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Comparison of Multiplex Real-Time PCR, Gram Stain, Latex and Culture for the Diagnosis of Bacterial Meningitis in Vietnamese Children

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Abstract— Bacterial meningitis is a life-threatening medical emergency. Cerebrospinal fluid (CSF) culture is a must to identify the pathogens of bacterial meningitis. Still, its result is usually late and sometimes shows low sensitivity, especially due to antibiotics previously given. Latex agglutination test and Gram staining are not sufficiently sensitive. Multiplex real-time PCR assay gives quick diagnostic results with high sensitivity, even with non-viable bacterial agents. The study aimed to evaluatethe validity of the combination of different assays in diagnosing bacterial meningitis in Vietnamese children.A cross-sectional study was conducted. CSF culture, latex agglutination test, Gram staining, and multiplex real-time PCR assays were performed. The Kappa statisticwas used to calculate the agreement between the methods.Totally, 108 eligible cases were recruited. There were five positive cases (4.6%) isolated by CSF culture, while the multiplex real-time PCR assays identified pathogensin fourcases (3.7%). Latex agglutination testing and Gram staining gave positive signals in three different cases.The agreement between multiplex real-time PCR and culture / latex agglutination testing / Gram staining was 0.652 / 0.852 / 0.262 correspondingly by the kappa statistics.The study showed that different diagnostic methods should be used to give accurate results in the determination of infectious agents in CSF specimens causing acute bacterial meningitis in Vietnamese children.

Keywords—Bacterial meningitis; multiplexreal-time PCR; Vietnamese children.

1. Introduction

Meningitis is an acute inflammation of the protective membranecovering the brain and spinal cord caused by infection with bacteria, viruses, or other microorganisms(1). The condition is classified as a medical emergency because it can be life-threatening, especially the bacterial meningitis(2). This disease can rapidly lead to death if appropriate antibiotics are not early given (2, 3). If patients survive, neurological sequelae persist in as many as one-third of all survivors, especially in newborns and children (1). For this reason, accurately and rapidly identifying the causes of bacterial meningitis in the treatment, prevention, and the prognosis is essential(4). There are several types of bacteria that can cause meningitis,and the etiological agent varies depending on the patient's age(5).*Listeria monocytogenes*, *Group B Streptococcus*, *Escherichia coli,* and other Gram-negative bacteria belonging to *Enterobacteriacae* are the leading causes in neonates, while *Haemophilusinfluenzae, Neisseria meningitidis,*and *Streptococcus pneumoniae*frequently cause bacterial meningitis in children with age from 1 month to 12 months. For children over 1 year old, *Neisseria meningitidis*and *Streptococcus pneumoniae*are also the common pathogens causing meningitis (1, 6).

Microbiological examination of the cerebrospinal fluid (CSF) specimen is crucial in order to determine the bacterial meningitisetiology(7). Methods include CSF culture, cell count and Gram staining, together with immunological diagnosis (latex agglutination test, immunochromatography) (8). However, some limits of these methods hamper the correct and quick detection of bacterial meningitis. Despite the fact that CSF culture, as a "gold standard",is required for the definitive diagnosis of the cause of meningitisand determination of bacterial sensitivity to antibiotics, it needs a long incubation period of time and usually results in a delay which takes about 48 – 72 hours after clinical sample collection, as well as may be negative if antibiotics are given prior to lumbar puncture, which could delay in the diagnosis and treatment (6, 9). The rapid tests such as latex agglutination and

Gram stainingare available to detect some limited etiological agents but with low sensitivity and cross-reaction possibility(4, 9, 10). Besides, all of the above methods are also seriously affected by the low bacterial titre in the CSF specimens(11).

Molecular diagnostic tests, typically including real-time polymerase chain reaction (PCR), has been suggestedas a rapid and accurate diagnostic test for bacterial meningitis, especially in cases withnegative culture results, owing to its ability to detect DNA from non-viable bacteria(5).Moreover, real-time PCR might be more sensitive than culture in case of sensitivity reduction of culture, because antibiotic treatment is applied prior to sample collection (9). Besides, multiplex real-time PCR assay,which can detect multiple targeted pathogens simultaneouslyin a reaction, is really useful to identify the etiologies of bacterial meningitis(12). Many multiplex real-time PCR assays have been developed and able to detect up to 12 common bacterial and viral pathogens of acute meningitis of suspected patients(10, 13, 14).However, in practice, multiplex real-time PCR assay also has a limitationof detecting only a few numbers of targeted pathogens incorporated in the assays and not being able to detect rare potential pathogens accidentally presenting in the CSF specimens.Thisstudy was conducted to evaluate the validity of the combined different diagnostic methods to facilitate their application routinely in the diagnosis and management of bacterial meningitis in pediatric patients in one of the biggest children hospitals in Ho Chi Minh City, Vietnam.

2. Methods

Patient selection and clinical sample collection

Patients wereadmitted to Children's Hospital 1 from August 2019 to May 2020.The inclusion criteria wereunder-15-year-old patients with a suspecteddiagnosis of bacterial meningitis, CSF examination withleukocyte count $\geq$ 10 cell/mm^3, granulocytes$\geq$ 50%, glucose level $\leq$ 40mg/dL, and protein level $\geq$40mg/dL.Samples were excluded in case the quantitation was not enough for doing all testings.Eligible CSF samples were collected forcytology, biochemical analysis, microbiological characteristics, and multiplex real-time PCR testing.The sample size was estimated to be 106 cases according to the study of Wang et al. (14).This study was approved by the ethics committees of the University of Medicine and Pharmacy at Ho Chi Minh City (597/DHYD-HDDD, 11/11/2019).

Bacteriological characteristic determination

CSF samples were subjected to direct microscopic examination,such as Gram staining which is used to identify and characterize bacteria through a heat-fixed smear on a slide treated with dyes and solvents in order. Gram-positive bacteria have purple of crystal violet stain while Gram-negative bacteria have red of safranin or fuchsine.

For culturing, CSF specimens were inoculated ontochocolate agar plates containing 5% sheep blood and incubated at 37^{o}C for 48 hours in the incubator (JOUAN, United Kingdom). Identification and antimicrobial susceptibility of the bacteria were carried out using a VITEK 2 Compact System (BioMerieux, Vitek, Inc., Hazelwood, MO, USA).

Latex agglutination testing (BioMerieux, Vitek, Inc.) is performed at the patient's bed. The test was carried out based on the manufacturer's instructions. The reaction is reported as positive when there is an agglutination of the beads evident by clumps. The reaction is reported as negative when there is no agglutination or formation of clumps.

For multiplex real-time PCR, total DNA from CFS specimens was extracted by the QIAampMinElute Virus Spin kit (Qiagen, Germany) according to the manufacturer's instructions. The Allplex™ Meningitis Panel 3 Assay kit using multiplex one-step real-time PCR was appliedto simultaneously detect 6 bacterial meningitis agents, including *N. meningitidis, L.monocytogenes, H.influenzae, S.agalactiae, S.pneumoniae, E. coli K1*from the extracted samples. The multiplex real-time PCR assay

was performed usingthe CFX96 real-time PCR detection system (Bio-Rad). The turn-around time is within5 hours. The assay results were analyzed with Seegene Viewer software (Seoul, Korea).

Statistical analysis

Qualitative variableswere described as frequency, percentage and quantitative variableswere described as mean and standard deviation (if standard distribution) or median and quartile interval (if unstandardizeddistribution). The Kappa statisticwas used to calculate the agreement between the results of the multiplex real-time PCR and other methods(15).Data were processed in SPSS software version 20.0.

3. Results

During the nine-month study period, 108 CSFspecimens were collected from suspected meningitis pediatric patients and included in the study. The morbidity rate in males (70.4%, 76/108)was about 2.4times higher than that in females (29.6%, 32/108). The age of the patients was commonly under 5 years old, accounting for 72.2% (78/108). There were 60.2% (65/108) of patients under 1-year-old. The rate of antibiotic use prior toCSF testing was 67.6% (73/108).The meanwhite blood cell counts in the CFS were110(32-401)cells/mm^3. The biochemical abnormalities of CSF consistent with that of bacterial meningitis werelowglucose level (27.0; 22.2-32.7 mg/dL), high protein level (20.6; 14.7-28.4 mg/dL) and high lactate level (8.8; 6.0-15.6 mg/dL) compared to normal range (Table 1).

Table 1. Demographic and CSF characteristics of patients

Study population	Number (n = 108)	Percentage (%)
Sex		
Male	76	70.4
Female	32	29.6
Age		
≤ 1 month	19	17.6
1 month – 1 year	46	42.6
1 year – 5 years	13	12.0
> 5 years	30	27.8
Antibiotic prior to lumbar puncture	73	67.6
CSF characteristics		
Cytology		
Leukocyte (cell/mm^3)*	110 (32 – 401)	
Granulocytes (%)*	75 (65 – 81)	
Biochemical testing		
Glucose (mg/dL)*	27.0 (22.2 – 32.7)	
Protein (mg/dL)*	20.6 (14.7 – 28.4)	
Lactate (mg/dL)*	8.8 (6.0 – 15.6)	

* Median (quartile interval)

There were fivepositive cases with bacteria isolated from CSF culture, accounting for 4.6% (5/108), including*Neisseria meningitidis*, *Escherichia coli*, *Acinetobacterbaumannii*and *Micrococcus luteus*(Table 2). All of these five cases were not indicatedantibiotics before performing a lumbar puncture.Four cases were determined positive by multiplex real-time PCR assay, in which *S.pneumonia* and*N. meningitidi*were detected in each case, and *E. coli*was presented in two cases. The positive rateof multiplex real-time PCRwas 3.7% (4/108). There were 3 cases in which CSF multiplex real-time PCR assay and culture showed similar results, accounting for50% (3/6), including one case positive with *N. meningitidis* and two cases positive with *E. coli*. Three other positive cases were discordant between the two tests. One case was positive with *A. baumannii,* and the other one was positive with *M. luteus* when isolated from CSF by culture. Gram stain also showed positive results on these two specimens. On the other hand, the last case gave positive with *S. pneumonia* by

multiplex real-time PCR assay but negative with culture. For this specimen, latex agglutination test and Gram staining also gave negative signals.Antimicrobial susceptibility testing was done on isolated bacteria, except for *S. pneumoniae* (not positive with culture) and *M. luteus*(not routinely antimicrobial susceptibility tested).

Table 2. Detection of bacterial pathogens in CSF (n = 108) by different methods

Infectious agents	Culture	Multiplex real-time PCR	LATEX**	GRAM**
S. pneumonia	0	1	0	0
N. meningitidis	1	1	1	0
E. coli	2	2	2	1
*A. baumannii**	1	0	0	1
*M. luteus**	1	0	0	1

* Not included in the multiplex real-time PCR panel.
** Positive signals only able to assert the existence of a certain bacterial agent in the CSF specimen without knowing the exact one.

There were six CSF samples positive for at least one of the methods applied to detect the etiological agents as well as presented some biochemical, microbiological, immunological or hematological abnormalities suggestive of bacterial meningitis (Table 4). The concordance between CSF multiplex real-time PCR and culture / latex agglutination testing / Gram staining were presented by the kappa value of 0.652 / 0.852 / 0.262, showing substantial / almost perfect / fair agreement respectively (Table 3).

Table 3. The agreement of multiplex real-time PCR assay with culture, latex agglutination test and Gram staining results in CSF specimens (n = 108) by Kappa statistics

Multiplex real-time PCR	Culture		LATEX		GRAM	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	3	1	3	1	1	3
Negative	2	102	0	104	2	102
Kappa value	0.652		0.852		0.262	

4. Discussion

This study assesses the performance of different testing methods for the detection of bacterial pathogens, causing meningitis. The proportion of positive CSF culture was 4.6%, while the corresponding rates in other studies vary, ranging from 0.3 – 32% (10, 11, 16). It was explained by different study subjects, collected specimen quality and antibiotics using prior lumbar puncture. Among these, antibiotic is most likely to affect the CSF culture positive rate(9, 17). It can reduce the bacterial growth rate of CSF culture at 30 – 60% (4-6). However, it was also noted that there were two cases positive with *A. baumannii* and *M. luteus* by CSF culture and Gram staining, but negative by multiplex real-time PCR assay and latex agglutination test. The multiplex real-time PCR system does not detect these bacteria. This proved that the CSF culture can determine a wide range of agents. Moreover, it is needed for the antimicrobial susceptibility testing to choose appropriate antibiotics for the treatment in clinical practice.

Table 4. Case study of positive clinical CSF specimens by culture and/or multiplex real-time PCR

Case study	Depart ment	Clinical suspicion	Clinical features and Microscopic examination result	Diagnostic results	Antimicrobial susceptibility
1-year-old	Infectio us	Bacterial meningitis	Clear CSF, leukocytes 45/mm^3, granulocytes	*Streptococcus pneumoniae*[1]	Unperformed

female	disease department		18%, glucose 2.84 mg/dL, lactic acid 1.67 mg/dL, protein 0.67 mg/dL		
6-year-old male	Infectious disease department	Bacterial meningitis	Clear CSF, leukocytes 158/mm^3, granulocytes 70%, glucose 3.95 mg/dL, lactic acid 2.43 mg/dL, protein 0.496 mg/dL	*Micrococcus luteus*[2]	Unperformed
Newborn male	Infectious disease department	Bacterial meningitis	Clear and yellow CSF with hemoglobin, leukocytes 2056/mm^3, granulocytes 93%, glucose 0.04 mg/dL, lactic acid 7.27 mg/dL, protein 3.434 mg/dL	*Escherichia coli*[1,2]	*Sensitive*: amikacin, imipenem *Resistant*: ampicillin, cefepime, ceftazidime, ceftriaxone, ciprofloxacin, Co-trimoxazole, gentamycin
5-year-old male	Pediatric intensive care department	Hydrocephalus	Opaque and yellow CSF with hemoglobin, leukocytes 1050/mm^3, granulocytes 90%, glucose 0.82 mg/dL, lactic acid 10.76 mg/dL, protein 4.308 mg/dL	*Acinetobacter baumannii*[2]	*Sensitive*: ciprofloxacin, gentamycin, imipenem *Intermediate*: cefotaxime *Resistant*: ceftazidime
Newborn male	Neonatal intensive care department	Sepsis	Opaque and yellow CSF, leukocytes 30/mm^3, granulocytes 80%, glucose 3.91 mg/dL, lactic acid 1.39 mg/dL, protein 0.257 mg/dL	*Escherichia coli*[1,2]	*Intermediate*: amikacin *Resistant*: ampicillin, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, Co-trimoxazole, ertapenem, gentamycin, imipenem, levofloxacin, meropenem, ticarcillin/clavulanic acid
4-year-old male	Infectious disease department	Septic shock	Opaque CSF with hemoglobin, leukocytes 110/mm^3, granulocytes 68%, glucose 6.53 mg/dL, lactic acid 16.64 mg/dL, protein 2.48 mg/dL	*Neisseria meningitidis*[1,2]	*Sensitive*: ceftriaxone *Resistant*: ciprofloxacin

[1] Multiplex real-time PCR, [2] Culture

The multiplex real-time PCR assay identified 4 positive cases. *S. pneumonia* was detected in one case given prior to lumbar puncture, whereas CSF culture was negative. This was consistent with recent

studies showing that antibiotics were themain reason resulting in positive PCR and negative culture (3, 5, 9, 17)because non-viable bacteria could be detected by multiplex real-time PCR. Furthermore, in this case, the latex agglutination test and Gram staining also gave a negative result with high possibility of not sufficient sensitivity(4). Moreover, multiplex real-time PCR provided a fast testingresult within 5 hours, much shorter than culture, usually around 48 hours. Therefore, multiplex real-time PCR can be used as an alternative diagnostic method in identifying the etiologic agents of bacterial meningitis in clinical settings(4, 18).

In this study, *N. meningitidis* infected a 4-year-old boy with suspected septic shock. The age of the patient was in line with other reports saying that *N. meningitidis*were the common etiologies of bacterial meningitis in under-5-year-old children (1, 10, 11, 16). Meanwhile, the Gram-negative bacteria, such as *E. coli* and *A. baumannii*, were only isolated from CSF cultures of neonatal bacterial meningitis. This results from neonates being much susceptible to Gram-negative bacterial infection. These bacteria are also the common etiologic agents, causing bacterial meningitis in the neonate that has been reported (1, 16). Especially, this study did not report any cases of bacterial meningitis caused by *H. influenzae*. The successful *H. influenzae* targeting vaccination program started 5 years ago in Vietnam could explain this trend. The declining incidence of diseases associated with *H. influenzae* recently has also been reported in other studies (10, 16, 19).

The study also showed that clinical features alone could not determine if meningitis is present, although a probable acute bacterial meningitis was defined by cytological and biochemical CSF characteristics as the reference standard (4). Therefore, it is necessary to use additional diagnostic methods, to give accurate and quick determination of etiological agents of acute bacterial meningitis in Vietnamese pediatric patients, especially in cases of low concentration of the microorganisms, patients treated with antimicrobials, or in case other methods give unsatisfactory results (20).

Our study has some limitations. Firstly, the positive cases identified by CSF culture and multiplex real-time PCR assay were small, so the diagnostic values (PPV, NPV) of each method could not be calculated. Furthermore, the small number of samples limited the evaluation of the accuracy of multiplex real-time PCR for each type of bacterium identified by culture. Secondly, for a large number of samples with negative results determined by different methods used in this study, there were high possibilities that they belong to viral meningitis, which is more common than bacterial meningitis. However, the strength of this study was that the patients were from one of the biggest pediatric hospitals in Ho Chi Minh City which admits patients from provinces nearby. Therefore, they might be representative for the general Vietnamese pediatric patients. Furthermore, combining different diagnostic methods could give satisfactory results because each method has its own advantages and disadvantages, as well as necessarily to exclude false-negative results for bacterial meningitis detection.

5. Conclusion

In conclusion, it is recommended that different diagnostic methods should be used to test infectious agents presenting in CSF samples to determine acute bacterial meningitis. Amongst these methods, multiplex real-time PCR can be used as a routine test in a clinical settingfor themanagement of bacterial meningitis in Vietnamese children.

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