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Molecular Analysis of Bacterial Meningitis in Suspected Cases

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Abstract

One hundred and seventy-six cases of suspected meningitis (SMN) were included in a cross-sectional study. Their ages ranged from less than 1 year to 80 years, of whom 44.3% were male. The aim was to assess bacterial meningitis (BMN) in terms of incidence and types of causative bacteria. Cerebrospinal fluid (CSF) specimens were collected and polymerase chain reaction (PCR) analysis was conducted with universal primers designed to amplify a DNA fragment (996 bp) of the 16S rRNA gene of eubacteria. Resolving PCR products in agarose-gel electrophoresis revealed that 37.5% of CSF specimens were PCR positive, while 62.5% of CSF specimens showed no band and were considered PCR-negative. Eighty percent of the latter specimens were noted to have pleocytosis, in addition to having protein and/or glucose concentrations lower or higher than those in the normal range (abnormal CSF). The remaining 20.0% of CSF specimens were considered normal regarding pleocytosis, protein, and glucose (normal CSF). When the amplified DNA of PCR-positive specimens was subjected to sequencing and alignment with reference sequences, both Gram-negative (GN) and Gram-positive (GP) bacteria were identified (23 and 9 types, respectively). *Acinetobacter* spp. and *Pseudomonas* spp. were the most common GN bacteria (each with 17.8%), while *Staphylococcus* spp. was the most common GP bacteria (43.75%). The study concluded that BMN presents an important public health challenge, and PCR analysis of the CSF was an effective method for diagnosing pathogenic bacteria in SMN. In addition, leukocytes, glucose, and protein are valuable CSF parameters that may aid in the diagnosis of BMN.

Keywords: Cerebrospinal fluid, Suspected meningitis, leukocytes, glucose, protein, Polymerase chain reaction.

التحليل الجزيئي لالتهاب السحايا الجرثومي في الحالات المشتبه بها

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الخلاصة

تم تضمين 176 حالة من حالات التهاب السحايا المشتبه بها في دراسة مقطعية. تراوحت أعمارهم بين أقل من سنة واحدة و 80 سنة ، منهم 44.3٪ ذكور. كان الهدف هو تقييم التهاب السحايا الجرثومي من حيث الإصابة وأنواع البكتيريا المسببة. تم جمع عينات السائل الدماغي–الشوكي وأجري تحليل تفاعل البلمرة المتسلسل (PCR) باستخدام بادئات شاملة مصممة لتضخيم جزء من الحمض النووي (996 زوج قاعدة) من جين 16S rRNAمن البكتيريا الحقيقية. أظهر تحليل منتجات PCR في الترحيل الكهربائي لهلام الاغاروز بأن 37.5٪ من عينات السائل الدماغي كانت موجبة PCR ، بينما 62.5٪ من عينات السائل الدماغي النخاعي لم تظهر أي حزمة واعتبرت سلبية PCR. وقد لوحظ أن 80٪ من العينات الأخيرة لديها ارتفاع بعد خلايا الدم البيض، بالإضافة إلى وجود بروتين و / أو تركيزات جلوكوز أقل أو أعلى من تلك الموجودة في النطاق الطبيعي (السائل الدماغي غير الطبيعي). واعتبرت 20.0٪ المتبقية من عينات السائل الدماغي–الشوكي طبيعية من حيث عد خلايا الدم البيض والبروتين والجلوكوز (السائل الدماغي–الشوكي الطبيعي). وعند تحديد تسلسل الحمض النووي المتضخم للعينات الإيجابية لـ PCR ومحاذاتها مع التسلسلات المرجعية المتاحة، تم تحديد كل من البكتيريا السالبة والموجبة لملون جرام (23 و 9، على التوالي). كانت *Acinetobacter* spp و *Pseudomonas* spp الأكثر شيوعً من البكتريا السالبة لملون جرام (كل منها 17.8٪) ، بينما كانت *Staphylococcus* spp الأكثر شيوعًا من البكتريا الموجبة لملون جرام (43.75٪). خلصت الدراسة إلى أن التهاب السحايا الجرثومي يمثل تحديًا مهمًا للصحة العامة ، وكان تحليل PCR للسائل الدماغي–الشوكي طريقة فعالة لتشخيص البكتيريا المسببة للأمراض في الحالات المشتبه اصابتها بالسحايا. بالإضافة إلى ذلك، تعد خلايا الدم البيض والجلوكوز والبروتين عوامل معايير مهمة في السائل الدماغي–الشوكي والتي قد تساعد في تشخيص التهاب السحايا الجرثومي.

1. Introduction

Meningitis (MN), a life-threatening disease associated with a high risk of permanent disability and death, is an inflammation of the membranes surrounding the brain and spinal cord and lining the vertebral canal and skull, mostly due to viral, bacterial or fungal agents [1]. However, it has been pointed out that bacteria are a significant cause of MN and there has been a global public health concern to determine the incidence rate of bacterial meningitis (BMN), as well as the causative bacteria [2]. A global estimate in 2016 revealed that there were 2.82 million BMN cases with a mortality rate of 11.3%. The incidence of BMN varies and depends on age, geographical region, and bacterial type. Australia recorded the lowest infection rate, while South Sudan was found to have the highest rate of BMN (0.5 and 207.4 per 100,000 population, respectively [3],[4]. Regarding the bacterial type, several bacteria have been identified as BMN-causing agents. A meta-analysis of 56 studies demonstrated that *Streptococcus pneumoniae* and *Neisseria meningitidis* were the most frequent cause of BMN in all ages and regions of the world. In addition, *S. pneumoniae* was the most frequent BMN-associated pathogen in European and African children, as well as Western Pacific and African adults. In African neonates, *S. pneumoniae* and *Escherichia coli* were the most frequently encountered bacteria associated with BMN. The analysis also revealed that *Haemophilus influenzae* is a further causative bacteria of BMN in all ages, particularly in children younger than 5 years [5]. *Mycobacterium tuberculosis* has also been recognized to cause BMN (in this case, tuberculous meningitis) as this bacteria can cross the protective barriers and enter the central nervous system (CNS) [6].

The laboratory diagnosis of BMN is primarily based on the evaluation of leukocyte count, glucose and protein concentrations and microscopic examination of CSF smears stained with Gram stain. In addition, CSF culture is performed in laboratory settings and is considered a gold diagnostic test for BMN [7]. However, these assessments may have limitations; for

instance, low rates of bacterial growth can occur due to the use of antibiotics [8]. Thus, there is a necessity to adopt more sensitive and reliable methods. The molecular detection of CSF bacterial DNA, such as the use of a polymerase chain reaction (PCR) assay, is probably the best approach to adopt in this regard. In fact, the speed and accuracy of PCR in diagnosing BMN have been largely recognized, particularly in CSF samples with culture-negative results. Thus, adopting a molecular approach may improve our epidemiological understanding of the BMN burden [9].

As BMN is associated with neurological comorbidities, sensitive, rapid and accurate methods are definitely needed and are a priority in determining BMN burden. In view of this, the present study aimed to determine the incidence of BMN in a cohort of patients suspected of having MN (SMN) of a wide age range (infant, children, adults, and elderly), and to identify the bacteria involved using a molecular approach (PCR-based method). Data on Iraqis for this topic is not overwhelming, and studies are warranted to understand the burden of BMN in SMN cases of all ages.

2. Patients and methods

2.1 Cases of suspected meningitis

Written consent was given by all participants or their legal guardians. The study protocol was also approved by the Ethics Committee of the College of Science, University of Baghdad. Patients were initially characterized in terms of age, sex, and antibiotic use. These data were recorded from the hospital records.

One hundred and seventy-six cases of SMN were included in a cross-sectional study from January-November 2020. These cases were administrated to Neurosurgery Teaching Hospital and Alwitri Neuroscience Teaching Hospital due to SMN (hydrocephalus in infants and children and high intracranial pressure in adults). A CSF sample was aseptically obtained by lumbar puncture (LP) and dispensed in a sterile plain tube. The CSF sample was initially evaluated with the conventional bacteriological cultural methods (blood, chocolate, and MacConkey agar media). Besides, leukocyte count and glucose and protein concentrations were determined. These laboratory tests were not carried out by the researchers and their results were provided by the hospital diagnostic laboratories.

Two categories of leukocyte were considered, normal or pleocytosis, based on the normal leukocyte count in CSF (0-15, 0-9, and 0-5 cells/mm^3 for infants $\leq$ 28 days, infants 29-60 days and children and adults, respectively) [10],[11]. Protein and glucose concentrations were categorized as low, normal, and high according to their reference range in CSF (15-45 and 40-70 mg/dL, respectively) [12].

2.2 PCR analysis

DNA was isolated from the CSF samples using a commercial kit (HiPurA *Mycobacterium tuberculosis* DNA purification kit, Himedia, India) and instructions of the manufacturer were followed. This kit can be used to isolate DNA from *M. tuberculosis* and all other bacteria. PCR analysis was conducted using a previously described protocol [13]. A universal PCR assay was established in this protocol to amplify a portion (996 bp) of the 16S rRNA gene in the identified eubacteria of CSF. In this assay, two primers were introduced and marked as U1 (5′-CCAGCAGCCGCGGTAATACG-3′) and U2 (5′-ATCGG(C/T)TACCTTGTTAGA-CTTC-3′). The two primers were online tested for specificity using the Primer-BLAST function (https://blast.ncbi.nlm.nih.gov/), and their efficiency and specificity were confirmed.

Isolated DNA was subjected to PCR amplification (OptiMax Gradient Thermal Cycler, (Labnet-Multi-Gene, USA), and 25 µL reaction mix was set up to include the following components: Maxime- PCR-PreMix, iNtRON Biotechnology, Inc., Korea (5 µL), U1 primer (1 µL), U2 primer (1 µL), DNA (1.5 µL) and deionized distilled water (16.5 µL). The following optimized conditions were adopted to run the thermocycling: an initial denaturation cycle for 3 minutes at 94°C, 45 cycles of denaturation for 45 seconds at 94°C, annealing for 45 seconds at 66 °C and extension for 1 minute at 72 °C and finally an extension cycle for 7 minutes at 72 °C. Agarose gel electrophoresis was used to resolve PCR products (1.5% agarose gel, 5 volts/cm^2 for 60 minutes). PCR positive CSF samples showed a 996 bp band. This band was eluted from the agarose gel (Zymoclean Gel DNA Recovery Kit, Zymo Research, USA) and shipped for Sanger sequencing (ABI-310 genetic analyzer, Macrogen, Korea). DNA sequences of PCR positive CSF samples were subjected to an alignment analysis against reference sequences available in the database of NCBI using the BLAST tool. According to this analysis, the bacteria were identified at the genus or species level.

2.3 Statistical analyses

Data of the current study was described as a number followed by a frequency (percentage). These frequencies were compared and significant differences were assessed using the Pearson Chi-square test. The level of significance was set at a two-tailed probability (*p*) less than 0.05, which was taken as statistically significant. These analyses were conducted with GraphPad Prism version 9.2.0 (San Diego, CA, USA).

3. Results

3.1 Baseline characteristics

Female frequency in the 176 SMN cases was higher than that in males (55.7 *vs*. 44.3%). In terms of age groups, the SMN cases were categorized into six groups, including ≤ 2, 3-12, 13-18, 19-39, 40-59, and ≥ 60 years. The age group 40-59 years was the most common and accounted for 29.0% of cases. This was followed by the age groups < 2, 19-39, and 13-18 years, which accounted for 27.8, 27.3, and 6.8% of cases, respectively. The least common age groups were 3-12 and ≥ 60, each with a frequency of 4.5%. Some CSF samples showed pleocytosis of leukocytes (39.4%), neutrophils (26.1%), and lymphocytes (20.9%). Further, 20.9% of CSF samples sowed neutrophil-to-lymphocyte ratio (NLR) greater than 1.0. In the case of CSF protein and glucose; low and high concentrations were found with frequencies of 6.9 and 52.9% for protein, and 27.0 and 27.6% for glucose, respectively. It was also observed that 39.2% of SMN cases were receiving antibiotics at the time of CSF collection (Table 1).

Table 1: Baseline characteristic information of suspected cases of meningitis

Characteristic		N = 176	
		N	%
Sex	Male	78	44.3
	Female	98	55.7
Age group; year	≤ 2	49	27.8
	3-12	8	4.5
	13-18	12	6.8
	19-39	48	27.3
	40-59	51	29.0
	≥ 60	8	4.5
Leukocyte count	Normal	106	60.6
	Pleocytosis	69	39.4
Neutrophil count	Normal	113	73.9
	Pleocytosis	40	26.1
Lymphocyte count	Normal	121	79.1

Neutrophil-to-lymphocyte ratio	Pleocytosis	32	20.9
	No cells	55	35.9
	≤ 1.0	66	43.1
	> 1.0	32	20.9
Protein concentration; mg/dL	Normal	70	40.2
	High	92	52.9
	Low	12	6.9
Glucose concentration; mg/dL	Normal	79	45.4
	High	48	27.6
	Low	47	27.0
Table 1 continued			
Antibiotic medication	No	107	60.8
	Yes	69	39.2

3.2 PCR analysis

Agarose gel electrophoresis of PCR products revealed that some CSF samples showed a band with a molecular size of 996 bp (Figure 1), and these samples were considered PCR positive (i.e. having bacteria in their CSF). They accounted for 66 samples out of the 176 CSF samples (37.5%). The remaining 110 CSF samples (62.5%) showed no band in the resolved agarose gel electrophoresis of PCR products and were considered PCR negative. However, when their CSF was examined for leukocyte count, as well as protein and glucose concentrations, some differences were observed. Eighty-eight (80.0%) of PCR negative CSF samples showed an abnormal distribution of pleocytosis, protein, and/or glucose. These samples were regarded as abnormal (abnormal CSF group). Twenty-two of PCR negative CSF samples (20.0%) did not show these variations and were considered normal (normal CSF group).

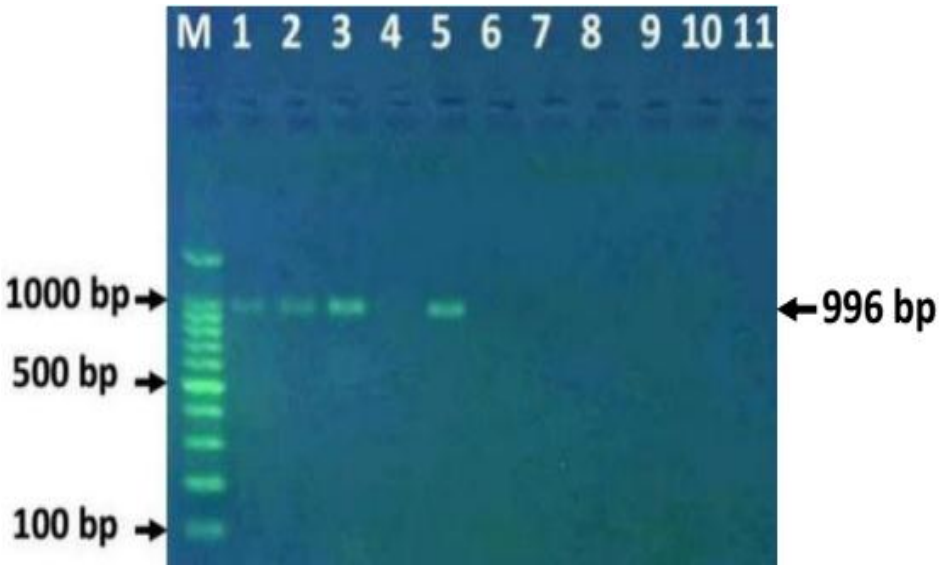

Figure 1: PCR products resolved by agarose gel electrophoresis (1.5% at 5 volts/cm^2 for 60 minutes). Products were amplified with the primer pair U1 (5′-C-C-A-G-C-A-G-C-C-G-C-G-G-T-A-A-T-A-C-G-3′) and U2 (5′-A-T-C-G-G-(C/T)-T-A-C-C-T-T-G-T-T-A-C-G-A-C-T-T-C-3′). Primers were designed to amplify a portion of the 16S rRNA gene in eubacteria (996 bp). Lane M: DNA ladder (100 bp); Lanes 1-3 and 5-6: PCR positive CSF samples; Lanes 4 and 7-10: PCR negative CSF samples; Lane 11: negative control.

A comparison was made between the PCR positive group and the abnormal CSF group with regard to the characteristics given in Table 1. Frequencies of males and females, as well as age groups in the two groups did not show significant variations (p = 0.327 and 0.128,

respectively). However, abnormal CSF samples showed a significantly higher frequency of pleocytosis than ~~in~~ PCR positive samples (52.3 *vs*. 35.4 %; p = 0.038). A similar trend was observed in the case of pleocytosis for neutrophils and lymphocytes but the difference did not reach a significant level. When the NLR was classified into no cell, ≤ 1.0 and > 1.0 subgroups, their frequencies showed no significant differences between PCR positive and abnormal CSF samples (p = 0.156). In the case of protein concentration, frequencies of normal, high and low frequencies in the PCR positive CSF samples were 43.1, 50.8 and 6.2%, respectively. The corresponding frequencies in the abnormal CSF samples were 23.0, 67.8 and 9.2%, respectively. These differences were significant (p = 0.031). With regard to glucose concentration, normal, high and low concentrations had frequencies of 35.9, 35.9 and 28.1%, respectively in the PCR positive CSF samples, while in the abnormal CSF samples, they were 38.6, 28.4, and 33.0%, respectively. However, these differences were not significant (p = 0.602). Antibiotic use was also observed to have some differences between PCR positive CSF samples and abnormal CSF samples (34.8 and 45.5%, respectively), but again the variation did not reach a significant level (p = 0.185) (Table 2).

Table 2: Suspected cases of meningitis stratified according to PCR analysis and characteristics.

Characteristic	PCR positive CSF N = 66		PCR negative CSF; N = 110				*p*-value
			Abnormal [a]; N = 88		Normal; N = 22		
	N	%	N	%	N	%	
Sex							
Male	27	40.9	44	50.0	7	31.8	0.327
Female	39	59.1	44	50.0	15	68.2	
Age group; year							
≤ 2	14	21.2	32	36.4	3	13.6	0.128
3-12	4	6.1	3	3.4	1	4.5	
13-18	5	7.6	4	4.5	3	13.6	
19-39	21	31.8	17	19.3	10	45.5	
40-59	17	25.8	29	33.0	5	22.7	
≥ 60	5	7.6	3	3.4	0	0.0	
Leukocyte count							
Normal	42	64.6	42	47.7	22	100.0	**0.038**
Pleocytosis	23	35.4	46	52.3	0	0.0	
Neutrophil count							
Normal	46	74.2	45	65.2	22	100.0	0.265
Pleocytosis	16	25.8	24	34.8	0	0.0	
Lymphocyte count							
Normal	51	82.3	48	69.6	22	100.0	0.091
Pleocytosis	11	17.7	21	30.4	0	0.0	
NLR							
No cells	26	41.9	18	26.1	11	48.5	0.156
≤ 1.0	24	38.7	33	47.8	9	48.5	
> 1.0	12	19.4	18	26.1	2	3.0	
Protein concentration; mg/dL							
Normal	28	43.1	20	23.0	22	100.0	**0.031**
High	33	50.8	59	67.8	0	0.0	
Low	4	6.2	8	9.2	0	0.0	
Glucose concentration; mg/dL							
Normal	23	35.9	34	38.6	22	100.0	0.602
High	23	35.9	25	28.4	0	0.0	
Low	18	28.1	29	33.0	0	0.0	
Antibiotic medication							
No	43	65.2	48	54.5	16	72.7	0.185
Yes	23	34.8	40	45.5	6	27.3	

3.3 DNA sequence analysis

PCR products of the 66 PCR positive CSF samples were subjected to DNA sequencing, and the sequences were then analysed through alignment with reference sequences in the database of NCBI. The analysis identified Gram-negative (GN) and Gram-positive (GP) bacteria. Forty-five (68.2%) were identified as GN bacteria, while 16 (24.2%) were identified as GP bacteria. Five PCR positive CSF samples (7.6%) were recognized as *Mycobacterium* spp. This bacterium was only found in the CSF of adults who were ~~at the~~ aged ~~of~~ 17 years and older (Table 3).

To understand whether the GN and GP bacteria are influenced by the characteristics given in Table 1, comparisons were conducted regarding these characteristics between the two types of bacteria. In fact, although some differences were found between GN and GP bacteria, no statistical significance was attended (Table 3).

Table 3: PCR-positive meningitis cases classified according to broad types of bacteria and characteristics.

Characteristic	Gram-negative bacteria; N = 45		Gram-positive bacteria; N = 16		*p*-value	*Mycobacterium* spp.; n = 5	
	N	%	N	%		N	%
Sex							
Male	20	44.4	5	31.2	0.357	2	40.0
Female	25	55.6	11	68.8		3	60.0
Age group; year							
≤ 2	12	26.7	2	12.5	0.582	0	0.0
3-12	3	6.7	1	6.3		0	0.0
13-18	4	8.9	0	0.0		1	20.0
19-39	12	26.7	7	43.8		2	40.0
40-59	11	24.4	5	31.3		1	20.0
≥ 60	3	6.7	1	6.3		1	20.0
Leukocyte count							
Normal	31	70.5	9	56.3	0.302	3	60.0
Pleocytosis	13	29.5	7	43.8		2	40.0
Neutrophil count							
Normal	34	81.0	10	66.7	0.258	2	40.0
Pleocytosis	8	19.0	5	33.3		3	60.0
Lymphocyte count							
Normal	37	88.1	11	73.3	0.178	3	60.0
Pleocytosis	5	11.9	4	26.7		2	40.0
NLR							
No cells	19	45.2	5	33.3	0.620	2	40.0
≤ 1.0	16	38.1	6	40.0		2	40.0
> 1.0	7	16.7	4	26.7		1	20.0
Protein concentration; mg/dL							
Normal	23	52.3	5	31.3	0.353	0	0.0
High	19	43.2	10	62.5		4	80.0
Low	2	4.5	1	6.3		1	20.0
Glucose concentration; mg/dL							
Normal	13	30.2	8	50.0	0.353	2	40.0
High	17	39.5	4	25.0		2	40.0
Low	13	30.2	4	25.0		1	20.0
Antibiotic medication							
No	29	64.4	11	68.8	0.756	3	60.0
Yes	16	35.6	5	31.3		2	40.0

NLR: Neutrophil-to-lymphocyte ratio; CSF: Cerebrospinal fluid; *p*: Pearson's Chi-square test probability.

3.4 Bacterial identification

DNA sequence analysis (section 3.3) was extended to identify the types of GN and GP bacteria at the genus or species level. Twenty-three types were identified as GN bacteria and nine types as GP bacteria. *Acinetobacter* spp. and *Pseudomonas* spp. were the most common types of GN bacteria, each with a frequency of 17.8%. Two species of *Pseudomonas* spp. were recognized and included *P. aeruginosa* (seven cases) and one *P. stutzeri*. *E. coli*, *Enterobacter* spp. and *Burkholderia* spp. were the next most frequent types, each with a frequency of 6.7%. Other types of GN bacteria were only found in one or two CSF samples (Table 4).

For GP bacteria, *Staphylococcus* spp. was the most frequent (43.75%) and the next was *S. pneumoniae* (12.50%). The remaining GP bacteria included one type of each of the following: *Lactococcus lactis*, *Bacillus* sp., *Peptoniphilus* sp., *Leuconostoc* sp., *Paenibacillus* sp., *Priestia megaterium* and *Rhodococcus* sp. (Table 4).

Table 4: Gram-negative and Gram-positive bacteria determined by PCR in the CSF of meningitis cases

Gram-negative bacteria; N = 45	N	%	Gram-positive bacteria; N = 16	N	%
***Acinetobacter* spp.**	8	17.8	*Staphylococcus* spp.	7	43.75
Pseudomonas aeruginosa	7	15.6	*Streptococcus pneumoniae*	2	12.50
Escherichia coli	3	6.7	*Bacillus* sp.	1	6.25
***Enterobacter* spp.**	3	6.7	*Lactococcus* lactis	1	6.25
***Burkholderia* spp.**	3	6.7	*Leuconostoc* sp.	1	6.25
***Acidovorax* spp.**	2	4.4	*Peptoniphilus* sp.	1	6.25
***Comamonas* spp.**	2	4.4	*Paenibacillus* sp.	1	6.25
***Neisseria* spp.**	2	4.4	*Priestia megaterium*	1	6.25
***Paracoccus* spp.**	2	4.4	*Rhodococcus* sp.	1	6.25
Alcaligenes faecalis	1	2.2			
***Brevundimonas* sp.**	1	2.2			
Delftia acidovorans	1	2.2			
***Mixta* sp.**	1	2.2			
***Pantoea* sp.**	1	2.2			
Proteus mirabilis	1	2.2			
Pseudomonas stutzeri	1	2.2			
***Ralstonia* sp.**	1	2.2			
Raoultella ornithinolytica	1	2.2			
***Roseomonas* sp.**	1	2.2			
***Serratia* sp.**	1	2.2			
***Sphingomonas* sp.**	1	2.2			
***Pseudomonadales* bacterium**	1	2.2			

3.5 PCR versus culture in the diagnosis of meningitis

Normal and abnormal PCR-negative CSF samples showed no bacterial growth, but what is striking was the PCR positive CSF samples. Only 10 CSF samples (15.2%) out of 66 showed bacterial growth, which included five GN isolates and a similar number of GP isolates. The

GN isolates included one of each of *Pseudomonas* sp. and *E coli*, while three isolates were unclassified GN bacilli, but the PCR analysis identified them as *Acinetobacter* spp. (two isolates) and *Acidovorax* sp. (one isolate). The five GP bacteria were all belong *S. aureus*. However, the current PCR method failed to identify two of these isolates.

4. Discussion

Bacterial meningitis is an important public health threat. Therefore, the adoption of accurate diagnostic methods may contribute to the development of appropriate and effective therapeutic strategies to reach a good outcome [2]. In the current study, 176 SMN cases were studied, and conventional culture methods showed that only 10 of them (5.7%) had BMN. Whereas using a PCR-based method for BMN diagnosis, the infection rate increased to 37.5%. Thus, the laboratory culture of CSF did not diagnose about 30% of suspected cases. Most recent studies agree that molecular methods are more accurate in diagnosing BMN, especially in cases of CSF negative cultures. Failure to diagnose some cases may be due to the use of antibiotics, which may affect bacterial growth [8] [9]. The current study may agree with this view because 39.2% of the cases were under antibiotic treatment at the time of CSF collection.

When applying other criteria in diagnosing BMN, such as counting leukocytes and measuring protein and glucose concentrations, PCR negative CSF samples were divided into two groups. The first group included samples with abnormal values for the three diagnostic criteria, and this group was considered abnormal and included 80% of the samples, while the second group was considered normal and accounted for 20%. This illustrates the importance of the above three criteria in aiding the diagnosis of CSF-MN. In fact, an elevated leukocyte count in the CSF (pleocytosis), increased protein concentration, and decreased glucose concentration are important criteria in diagnosing MN [7]. Regarding pleocytosis, 35.4% of PCR positive CSF samples had elevated cell count, but the increase was greater in abnormal PCR negative CSF samples (52.3%). This indicates that some CSF samples did not show pleocytosis, and although this phenomenon is not common in MN, studies have indicated the absence of pleocytosis in the CSF of some MN cases. A study reviewed 218 MN cases published in 51 studies, and it turned out that 57% of them had no pleocytosis, but when a second LP was conducted for 37 of these cases, the number of cases with no pleocytosis was reduced to only four. Therefore, one LP sample may not be sufficient to diagnose CSF pleocytosis[14]. Another study reached the same conclusion and attributed this to a combination of factors such as the severity and duration of the disease, as well as the CSF inflammatory status [15].

In addition to elevated white blood cell count in the CSF, measurement of protein and glucose concentrations is a further important biochemical parameter in the diagnosis of MN in general and BMN in particular [7]. This study shares this view, and protein concentrations were high in 50.8% of PCR positive CSF samples, but the most important observation is that this elevation in protein concentration was more frequent in PCR negative CSF samples (67.8%). Most studies indicate a high protein concentration ~~of~~ in the CSF of BMN cases, particularly tuberculous meningitis, but the CSF protein concentrations range from normal to moderately elevated in viral or fungal MN[16]. The high concentration of protein in the CSF is suggested to be due to several factors, the most important of which is the change in the permeability of the blood-brain barrier due to BMN, and this allows the entry of proteins. Besides, inflammatory reactions in the CSF may also contribute to the increased CSF protein concentrations [17].

Glucose concentration is the second important biochemical assessment in the diagnosis of MN. The results of the current study showed that only 28.1% of PCR positive CSF samples had lower glucose concentrations, while the percentage was higher in PCR negative CSF samples (33.0%). In general, glucose concentrations in the CSF of patients with MN tend to be low, but some differences appear when the type of infection is considered. It has been shown that glucose concentrations are low in BMN and tuberculous MN, while they range from normal to moderately low or low in viral and fungal MN [16]. Decreased concentrations of glucose in the CSF, also termed hypoglycorrhachia, are mainly due to increased consumption of glucose and this is positively correlated with the abundance of leukocytes in the CSF, as well as inflammatory reactions of the CSF [18]. However, the sensitivity of measuring the CSF glucose concentration remains low or limited in MN diagnosis. It has been observed that there are about 50% of MN cases where the glucose concentrations are normal or high. It has also been pointed out that there are two types of limitations that we must pay ~~an~~ attention in this regard. The first is the tendency for the CSF glucose concentrations to decrease in the CSF if it is not immediately assessed due to ongoing metabolic processes. The second limitation is that the CSF glucose concentration depends on its concentration in the blood, and therefore this concentration may be affected in patients with diabetes [17].

Regardless of the limitations accompanying the aforementioned three diagnostic criteria in MN, it was evident in this study that although some SMN cases showed a negative PCR test, their leukocyte counts, and protein and glucose concentrations were abnormal. This finding suggests that MN in these cases may not be due to a bacterial infection, but that there may be other infectious agents that contributed to the abnormal values of the three assessments in the CSF. In line with this view, it has recently been pointed out that viruses, such as enteroviruses, herpesviruses, and influenza viruses, are also an important cause of MN [19]. In addition, over the past two decades, studies have shown that fungal infections can also cause MN (for example, cryptococcal MN), especially in immunocompromised patients [20]. Other studies have also revealed that both viruses and fungi can affect leukocyte count and protein and glucose concentrations in the CSF [16],[17]. Unfortunately, the present PCR analysis targeted only bacterial DNA and do not diagnose viruses or fungi.

Sequence analysis of DNA-PCR products identified 45 GN and 16 GP types of bacteria. *Acinetobacter* spp. (17.8%) and *P. aeruginosa* (15.6%) were the most frequent GN bacteria, and although they are not a common cause of community-acquired BMN, both types have been indicated to be associated with post-neurosurgical BMN in children and adults. In addition, *Acinetobacter* spp. and *P. aeruginosa* are associated with a high degree of multi-drug resistance and pan-drug-resistance. Further, they are linked to high mortality rates [21]–[23]. The next identified GN bacteria in MN cases were *E. coli* and *Neisseria* with a frequency of 7.6 and 4.4%, respectively, and their role in causing BMN has been indicated [5]. With regard to GP bacteria, the cause of BMN was dominated by *Staphylococcus* spp. (43.75%) and in ~~a~~ second place came *S. pneumoniae* (12.5%). Although the incidence of staphylococcal MN is low (0.3-8.8%) compared to other bacterial causes of MN, an increasing number of cases has been reported in recent years with a global mortality rate of 31-56% [24],[25]. In the case of *S. pneumoniae*, it has been indicated that this bacteria is responsible for 25-41% of BMN in cases of all age groups, and it is also linked to a mortality rate of 16-37%. Importantly, this type of BMN was associated with permanent neurological impairments in 30-52% of patients who survived the disease [26], [27].

Sequence analysis of DNA-PCR products showed an additional important finding. Five cases of tuberculous MN were identified in 7.6% of PCR positive cases and 2.8% of all SMN cases. Tuberculous MN is the most dangerous type of extra-pulmonary tuberculosis, as the associated mortality rate is reported to be 30-40%. The most susceptible group is children, particularly those in the age range of 2-5 years, but adults and the elderly are also at risk [28]. The identified tuberculous MN cases in this study were older than 17 years. Data on the burden of tuberculous MN has not been well presented, but there have been some estimates in populations of low tuberculosis incidence that suggest that tuberculous MN may develop in about 1% of cases with tuberculosis. However, in hospitalized children with tuberculosis, the frequency may increase to 10% [29]. It has been shown that the CSF of tuberculous MN cases manifest high protein and low glucose concentrations with pleocytosis characterized by a predominance of lymphocytes over neutrophils [28]. Low glucose concentrations were observed in 20% of the current tuberculous MN cases. With respect to protein, most cases (80%) showed high concentrations. In addition, pleocytosis was encountered in 40% of cases, but it was more connected to neutrophils (60%) than lymphocytes (40%). However, 40% of tuberculous MN cases showed NLR equal to or less than 1.0. These differences from previous studies may be due to the small size of patients included (only five tuberculous MN cases).

5. Conclusions

The study concluded that BMN presents an important public health challenge, and PCR analysis of the CSF was an effective method for diagnosing pathogenic bacteria in SMN. In addition, leukocytes, glucose, and protein are valuable CSF parameters that may aid in the diagnosis of BMN.

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