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Comparison of Multiplex Quantitative Real-Time PCR and Culture Methods for the Diagnosis of Bacterial Meningitis in Patients with Suspected Meningitis

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ABSTRACT

Background: This study aimed to compare the diagnostic efficacy of standard culture method with multiplex quantitative real-time polymerase chain reaction (qPCR) in examining cerebrospinal fluid (CSF) samples collected from patients with suspected meningitis. **Materials & Methods:** A retrospective evaluation was conducted on 166 patients with suspected meningitis, who were treated in Vali-Asr hospital in Birjand, Iran between 2011 and 2020. Diagnosis of bacterial meningitis was based on CSF culture and multiplex qPCR results. **Findings:** Among 166 patients, conventional methods identified causative pathogens in only 10.3% of cases, while multiplex qPCR detected pathogens in eight out of 25 culture-negative cases as well. The most common pathogens identified were enterovirus, Epstein-Barr virus, herpes simplex, *Haemophilus influenzae*, and *Streptococcus pneumoniae*. **Conclusion:** Multiplex qPCR appears to be a more effective method than conventional culture in identifying bacterial and viral pathogens that most commonly cause meningitis. The incorporation of qPCR as a routine diagnostic method for meningitis in clinical practice could significantly enhance clinical decision-making and patient care.

Keywords: Bacterial meningitis, Culture, Real time polymerase chain reaction

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Introduction

Meningitis is an inflammation of the protective tissues surrounding the brain and spinal cord [1]. Bacteria, fungi, viruses, and protozoa are common causes of meningitis. Most cases are caused by bacterial infections. Despite advances in medical treatment and vaccine development in recent years, it remains as a significant cause of morbidity and mortality, especially in developing countries [2]. It is estimated that about 1.2 million cases of bacterial meningitis (BM) occur annually worldwide. The case fatality rate associated with BM is between 19 to 37%, and most of them are children and young adults [3]. The most common bacterial causes of BM are *Streptococcus pneumonia, Neisseria meningitidis, Haemophilus influenzae* type b (Hib), and *S. agalactiae*. Among patients, key clinical signs suggestive of BM commonly include fever, headache, cerebral dysfunction, and meningismus. But these meningitis symptoms are not always reliable, and laboratory support is necessary to establish an etiological diagnosis. Moreover, expeditious and accurate identification of responsible bacteria is essential for effective health surveillance and implementation of prophylaxis measures to avoid transmission and mitigate potential adverse neurological sequels [4]. Conventional methods like direct microscopy, Gram staining, and culture have low sensitivity. Several factors could influence their accuracy, such as prior use of antibiotics reducing culture yield, low bacterial counts, the presence of non-culturable and fast/ slow-growing microbes that are challenging to detect, and the time-consuming nature of these tests, taking 36 hours or more. These limitations have led to the development of more rapid tests. The use of molecular diagnostic techniques has enhanced accuracy and speed in detecting clinically-relevant causative agents of infections.

Molecular assays like polymerase chain reaction (PCR) have high detection rates as they could detect both dead and viable microorganisms. They could detect specific DNA of different pathogens including viruses, bacteria, and protozoa in human or animal clinical samples. Higher sensitivity and faster resolution make them a rapid, sensitive, and specific method for obtaining reliable results. During the last two decades (2002-2022), PCR has been recommended as a molecular strategy for the detection of BM [2, 4-9]. In two studies conducted in 2002 and 2007, the sensitivity and specificity of the PCR method have been reported to be100 and59% as well as 98.2 and97% , respectively. However, in these studies, the sensitivity of the culture method has been reported to be very low (43%) [4, 6]. In studies performed in Turkey and India, it has been shown that the multiplex PCR method has a higher detection rate than conventional culture approaches [2, 7]. PCR is a highly accurate method for diagnosing BM. However, the high cost of reagents and equipment may limit the widespread use of this method in resource-limited settings such as Iran. In such budget-limited settings, the utilization of diagnostic gold standard methods like cerebrospinal fluid (CSF) and blood cultures could still be more suitable for achieving diagnostic objectives. To the best of our knowledge, this study was the first research conducted in Iran to assess the success rate of both conventional and molecular methods in diagnosing the etiological agents of BM across all age groups. Therefore, significant progress was made in the effective management of meningitis. This study compared the effectiveness of molecular with GA 21 plex MeningitEncephalit qPCR commercial kit and conventional procedures in identifying the causes of the disease and introduced a fast and accurate identification method for

rapid detection of specific pathogens in CSF. This method allows the prompt initiation of appropriate treatment based on the type of pathogen (either bacteria or viruses). Timely and accurate diagnosis is crucial in reducing the risk of neurological complications and mortality associated with meningitis.

Objectives: The objective of this research was to evaluate the comparative effectiveness of molecular assay using the GA 21 plex MeningitEncephalit qPCR commercial kit and conventional methods in diagnosing the etiological agents of bacterial meningitis, aiming to introduce a rapid and accurate identification method for prompt initiation of appropriate treatment based on the type of pathogen.

Materials and Methods

This retrospective study was conducted with a sample size of 166 patients. These patients were selected based on their medical files, which indicated that they were subjected to both molecular and conventional diagnostic methods for meningitis. Additionally, there were cases for whom only one of the diagnostic methods was administered, typically based on clinical suspicion. Notably, in cases with suspected viral meningitis, only molecular tests were requested.

The aim of this study was to compare the diagnostic efficiency of molecular and conventional methods in detecting the causative agents of meningitis when both methods were available for a single patient. Thus, this study provided a unique opportunity to directly compare these two approaches under identical clinical conditions. In this study, inclusion criteria were applied only to patients with confirmed BM. Patients who were hospitalized in the infectious and pediatric departments of Vali-Asr hospital between 2011 and 2020 were eligible. Additionally, all patients had to exhibit clinical symptoms suggestive of meningitis following the guidelines of the Infectious Diseases Society of America (IDSA). Confirmation of meningitis was based on microscopic examination, presenting more than 20 WBC/mm^3 with neutrophils dominance, and CSF protein level more than 45 mg/dl. Other decisive factors were microorganism growth in CSF culture or positive CSF PCR. Conversely, patients with central nervous system (CNS) malformations were excluded from this study.

The CSF samples were centrifuged at 4000 rpm for 5 minutes. Afterwards, the samples were inoculated onto 5% sheep blood agar, EMB agar, and chocolate agar and then incubating them at 37°C in a 5% CO2 environment for 24 and 48 h. The genus and species of the isolates were identified based on by colony morphology, latex agglutination of specific antisera (Slidex Meningitis – Biomerieux), Gram staining and optochin susceptibility test and biochemical analysis [2, 10]. DNA extraction was performed using the QIAamp DNA mini kit (Qiagen, USA) according to the manufacturer's instructions. The extracted DNA samples were amplified using the GA 21 plex MeningitEncephalit qPCR commercial kit (Geneova, Iran). This kit utilizes the TaqMan Real-Time PCR method to detect various microorganisms including *S. pneumoniae*, group B Streptococcus, *N. meningitidis*, *H. influenzae*, *Listeria monocytogenes*, *Escherichia coli*, as well as parcovirus, measles, MUMPS, parvovirus B19, polyomavirus JC, HSV-1,2, CMV, VZV, EBV, HHV-6A/6B, HHV-7, HHV-8. By employing a multiplex 21-plex primer and probe mixture, it can simultaneously identify and differentiate these bacterial and viral causes of meningitis. The reaction mixture in the kit amplifies the specific target regions of the microorganisms, and the resulting

fluorescence signal is measured using a Real-Time PCR device (LightCycler® 96 System (Roch, Germany)) Table 1. Thermal cycling conditions are displayed in Table 2.

The test procedures and the assessment of the results were performed according to the manufacturer's instructions. Viral and bacterial causes of meningitis.

Statistical analysis: SPSS software Version 18.0 was utilized for statistical analysis.

Table 1) Rea Reaction mixture in quantitative real-time polymerase chain reaction (qPCR) test using GA 21 plex MeningitEncephalit RT-qPCR kit in the present study

Reagent	volume
GA 21plex MeningitEncephalit MasterMix T1 GA 21plex MeningitEncephalit MasterMix T2 GA 21plex MeningitEncephalit MasterMix T3 GA 21plex MeningitEncephalit MasterMix T4 GA 21plex MeningitEncephalit MasterMix T5	10 µl
Template DNA	10 µl
Total	20 µl

Comparison of variables was done using Fisher's exact test when appropriate. Statistical significance was ascertained by a *p*-value of less than 0.05.

Findings

During the ten-year study period, a total of 166 patients with meningitis were admitted to the hospital. Out of all patients, 60.24% (n=100) were male, and 39.76% (n=66) were female with an average age of 8 ±16.5 (SD) years (range: < 1 month– 21 years <). All patients with suspected meningitis received antibiotics. The most frequently prescribed antibiotic was vancomycin, followed by ceftriaxone.

Statistical analysis revealed no significant correlation between pathogenic agents confirmed by smear and conventional culture methods in hospitalized meningitis patients and the occurrence of fever (*p*= .32). Similarly, Fisher's exact test findings indicated that there was no notable connection between meningitis patients' qPCR results and the incidence of fever.

Upon analyzing 166 patients with suspected BM, it was found that conventional microbiological methods (culture and smear) were performed for all 166 patients, and 156 cases were diagnosed as negative in CSF Gram staining/culture.

Table 2) Temperature and time program in quantitative real-time polymerase chain reaction (qPCR) test using GA 21 plex MeningitEncephalit RT-qPCR kit in the in the present study

	Stage		Temperature	Time	Cycle
1	Holding		53°C	15 min	1
2	Holding		95°C	3 min	1
3	Cycling	Denaturation	94°C	5s	46
		Annealing, Extension and fluorescence measurement	60°C	30s	

Table 3) Laboratory results of the patients

Characteristics	All the Patients (N =166) N(%)
CSF	
WBC count (mm^3)	
0-100	5 (29.4)
101-500	2 (11.8)
501-1000	4 (23.5)
>1000	6 (35.3)
Protein (mg/dL)	
>45	4 (23.5)
Blood	
Serum C-reactive protein (CRP) (mg/dL)	Positive: 4 (22.2)
	Negative: 14 (77.8)
Erythrocyte sedimentation rate(ESR) (mm/h)	2 (11.1)
	16 (88.9)

Laboratory findings of 166 patients with suspected meningitis in this study.
CSF: cerebrospinal fluid, WBC: white blood cells, ESR and CRP are very old biomarkers of inflammation.

Bacterial growth was observed in CSF cultures of ten patients. The traditional methods identified the causative pathogens only in 10.3% of cases (based on the detection rate calculated for the test according to the formula used in a previous study) [11]. These laboratory findings are systematically detailed in Table 3, and the distribution of pathogens identified by smear and conventional culture methods in patients with meningitis is shown in Table 4. Interestingly, multiplex qPCR was not performed for any of the culture-positive cases, it was performed only on a subset of 25 out of 156 culture-negative patients. According to the multiplex qPCR test results,

Table 4) Distribution of pathogens in meningitis patients by smear and conventional culture methods

Pathogen	Number	(%)
Escherichia coli (E. coli)	1	0.6
Haemophilus influenza	1	0.6
Methicillin-resistant *Staphylococcus aureus* (MRSA)	1	0.6
Staphylococcus epidermidis	1	0.6
Streptococcus agalactiae	1	0.6
Streptococcus pneumoniae	3	1.8
Gram-positive diplococci	1	0.6
Gram-negative bacilli	1	0.6
Negative	156	94
Total	166	100

Bacterial pathogens detected only by smear and culture of cerebrospinal fluid (CSF) samples collected from patients in this study.

Table 5) Distribution of pathogens in meningitis patients by multiplex PCR test

Pathogen	Number	(%)
Epstein-Barr virus (EBV)	1	4
Enterovirus	2	8
Herpes simplex virus (HSV)	1	4
H. influenza	3	12
Strep. Pneumoniae	1	4
Negative	17	68
Total	25	100

Bacterial and viral pathogens detected by multiplex RT-qPCR of cerebrospinal fluid (CSF) samples of 25 suspected meningitis cases with culture-negative results in this study.

meningeal pathogens were identified in eight out of 25 culture-negative patients tested by qPCR (Table 5).

The detection rate of multiplex qPCR was about 100%. It should be noted that all 25 patients who were tested by qPCR were also examined with conventional methods, and none of them were diagnosed as positive by culture and smear techniques. For 17 multiplex
qPCR negative cases, the culture test results were also negative. Table 6 compares molecular and conventional methods in the diagnosis of meningitis causative agents in the cases that were investigated with both techniques.

Discussion

Rapid identification of meningeal pathogens in CSF and subsequent initiation of appropriate treatment are pivotal steps in the effective management of meningitis. Timely diagnosis and prompt intervention play a vital role in mitigating the risk of neurological complications and mortality associated with this disease. Therefore, any delay in the diagnosis process and initiation of treatment may significantly increase the likelihood of adverse outcomes [12].

The age of patients has a significant impact on the prevalence of infections caused by meningeal pathogens. In the present study, the incidence of meningitis infection in younger age groups (≥ 5 years) was higher than in other age groups (46.5%). The results of published studies in different parts of Iran support our findings [1, 10, 13]. The reason for this could be attributed to multiple factors, such as the decrease in immunity acquired from mothers in children less than one-year-old, the lack of physical defense of mucous surfaces in children between six months and two years old, and the absence of a widespread immunization program in our country against the main meningeal pathogens [1, 14, 15]. The current study showed that men were more susceptible to meningitis, which is consistent with the results of previous research in Iran, occupied Palestinian territory, and Cuba. This may be due to biological or social factors [1, 16, 17].

Table 6) Comparison of molecular and conventional methods in detecting the causative agents of meningitis

Culture Results	Multiplex RT-qPCR					
	Negative	*H. influenza*	*S. pneumoniae*	EBV	Enterovirus	HSV
Negative	17(68)	3(12)	1(4)	1(4)	2(8)	1(4)
E. coli	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
H. influenza	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Enterovirus	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. agalactiae	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. epidermidis	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. pneumoniae	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Gram-positive Diplococci	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Gram-negative bacilli	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total	17(68)	3(12)	1(4)	1(4)	2(8)	1(4)

Diagnosis of the causative agents of meningitis is based on blood and CSF cultures and microscopic and chemical analysis of CSF samples. Although CSF culture is considered as the best way to diagnose BM, its accuracy could be compromised by several factors. These factors include: use of antibiotics before culture, failure to follow proper guidelines for sample storage and transportation, use of incorrect sampling techniques, and insufficient microbiological culture. The presence of these issues could impede clinicians from promptly diagnosing and treating bacterial meningitis, especially in developing countries [1, 2, 12]. For over ten years, PCR has been considered as a valuable diagnostic tool in the field of clinical microbiology. This technique demonstrates remarkable proficiency in identifying even low quantities of infectious agents in cerebrospinal fluid (CSF) while also enabling detection of nonviable etiological agents [18, 19]. In this research, the standard culture method was compared with multiplex qPCR test in examining CSF samples of patients with suspected meningitis. The findings of the conventional culture method revealed a relatively low positivity rate for CSF cultures, with only 10.3% of the samples yielding positive results. Among the isolated bacteria, *S. pneumoniae* was the most frequently identified species using conventional culture method. It is worth noting that previous studies conducted in Iran have reported a lower positivity rate for CSF cultures, with only 10% of patients being culture-positive. In contrast, studies conducted in other countries have indicated significantly higher positivity rates, with approximately 70% of cases being culture-positive [15, 20-22]. The observed discrepancy could be attributed to several factors. Firstly, the use of antibiotics before sample culture may influence the growth and identification of microbial species. Secondly, non-compliance with technical guidelines during sample storage and transfer could compromise the integrity and viability of microorganisms. Furthermore, an improper sampling method might result in inadequate representation of the microbial population. Lastly, the insufficiency of microbial culture medium may limit the growth and detection of certain microorganisms [23].

There are several methods for molecular detection of meningeal pathogens. In the present study, the effectiveness of multiplex qPCR in detecting the etiological agents of this infection was investigated in 25 suspected cases with negative culture results. Interestingly, there were eight cases whose CSF culture results were negative, but their multiplex qPCR results were positive. *H. influenzae* was the most frequently identified pathogen using multiplex qPCR method. Multiplex PCR as a PCR-based method exhibited higher sensitivity and outstanding specificity for the identification of meningitis compared to culture. This finding is in line with the findings of previous studies conducted in Iran, India, Turkey, and Sweden [2, 7, 9, 12, 24, 25]. In the current study, *S. pneumoniae* and *H. influenzae* were identified as the most common pathogens causing BM. Culture and multiplex qPCR methods were employed to accurately detect these pathogens. The consistency of this study findings with previously published works in diverse geographic regions such as Iran, Turkey, Sweden, Nepal, and India further supports the validity of the obtained results [2, 8-10, 13, 26]. The molecular diagnostic technique, specifically qPCR, demonstrated high sensitivity and specificity in detecting a broad range of bacterial and viral pathogens commonly associated with meningitis, including enterovirus, Epstein-Barr virus, herpes simplex, *H. influenzae*, and *S. pneumoniae*. Enterovirus was the most

common viral cause of meningitis in analyzed cases, which is in line with the findings of global studies and previous research in our country [27, 28]. This consistency highlights the prominence of enterovirus as a significant etiological agent of viral meningitis, thereby emphasizing its vital role in the differential diagnosis and management of meningitis. Moreover, the efficiency of qPCR in successfully identifying these diverse pathogens highlights its potential as an accurate diagnostic tool for the diagnosis of meningitis. Contrary to conventional methods, qPCR could detect fastidious organisms that are often challenging to culture. Its high sensitivity and specificity as well as its rapid turnaround time make it an ideal tool for early detection and prompt treatment. Therefore, this study findings contribute to the growing body of evidence endorsing the incorporation of qPCR as a routine diagnostic instrument for meningitis in clinical practice, a move that could significantly enhance clinical decision-making and patient care.

This study had certain limitations. It was conducted in a single location and reviewed past data, which could potentially constrain the broad applicability of the findings. Furthermore, only a small subset of the samples (25 cases) underwent molecular testing due to their initial negative culture results, and the majority of the samples were not tested by this method. This might have resulted in undetected meningitis cases. Despite these constraints, the survey offers valuable insights into common meningeal pathogens and the comparative efficacy of conventional versus molecular diagnostic methods. These findings could be effective in shaping enhancements in meningitis prevention and control programs. Additionally, the study outcomes could contribute to the improvement of the existing meningitis surveillance system by underscoring the potential advantages of more extensive use of molecular diagnostic techniques.

Conclusion

S. pneumoniae, *H. influenzae*, and Enterovirus detected by both conventional culture and multiplex qPCR methods were identified as the three most common causes of meningitis in patients. The use of multiplex qPCR demonstrated a high level of accuracy in the diagnosis of eight common bacterial and viral causes of meningitis. Hence, multiplex qPCR technique could be considered as a rapid, precise, and reliable approach, able to detect various types of pathogens simultaneously. Additionally, this method offers a promising potential in the diagnosis of fastidious bacteria and viruses and is appropriate for use in therapeutic strategies.

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Ethical permissions: The Ethics Committee of Birjand University of Medical Sciences (IR.BUMS.REC.1400.044) approved this study.

Author's contributions: Data collection was led by KAR, while AP was responsible for drafting and editing the manuscript. ZM and GHSH designed the study, supervised data collection, and reviewed the manuscript thoroughly. All authors read and approved the final version of the manuscript.

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