified by using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The eluted PCR products were cloned into the *pTG19* vector according to routine protocol. Then, it was transformed into *E. coli DH₅*. The *pTG19* plasmid was extracted using the QIAprep® plasmid Miniprep Kit (Qiagen, Hilden, Germany) and serially diluted from cultured *E. coli DH₅* in LB broth containing 100 µg/ml ampicillin, 40 µg/ml X-Gal, and 40 µg/ml IPTG.

Clinical specimens. A total of 212 CSF samples were collected from the patients (median 0-80 years) with meningitis symptoms, admitted to hospital from April 2017 to April 2019. All samples from patients who had taken antibiotics were excluded from the study. According to routine clinical care protocol, lumbar punctures were performed on patients with suspected acute meningitis, defined as sudden onset of fever more than 38°C and one of the following signs: neck stiffness altered consciousness and neck stiffness. CSF samples were collected and processed

Table 1. The list of designed primers and probes used in the present study

Standard strain	Specific gene	Primer Sequence 5'→3'	Length of product
E. coli K₁ JM101	*neuC*	F: ATGGTGATGTTTCTGTTGGAGAAG	81
		R: TCTTTGAGCCTAGCTTCAACTGG	
		Probe: FAM-ACAGTCACGCTACCAA-TAMRA	
Streptococcus agalactiae 2603V/R	*cfb*	F: GACTTCATTGCGTGCCAACTC	88
		R: TGCTTCAATCACATCTGTTAAGGC	
		Probe: HEX-TCTACACGACTACCAAT-BHQ1	
N. meningitides MC58	*ctrA*	F: TCGTCCAGTTTGCCGGTTTG	140
		R: TTTGAGTCCGATCCGCAAGG	
		Probe: FAM-CGCACTGCCCGCCGTTTGGTCG-BHQ1	
S. pneumonia R6	*lytA*	F: AGTGTTCCGTCTGGTTTGAGG	150
		R: CAACGAAGAAGGTGCCATGAAG	
		Probe: VIC-AGCCTGTTCCGTCCGCTGACTGGA-BHQ1	

immediately upon receipt by the biochemistry and microbiology laboratories using methods based on WHO recommendations.

In summary, samples were examined macroscopically prior to centrifugation. Differential cell counts and Gram staining were performed by light microscopy. Also, CSF, white blood cell (WBC) count, protein, and glucose levels were analyzed using auto-analyzer Hitachi model 917 (Hitachi, Japan) and CytoFLEX Flow-Cytometer (Beckman, USA).

Direct antigen testing for *E. coli K₁*, *N. meningitidis* groups A, C, Y, W135, *S. pneumoniae* and *S. agalactiae* was performed using a bacterial antigen rapid latex agglutination test (Pasteur institute, Tehran, Iran). All procedures were performed at microbiology and biochemistry laboratories and individual informed consent was not required as sample collection and analysis were conducted as part of routine diagnostics. An ethical clearance certificate was approved by Ethical Committee in Qazvin University of Medical Sciences (ref. no. IR.QUMS.REC.1399.257).

qPCR assay. The samples of DNA were extracted using QIAamp® genomic DNA kits (Qiagen, Hilden, Germany). The genomes extracted as template as well as the cloned genomes of standard strains were tested as positive controls in a multiplex qPCR reaction using QuantiNova Pathogen +IC Kit (plus internal control) (Qiagen, Hilden, Germany).

All reactions were performed in triplicate and according to the manufacturer's protocol. The standard curve was plotted and according to the standard samples C_T, the number of targets in each sample was obtained. The multiplex qPCR was carried out using a CFX96 Real-Time System (Bio-Rad, Hercules, USA) under the following conditions: 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 5 seconds), annealing (60°C for 10 seconds), and extension (72°C for 15 seconds).

RESULTS

Out of total 212 samples, 81 samples were found to be positive by qPCR test, for which the results of laboratory tests were as follows based on age and sex, shown in Table 2.

The results of microbiological method on 212 suspected meningitis specimens demonstrated that only 14 specimens were due to *N. meningitidis* whereas multiplex qPCR confirmed the presence of 28 samples positive e for this bacterium. Among the 28 cases of *N. meningitidis*, approved by qPCR, 14 samples were confirmed by microbiological method. Also, by performing culture on 212 suspected meningitis specimens, 7 specimens were found to be *S. pneumoniae* while 15 positive pneumococcal samples detected by multiplex qPCR. In *S. pneumoniae* cases, of the 15 samples approved in qPCR, 7 samples were confirmed by microbiological method.

Similarly, applying the standard microbiological method on 212 specimens, suspicious of having the causative agent of meningitis, revealed 12 specimens detected as *E. coli K₁* while multiplex qPCR identified *E. coli K₁* in 17 samples. In *E. coli K₁* cases, of

Table 2. The sex and age groups profile of patients with bacterial meningitis

Specification			Age						
			0-1	1-10	10-20	20-40	40-60	< 60	Total
Gender	Male	Number	15	17	0	1	3	6	42
		Percentage	35.7	40.5	0	2.4	7.2	14.2	100%
	Female	Number	14	15	1	0	2	7	39
		Percentage	35.8	38.5	2.6	0	5.2	17.9	100%
Total		Number	29	32	1	1	5	13	81
		Percentage	35.9	27.2	1.3	1.3	6.2	16.1	100%

the 17 samples approved in qPCR, 12 samples were confirmed by microbiological method. Finally, employing the routine microbiological method on total specimens, suspect for bearing meningitis causing organism, resulted in identifying 13 specimens of *S. agalactiae* whereas this organism was found in 21 samples when multiplex qPCR used. In *S. agalactiae* cases, of the 21 samples approved in qPCR, 13 samples were confirmed by culture. A total of 46 samples were detected by culture out of 81 samples that multiplex qPCR were able to detect. Table 3 shows the sensitivity, specificity, predictive values and accuracy of the multiplex qPCR for each bacterium.

Table 4 also shows the mean C_T bacterium according to the dilution obtained in each bacterial sample cloned in the *pTG19* plasmid. Using the data obtained in Table 4, the number of meningitis-causing bacteria for each bacterium was obtained. Using the C_T of each bacterium and qPCR reactions efficiency, once the primers and probes designed in this project were used, led to the detection of at least 56 bacteria per milliliter for *N. meningitidis*. These values were 62, 58, and 56 bacteria per milliliter for *S. pneumoniae, S. agalactiae,* and *E. coli K_1*, respectively. Finally, statistical analysis produced values of 100%, 92.9%, 50% and 100%, for sensitivity, specificity, positive predictive value, and negative predictive value, respectively.

DISCUSSION

One of the vital challenges in the field of medicine is the accurate and rapid diagnosis of bacterial meningitis, if done correctly, can lead to appropriate treatment and prevention of close contact with other patients. Conventional microbial culture techniques, which are considered as gold standard diagnostic method, have some limitations due to delayed results and in some cases low sensitivity, especially if the patient has already taken antibiotics (8-10). In previous studies carried out in Iran, negative culture results were reported, which claimed to be associated with easy access to antibiotics, its improper use, and delay in CSF sampling (11). For these reasons, we developed a convenient and rapid molecular approach using a multiplex qPCR-based assay that improves the diagnosis of bacterial meningitis due to *N. meningitidis, E. coli K_1, S. pneumoniae,* and *S. agalactiae.* Data analysis showed that our patients had almost the same gender distribution (51% male and 49% female) and also the majority of patients were in the age range of 0-10 years. These results did not agree with the studies performed by Fouad et al. and Khater et al. because the conditions mentioned in their studies were somehow similar to Iran (12, 13). Studies in Europe and the United States revealed the commonest etiologic agents of bacterial meningitis

Table 3. Sensitivity, specificity, predictive values and accuracy of the multiplex qPCR for each bacterium

Name of bacteria	Based on the number of samples detected:		Percent of Specificity & Sensitivity	NPV* & PPV** respectively	Percent of Accuracy
	Microbiological method	Multiplex qPCR			
N. meningitidis	14	28	100, 85.8	91.4, 50	86.7
S. pneumoniae	7	15	100, 92.7	100, 31.8	92.9
E. coli K_1	12	17	100, 91.5	100, 41.4	92
S. agalactiae	13	21	100, 89.4	100, 38.2	90.1

*NPV: Negative Predictive Value, **PPV: Positive Predictive Value, ( )

Table 4. Specifications obtained based on the standard curve for standard strains of *S. pneumoniae, N. meningitides, S. agalactiae,* and *E. coli K_1*

Bacterial standard strain	PCR product dilution level per ml			
	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Mean C_T (bacterial count) of *S. pneumoniae R6*	28.94(24000)	30.44(2400)	32.56(240)	36.41(24)
Mean C_T (bacterial count) of *N. meningitides MC58*	29.60(19000)	31.77(1900)	33.19(190)	37.71(19)
Mean C_T (bacterial count) of *S. agalactiae 2603V/R*	29.11(17000)	30.78(1700)	33.06(170)	36.98(17)
Mean C_T (bacterial count) of *E. coli K_1JM10*1 strain	28.32(20000)	30.54(2000)	32.77(200)	36.80(20)

*Polymerase Chain Reaction   **Cycle of Threshold

including *S. pneumoniae. N. meningitidis, S. pneumoniae,* and *H. influenzae* which comprise about 80% of cases of bacterial meningitis in developing and developed countries (14). However, considering the problems that may occur in universal PCR assay, a single tube multiplex PCR was developed to detect these agents (15). Most cases of infant meningitis are reported to develop by *S. agalactiae* and *E. coli K_1* although in children aged 1-3 months, this ratio is reversed. Also, in those aged 3-48 months, the most common cause of meningitis is pneumococcus followed by meningococcus (16). However, this ratio is reversed in people aged 5-50 years. Interestingly, in our present study, 34.5% of the isolates belonged to *N. meningitidis* followed by *Streptococcus agalactiae* (25.9%). One possible reason for this difference may be due to the age range of the patients. It is reported that 50% of *N. meningitidis* negative cultures were found to be positive by multiplex qPCR-based assay design, a finding consistent with a study by Khater et al. (13). Also, 53% of *S. pneumoniae*, 29.4% of *E. coli K_1*, and 38% of *S. agalactiae* with negative culture results were reported positive by this method. In two studies by Wu et al. and Sacchi et al. the authors reported increased number of bacterial meningitis cases using PCR, which agrees with our results (6, 17). Considering the results of this study and those of previous studies, which show high rate of negative culture results in developing countries, the use of this technique can reduce the number of false negatives compared to traditional diagnostic method (14, 18). Also, regarding the patients with self-medication prior to sampling, the presence of bacterial genomes in the CSF can be easily identified by this rapid molecular technique. In this project, the preparation of a patient sample and transfer of the sample was the most important limitations.

CONCLUSION

In view of what described above, it seems that the design of a molecular platform, for rapid detection of deadly infections, is essential. In addition, the success of the project shows that such a platform can be designed for other important human infections. Finally, considering the high specificity and sensitivity of the designed primers, this technique can be an auxiliary method for bacterial diagnosis that takes at least 24 to 48 hours.

The most significant benefit of this method is shortening the time required for the test to be completed (up to 3 hours). It is also worth noting that this method can reduce the personnel error of those working in the laboratory. On the other hand, as this method identifies four common bacterial agents involved in causing meningitis, eye infections, septicemia agents, therefore, it could be used as an ideal alternative diagnostic kit in cases of emergencies.

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REFERENCES

1. Tubiana S, Varon E, Biron C, Ploy MC, Mourvillier B, Taha MK, et al. Community-acquired bacterial meningitis in adults: in-hospital prognosis, long-term disability and determinants of outcome in a multicentre prospective cohort. *Clin Microbiol Infect* 2020;26: 1192-1200.

2. Principi N, Esposito S. Bacterial meningitis: new treat-

ment options to reduce the risk of brain damage. *Expert Opin Pharmacother* 2020;21: 97-105.

3. Obaro S. Updating the diagnosis of bacterial meningitis. *Lancet Infect Dis* 2019;19: 1160-1161.

4. Kourna Hama M, Khan D, Laouali B, Okoi C, Yam A, Haladou M, et al. Pediatric bacterial meningitis surveillance in Niger: increased importance of *Neisseria meningitidis* serogroup C, and a decrease in *Streptococcus pneumoniae* following 13-valent pneumococcal conjugate vaccine introduction. *Clin Infect Dis* 2019;69(Suppl 2): S133-S139.

5. Jing-Zi P, Zheng-Xin H, Wei-Jun C, Yong-Qiang J. Detection of bacterial meningitis pathogens by PCR-Mass spectrometry in cerebrospinal fluid. *Clin Lab* 2018;64: 1013-1019.

6. Sacchi CT, Fukasawa LO, Gonçalves MG, Salgado MM, Shutt KA, Carvalhanas TR, et al. Incorporation of real-time PCR into routine public health surveillance of culture negative bacterial meningitis in São Paulo, Brazil. *PLoS One* 2011;6(6): e20675.

7. Papavasileiou K, Papavasileiou E, Tzanakaki G, Voyatzi A, Kremastinou J, Chatzipanagiotou S. Acute bacterial meningitis cases diagnosed by culture and PCR in a children's hospital throughout a 9-Year period (2000-2008) in Athens, Greece. *Mol Diagn Ther* 2011;15: 109-113.

8. Nigrovic LE, Malley R, Macias CG, Kanegaye JT, Moro-Sutherland DM, Schremmer RD, et al. Effect of antibiotic pretreatment on cerebrospinal fluid profiles of children with bacterial meningitis. *Pediatrics* 2008;122: 726-730.

9. Bryan JP, Desilva HR, Tavares A, Rocha H, Scheld WM. Etiology and mortality of bacterial meningitis in northeastern Brazil. *Rev Infect Dis* 1990;12: 128-135.

10. Ragunathan L, Ramsay M, Borrow R, Guiver M, Gray S, Kaczmarski EB. Clinical features, laboratory findings and management of meningococcal meningitis in England and Wales: report of a 1997 survey. meningococcal meningitis: 1997 survey report. *J Infect* 2000;40: 74-79.

11. Motamedifar M, Sedigh Ebrahim-Saraie H, Mansury D, Nikokar I, Hashemizadeh Z. Prevalence of etiological agents and antimicrobial resistance patterns of bacterial meningitis in Nemazee hospital, Shiraz, Iran. *Arch Clin Infect Dis* 2015;10(2): e22703.

12. Fouad R, Khairy M, Fathalah W, Gad T, El-Kholy B, Yosry A. Role of clinical presentations and routine CSF analysis in the rapid diagnosis of acute bacterial meningitis in cases of negative gram stained smears. *J Trop Med* 2014;2014: 213762.

13. Khater WS, Elabd SH. Identification of common bacterial pathogens causing meningitis in culture-negative cerebrospinal fluid samples using real-time polymerase chain reaction. *Int J Microbiol* 2016;2016: 4197187.

14. Brouwer MC, Tunkel AR, van de Beek D. Epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis. *Clin Microbiol Rev* 2010;23: 467-492.

15. Eskola J. Use of conjugate vaccines to prevent meningitis caused by Haemophilus influenzae type b or Streptococcus pneumoniae. J Hosp Infect 1995;30 Suppl:313-321.

16. Brink M, Welinder-Olsson C, Hagberg L. Time window for positive cerebrospinal fluid broad-range bacterial PCR and *Streptococcus pneumoniae* immunochromatographic test in acute bacterial meningitis. *Infect Dis (Lond)* 2015;47: 869-877.

17. Wu HM, Cordeiro SM, Harcourt BH, Carvalho M, Azevedo J, Oliveira TQ, et al. Accuracy of real-time PCR, gram stain and culture for *Streptococcus pneumoniae, Neisseria meningitidis* and *Haemophilus influenzae* meningitis diagnosis. *BMC Infect Dis* 2013;13: 26.

18. Afifi S, Wasfy MO, Azab MA, Youssef FG, Pimentel G, Graham TW, et al. Laboratory-based surveillance of patients with bacterial meningitis in Egypt (1998–2004). *Eur J Clin Microbiol Infect Dis* 2007;26: 331-340.