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Original Article

COMPARISON OF MULTIPLEX PCR, GRAM STAIN, AND CULTURE FOR DIAGNOSIS OF ACUTE BACTERIAL MENINGITIS

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ABSTRACT

Objectives: to compare characteristics of the multiplex PCR and gram stain with bacterial culture as reference method for detection of five pathogens which multiplex PCR can detect: Streptococcus pneumoniae, Haemophilus influenzae type b, Neisseria meningitides, Group B streptococcus, and Listeria monocytogenes in cerebrospinal fluid (CSF) samples of patients were suspected of acute bacterial meningitis.

Methods: 110 CSF samples were obtained from 110 patients were suspected of acute bacterial meningitis as defined by WHO. Gram stain, bacterial culture, and multiplex PCR tests were done for all samples.

Results: Gram stain for any bacteria was positive in 32 cases (29.1%), including the five pathogens in 11 cases (10%). Bacterial culture was positive in 38 cases (34.5%), including the five pathogens in 8 cases (7.2%). Multiplex PCR was positive in 60 cases (54.5%) and the most bacteria detected was Streptococcus pneumoniae in39/60 cases(65%), followed by Neisseria meningitides in 8/60 cases (13.3%). The sensitivity of multiplex PCR was 100%. 50 cases of acute bacterial meningitis were diagnosed by multiplex PCR, while both gram stain and bacterial culture were negative in it.

Conclusions: The PCR method is a rapid, sensitive, and specific diagnostic test for acute bacterial meningitis. PCR is particularly useful for analyzing CSF of patients who have been treated with antibiotics before lumbar puncture.

Keywords: Comparison, Bacterial meningitis, Multiplex PCR, Five pathogens.

INTRODUCTION

Bacterial meningitis is a medical emergency, and immediate steps must be taken to establish the specific cause and initiate effective therapy. The mortality rate of untreated disease approaches 100 percent and, even with optimal therapy, there is a high failure rate [1,2].

Over 1.2 million cases of bacterial meningitis are estimated to occur worldwide each year [3] and accounting for an estimated 171,000 deaths worldwide per year [4, 5, 6].

Acute bacterial meningitis is one of the most severe infectious diseases, causing neurologic sequelae [4,5].

Beyond the perinatal period, three organisms transmitted from person to person through the exchange of respiratory secretions [3,7,8,9], are responsible for most cases of bacterial meningitis: Neisseria meningitidis, Haemophilus influenzae, and Streptococcus pneumoniae [3,7,8,9,10,11,12,13,14].

Rapid diagnosis and treatment are critical [15, 16] because permanent neurological sequelae (suchas hearing loss, mental retardation, seizures, and behavioral changes) may occur in up to one-half of survivors [7, 9, 15, 16].

Traditional laboratory diagnostic methods of culture for the identification of bacterial meningitis pathogens take up to 36 h or more. Furthermore, it has been observed that following an increase in the practice of starting antimicrobial therapy prior to clinical sample collection [11,16,17,18], the ability to confirm the pathogenic microorganisms of bacterial meningitis and septicemia has decreased by approximately 30% [11,16].

Moreover, culture results may be false negative when fastidious or culture-resistant bacteria are involved or when patient samples are obtained after antimicrobial therapy has started [10].

Bacterial concentration in the Cerebrospinal fluid (CSF) has a profound effect on the results of microscopy. Regardless of the type of organism in the CSF, the percentage of positive microscopic results is only 25% with 10^3 cfu/ml [19,20] and 60% in the range of 10^3 to 10^5 cfu/ml [19] and in 75 percent of cases if more than 100,000 cfu/ml are present [20].

Recently, PCR-based assays have become available to provide an early and accurate diagnosis of bacterial meningitis [19]. This assay can detect as few as 10–100 cfu/ml of bacteria in CSF [21].

The usefulness of this diagnostic test would be to determine whether empirical antimicrobial therapy should or should not be administered and thus potentially eliminate unnecessary administration of antimicrobial therapy to some patients [15].

Our purpose was to determine the utility of a multiplex PCR as a rapid diagnostic technique for detection of bacterial meningitis and to delineate test characteristics, including sensitivity, specificity, positive predictive value, and negative predictive value.

According To these studies, immunization can be planned against the common causes of meningitis.

To the best of our knowledge, this research is done for the first time in Syrian Arab Republic.

MATERIALS AND METHODS

From November 2011 to November 2013, the suspected cases of acute bacterial meningitis among patients admitted to Aleppo University hospital were undertaken.

The suspected cases of acute bacterial meningitis were identified by a clinician, based on the following criteria:

Acute onset of fever (usually > 38.5 °C rectal or 38.0 °C axillary), headache and one of the following signs: neck stiffness, altered consciousness or other meningeal signs [22]. Newborns were enrolled in the study if the newborn has a fever accompanied by nonspecific symptoms (eg, poor feeding, vomiting, diarrhea, rash) [23].

Patient's samples were included in further analyses if the CSF examination showed at least one of the following:

- Turbid appearance.

-Leukocytosis (> 100 cells/mm³).

- Leukocytosis (10-100 cells/mm³) AND either an elevated protein (> 100 mg/dl) or decreased glucose (< 40 mg/dl) [22].

CSF samples were obtained via lumbar puncture or from CSF shunts and were submitted to routine microbiology, chemistry, cell count, and multiplex PCR.

Gram stain: if the volume of the specimen is more than 1 ml, we performed gram stain with a centrifuge specimen for 20 minutes at 2000 rpm. If CSF is less than 1 ml, we do not centrifuge [7].

Bacterial culture: CSF specimens were inoculated on chocolate agar and blood agar. Both plates were incubated in a carbon dioxide enriched atmosphere at 35–37 °C for up to 48 hours; plates were checked for growth after overnight incubation. When patient is a newborn, we also inoculated the specimen on MacConkey agar plate which was incubated aerobically at 35–37°C overnight [24].

Identification test: isolated bacteria were identified by BD Phoenix™ Automated Microbiology System (BD Diagnostic Systems, Sparks, MD).

Listeria monocytogenes, Neisseria meningitides and Haemophilus influenzae were identified by Api Microsystems [Api NH and Api Listeria] (bioMérieux SA, France).

Multiplex PCR: A CSF samples (minimum 0.5 mL) were kept at –20°C until transportation to the Research Laboratory-Faculty of Medicine (Aleppo University, Aleppo, Syria) for PCR analysis.

DNA extraction: CSF specimens were centrifuged at 15,000 rpm for 5 min. DNA was extracted from the cell pellet by using a nucleic acid purification column according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). The DNA extraction was stored at -20°C for DNA amplification.

DNA amplification: We used Seeplex Meningitis ACE Detection kit (Seeplex Meningitis-B, Korea) which detects five pathogens: Streptococcus pneumoniae (SP), Haemophilus influenzae type b (Hib), Neisseria meningitides (NM), Group B streptococcus (GBS), and Listeria monocytogenes (LM). [Table 1]

Each PCR amplification was performed using 5 µL of isolatednucleic acid solution, 2 µL of 10 x primer mixture, 10 µL of 2 x Multiplex Master Mix (Seegene Inc.), and 3 µL of 8-MOP solution in a total volume of 20 µL. The amplification protocol was as follows [Table.2]

Table 1: shows target genesused in theSeeplex Meningitis-B ACE Detection kit

	Organism	Target gene	Target protein	Size of product (bp)
Seeplex Meningitis-B	Internal control			1,000
	SP	GyrB	DNA gyrase, subunit B	669
	Hib	P6	Outer membrane protein (OMP) P6	532
	NM	ctrA	Capsule polysaccharide export outer membrane protein CtrA	410
	GBS	Cfb	CAMP-factor (cfb) gene	315
	LM	Hly	Pore-forming Cytolysin listeriolysin	213

-Multiplex PCR conditions

Table.2: shows the protocol of DNA amplification

Segment	No. of cycles	Temperature	Duration
1	1	94°C	15 min
2	40	94°C	0.5 min
		63°C	1.5 min
		72°C	1.5 min
3	1	72°C	10 min

The amplified PCR products were electrophoresed in 2% (w/v) agarose gels and were stained with ethidium bromide.

An example of PCR positive sample is shown in Fig. 1(A, B).

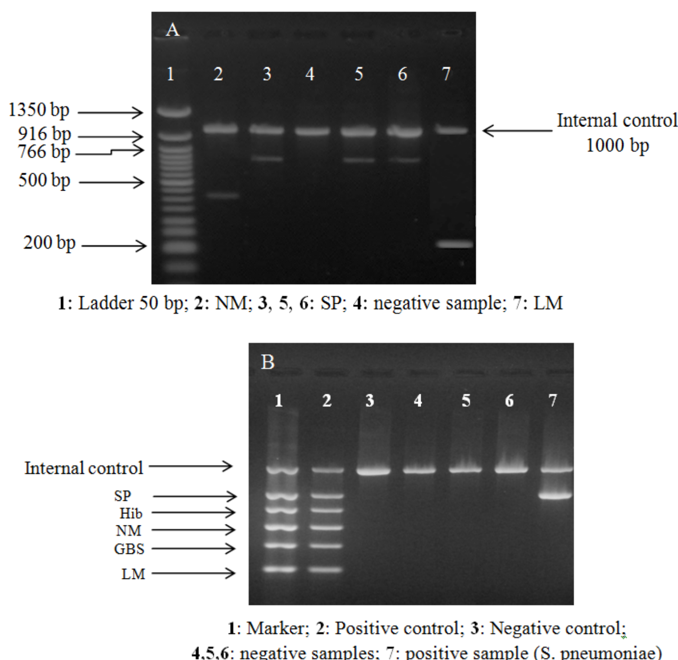

Fig. 1(A, B): Shows Amplification of standard DNA on clinical samples

A multiplex PCR kit uses dual priming oligonucleotide (DPO) methods, which consist of 2 separate priming regions joined by a polydeoxyinosine linker, yield 2 primer segments with distinct annealing properties. The long 5′-segment initiates stable priming, whereas the short 3′-segment controls target-specific extension; the use of these 2 primer segments effectively eliminates non-specific priming and yields consistently high PCR specificity even under less-than-optimal PCR conditions.

Statistical Analysis

All statistical analysis was performed with SPSS version 11.5 (SPSS Inc, Chicago, IL, USA) [25].

Sensitivity, specificity, positive predictive value, and negative predictive value were determined on the basis of standard definitions [26].

RESULTS

A total of 110 CSF samples were collected from 110 patients who were diagnosed with acute bacterial meningitis, based on clinical symptoms and CSF findings as described above.

The demographic and laboratory characteristics of patients were included in this study are shown in table 3.

Gram stain for the five pathogens, which multiplex PCR can detect, was positive in 11/110 cases (10%), of which 10 (90.9%) was S. pneumoniae and 1 (9.1%) was N. meningitidis.

Table 3: shows demographic and laboratory characteristics of patients

Demographic and laboratory characteristics	Patients (n =110)
Mean age, years (range)	4.2 (3 days-52 years)
Male (%)	60.9
Mean CSF leukocyte count, cells/mm^3 (range)	2500 (25-25000)
Neutrophils (%)	62.7
Mean CSF Glucose, mg/dl (range)	30.9 (4-239)
Mean CSF Protein, mg/dl (range)	234.6 (26-492)
Receipt of antibiotics before lumbar puncture (%)	65.5

Bacterial culture for the five pathogens, which multiplex PCR can detect, was positive in 8/110 cases (7.2%) and all these cases were S. pneumoniae.

Multiplex PCR test was positive in 60/110 cases (54.5%). Among the multiplex PCR positive cases, S. pneumoniae was detected at the highest incidence of 39 cases (65%), followed by N. meningitidis in 8 cases (13.3%), Listeria monocytogenes in 7 cases (11.7%), Group B streptococcus in 4 cases (6.7%), and Hib in 2 cases (3.3%) [table 4].

Multiplex PCR was negative in all 30 CSF specimens that with culture isolation of bacteria other than S. pneumoniae, N. meningitidis, Listeria monocytogenes, Group B streptococcus, or Hib.

Table 4: Shows the results of bacterial culture, gram stain and multiplex PCR tests

laboratory test	No. of positive (total 110)
Gram stain	
for any bacteria	32[(a)] (29.1%)
SP	10 (30.3%)
NM	1 (3.05%)
Gram negative bacilli	20 (60.6%)
Gram positive cocci	2 (6.05%)
Bacterial culture	
for any bacteria	38[(a)](34.5%)
SP	8 (20.6%)
Pseudomonas aeruginosa	8 (20.6%)
Acinetobacter spp.	7 (17.9%)
E. coli	6 (15.4%)
Enterobacter aerogenes	3 (7.7%)
Citrobacter freundii	2 (5.1%)
Serratia marcescens	2 (5.1%)
staphylococcus spp.	2 (5.1%)
Burkholderia cepacia	1 (2.5%)
Multiplex PCR	
Any positive	60 (54.5%)
SP	39 (65%)
NM	8 (13.3%)
Hib	2 (3.3%)
LM	7 (11.7%)
GBS	4 (6.7%)

(a) *one patient showed simultaneous infection with 2 bacterial (Acinetobacter spp. and Citrobacterfreundii) isolated from two ventricular shunts, right and left.

The results of the three tests (gram stain, bacterial culture, and multiplex PCR) for the five pathogens, which multiplex PCR can detect, are summarized in the table 5.

Table 5: shows the results of the three tests (gram stain, bacterial culture, and multiplex PCR) for the five bacteria studied by multiplex PCR

Bacterial culture	Gram stain	multiplex PCR	N= 110
+	+	+	8
-	+	-	1
+	-	-	0
-	-	+	50
+	+	-	0
-	+	+	2
+	-	+	0
-	-	-	49

Performance characteristics of gram stain and multiplex PCR for the five pathogens which multiplex PCR can detect:

A positive result for bacterial culture was defined as the isolation of one of the five pathogens.

A Gram stain result was defined as positive if bacteria have been seen in the Gram-stained smear with the same morphology of one of the five pathogens.

All other culture and gram stain results, including the absence of bacteria or the presence of other bacterial species (in the absence of S. pneumoniae, N. meningitidis, Listeria monocytogenes, Group B streptococcus, and H. influenzae), were considered a negative test result.

For the evaluation of performance characteristics of the gram stain and multiplex PCR, results were compared with CSF cultures as current gold standard [table 6].

Table 6: Shows performance characteristics of the gram stain and multiplex PCR

		Bacterial culture		Total
		Positive (n = 8)	Negative (n = 102)	(n = 110)
Gram stain	Positive	8	3	11
	Negative	0	99	99
Multiplex PCR	Positive	8	52	60
	Negative	0	50	50

According to these data, performance characteristics: Sensitivity, specificity, Positive predictive value (PPV), and Negative predictive value (NPV) were: 100%, 97.1%, 72.7%, 100% respectively for gram stain, and 100%, 49%, 13.3%, 100% respectively for multiplex PCR.

However, this specificity of the multiplex PCR does not reflect the true percentage, because in many cases with a negative bacterial culture an antibiotic had been prescribed before the bacterial cultivation of the CSF. [Table 7]

Table 7: Shows performance characteristics of multiplex PCR among untreated patient before lumber puncture.

		Bacterial culture		Total
		Positive	negative	
Multiplex PCR	Positive	6	2	8
	Negative	0	30	30
Total		6	32	38

According to these data, performance characteristics: Sensitivity, specificity, Positive predictive value (PPV), and Negative predictive value (NPV) of the multiplex PCR were 100%, 93.8%, 75%, 100% respectively

DISCUSSION

Bacterial meningitis is a life-threatening disease [13, 27], however, it is proven that early diagnosis improves clinical outcomes [13].

Although bacterial culture is considered to be the standard method, the negative effect of prior antimicrobial drug usage on its sensitivity makes necessary to search for non-culture techniques for diagnosis. Among non-culture diagnostic tests, PCR is the most accurate and reliable method, especially among patients with a history of antimicrobial drug use before spinal tap [5].

In this research, 50 cases (45.5%) of acute bacterial meningitis were diagnosed by multiplex PCR - in 29 cases for S. pneumoniae, in 8 cases for N. meningitidis, in 7 cases for Listeria monocytogenes, in 4 cases for Group B streptococcus, and in 2 cases for Hib, while both gram stain and bacterial culture were negative.

The bacterial meningitis epidemiologic landscape is not static [5], and the causative agents change with time and across regions of the world [5,8].

In the present study, among the multiplex PCR positive cases, S. pneumoniae was detected at the highest incidence of 39 cases (65%). This result is similar to that was found by Bøving et al. [18] (50.9%), Schuurman et al. [19] (36.7%), Welinder-Olsson et al. [28] (31.7%), Khan et al. [29] (73.8), and Ghotaslou et al. [16] (42.1%).

The second agent detected by multiplex RCR was N. meningitides in 9 cases (13.3%), this result is similar to that had seen by Bøving et al. [18] (14.5%).

In Turkey, Ceyhan et al [5] found that N. meningitis was the most common cause of acute bacterial meningitis, they highlighted the emergence of serogroup W-135 diseases in Turkey, and they concluded that meningococcal vaccine in this region must provide a reliable defense [5].

In the present study, there were few Hib influenza cases (3.3%) were detected by multiplex PCR; this can be due to routine Hib vaccination for infants. This result is similar to that was found by Welinder-Olsson et al. [28] (5.9%), and Schuurman et al. [19] (6.7%).

In Japan, Chiba et al. [8] found that H. influenzae was detected at the highest incidence (45.2%), followed by S. pneumoniae (21.4%).

In this study, Listeria monocytogenes was detected in (11.7%) cases by multiplex PCR. this prevalence is higher than what was detected by Bøving et al. [18] (1.8%) and by Chiba et al. [8] (0.8%), but it is lower than what was found by Favaro et al. [30] (24.4%).

In this study, group B streptococcus was detected in (6.7%) cases by multiplex PCR. This prevalence is higher than what was detected by Schuurman et al. [19] (3.3%) and by Welinder-Olsson et al. [28](1.9%).

In the present study, the sensitivity and specificity of the multiplex PCR are 100%, 49.0% respectively, which are similar to that was found by Sarookhani et al. [31] (100%, 40.6%) and Chiba et al. [8] (100%, 54%).

In this research, the bacterial culture was positive in 34.5%, which is similar to that was found by Sarookhani et al. [31] (36%), and it is higher than what was found by Saravolatz et al. [15] (20.3%), and Schuurman et al. [19] (16.3%), but it is lower than what was found by Welinder-Olsson et al. [28] (52.7%).

The low rate of positive bacterial culture may be due to the preceding antibiotic therapy of the patients before lumber puncture.

The high rate of isolation of pseudomonas aeruginosa and Acinetobacter spp. is consistent with the presence of head trauma or neurosurgically implanted devices.

H. influenzae, or Listeria monocytogenes were not isolated by bacterial culture in the present study, which is similar to that was found by Chakrabarti et al. [32], Saravolatz et al. [15], and Bøving et al. [18].

N. meningitidis was not isolated by bacterial culture in the present study, which is similar to that was found by Chakrabarti et al. [32], and Chiba et al. [8].

Group B streptococci was not isolated by bacterial culture in the present study, which is similar to that was found by Chakrabarti et al. [32], Welinder-Olsson et al. [28], Ghotaslou et al. [16], and Schuurman et al. [19].

In this study, the gram stain was positive in 29.1%, which is higher than that was found by Saravolatz et al. [15] (14.9%), and Schuurman et al [19] (9.3%), but is lower than that was detected by Favaro et al. [30] (75%).

Sensitivity and specificity of gram stain are 100%, 97.1% respectively,which are higher than that for multiplex PCR (100%, 49% respectively), although gram stain was positive only in 1 case, while multiplex PCR was positive in 50 cases.

However, the above information denotes that the gram stain and bacterial culture cannot be a considered as a method for early and exact diagnosis of meningitis

In conclusion, The PCR has high sensitivity and specificity for the detection of bacterial pathogens such as S. pneumoniae in the CSF. Further refinements in this technique may make it useful for the diagnosis of bacterial meningitis, especially when results of CSF gram stain and bacterial culture are negative and when patients had received antibiotics before the lumbar puncture was done.

Due to the need to antimicrobial susceptibility testing, PCR should be considered as an adjunct to bacterial culture and antimicrobial susceptibility testing.

Finally, we recommend the introduction of pneumococcal conjugate vaccine (PCV) in Aleppo to prevent pneumococcal disease in this region

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