Identification of bacterial aetiology in acute meningitis

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Abstract

Background: The lack of rapid and sensitive tests remains a key issue in diagnosing meningitis and affordability impedes using the molecular techniques. However, conventional PCR is currently becoming more affordable.

Objectives: Optimize and establish a multiplex PCR and to compare the above PCR to Cerebrospinal fluid (CSF) culture and antigen detection in sensitivity and specificity for the detection of bacterial meningitis.

Methods: CSF specimens were collected from patients with suspected acute meningitis admitted to Teaching Hospital, Peradeniya from December 2016 to March 2017. A multiplex PCR was used to detect *Neisseria meningitidis, Streptococcus pneumonia* and *Haemophilus influenzae*.

Results: Eighty specimens of CSF were collected during the study period. The mean duration to sample collection was 4.78 (SD 2.6) from the onset of symptoms. None of the samples gave positive CSF culture results. CSF antigen detection was performed on 50 specimens, and all were negative. Of the total samples, eight yielded positive PCR results. In two of the positives, the full report was normal, one was suggestive of viral aetiology and five were suggestive of bacterial aetiology. Three were positive for *S.pneumoniae* and five for *H.influenzae*. Positive PCR results were associated with a shorter time gap between hospitalization and sample collection and a larger CSF volume.

Conclusion: Findings of the study highlight the usefulness and recommendation of multiplex PCR in the diagnosis of pathogens causing acute bacterial meningitis. Collection of an adequate volume of CSF early in the illness, without delay may improve the diagnosis

Introduction

Streptococcus pneumoniae and *Neisseria meningitidis* are identified as the commonest pathogens of bacterial meningitis globally since the start of vaccination against *Haemophilus influenzae* type b (Hib) [1,2]. Introduction of *S.pneumoniae* vaccine into the expanded programme of immunization (EPI) might change the epidemiological pattern in Sri Lanka [3]. Clinical features alone are not specific enough to identify aetiological agents. Gram stain and culture methods are routinely used in aetiological diagnosis of bacterial meningitis. Despite the high specificity of these, the use of antibiotics prior to specimen collection, delay in performing the lumbar puncture (LP), incorrect storage and transport methods, inadequate volume and paucity of trained staff in the laboratory, contribute to false negative results [4].

Nucleic acid-amplification tests, such as polymerase-chain-reaction (PCR), have been assessed for their efficiency in identifying the aetiological agents of meningitis. Despite high sensitivity and specificity, factors such as time of collection of sample, volume, transport condition, storage condition, nucleic acid extraction method and number of times sample can be thawed can affect the results of these tests [5]. With the development of virological diagnostic services in Sri Lanka, molecular detection panels for viral aeitiologies of meningitis are starting to be offered at provincial hospitals. However, routine molecular based diagnostic services are not offered to all hospitals for detecting bacterial aeitiologies of meningitis. Therefore, this study focused on using a multiplex PCR to identify aetiological agents of bacterial meningitis as well as to analyze the CSF parameters of patients with clinical meningitis.

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Methods

This was a laboratory based descriptive study, including patients from medical and paediatric wards of the Teaching Hospital, Peradeniya (THP). All CSF samples sent for culture to the laboratory during a four-month period (1st December 2016 to 31st March 2017) were included in the study. Only the first CSF sample from each patient was included.

Inclusion criteria: All patients aged one year and above who were clinically suspected to have acute meningitis by the treating physician based on the surveillance case definition of the Ministry of Health were included [6].

Exclusion criteria: Children below one year of age, patients who had undergone cranial surgeries or with shunts were excluded from this study.

Assuming an unknown prevalence for meningitis in Sri Lanka, using the nomogram developed by Malhotra and Indrayan in 2010, the minimum sample size needed is approximately 75, assuming a test sensitivity of 80% and a precision of 0.1 [7].

Sample collection

Ethical clearance for the study was taken from the Ethics Review Committee, Faculty of Medicine, University of Peradeniya (No 2016/EC/09). Informed written consent was obtained from the participant or a guardian in the case of children. Eighty consecutive CSF samples were collected. Instructions were given to obtain a sufficient volume of CSF prior to antibiotic treatment into a sterile Bijou bottle. After obtained CSF for routine investigations, the remaining CSF was stored at -80°C.

A CSF volume of > 1 mL was considered as sufficient and < 1 mL was considered as insufficient. Samples which had more than 1 mL of CSF were centrifuged at 1500g for 15 minutes. From the supernatant, minimum 0.5mL were transferred to cryovials and kept at -80°C. From the supernatant a minimum of 0.2mL was transferred to sterile test tubes for antigen testing and heated for 5minutes in a boiling water bath. The samples were brought down to the room temperature (18 to 30°C) and tested by Wellcogen™ test for antigen. Less than 1ml samples were not centrifuged.

In addition to CSF full report, culture, antigen and Gram stain, we tested for CSF antibiotic activity by placing one drop of CSF onto Muller Hinton agar which had been inoculated with *S.aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. After 24 hours incubation at 35°C, we checked the antibiotic activity by measuring the diameter of the zone of inhibition. Presence of any zone of inhibition was taken as indicative of antibiotic activity.

Sample processing

DNA was extracted from *N.meningitidis*, *S.pneumoniae*, and *H.influenzae* positive controls by crude boil lysis. Genomic nucleic acids from samples were extracted using QIAampR Viral RNA minikit (Germany) without using carrier RNA.

Previously published primers were selected based on a CDC protocol [8]. Multiplex PCR mixtures contained 0.5µL of 10µM primer (IDT, USA), 3µL of 25mM MgCl2, 5 µL of x5 buffer, 1µL of 10µM dNTP, 0.4µL of 5u/µLTaq polymerase, 10.6µL PCR water and 2µL template. Singleplex PCR mixtures contained 1.0µL of 10µM primer, 2.5µL of 25mM MgCl2, 5µL of x5 buffer, 1 µL of 10µMdNTP, 0.2µL of 5u/µLTaq polymerase, 12.3µL PCR water and 2µL template.

The optimized PCR run parameters were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 49°C for 40 sec and extension at 72°C for 1 min. Final extension was at 72° C for 10minutes.

Ten microliters of the PCR reaction was loaded onto a 2% agarose gel containing GelRed™ (Biotium, USA).

Results

Demographic and clinical characteristics of the study population

Eighty CSF specimens were collected during this period from patients with suspected meningitis (0.95% of total admissions). Of these, 57 (71.2%) patients were from adult medical wards and 23 (28.8%) patients were from the paediatric ward.

Comorbid conditions among meningitis patients are mentioned in Table 1.

CSF collection

A delay was defined as more than 48 hours taken for the CSF collection from the time of admission and it was observed in 55(68.75%) patients. The mean time between hospital admission and sample collection was 4.78 days (SD 2.6).

Antibiotic treatment was commenced after hospitalization before CSF specimen collection in 48 (60%) patients and among them 47 (97.9%) were treated with β lactam antibiotics. Of the 80 participants, 21 (26.2%) were treated with antibiotics prior to hospital admission.

Sixty six (82.5%) samples had a volume of < 1 mL while sufficient volume was available only in 14 (17.5%) specimens.

CSF analysis and case classification

The CSF full report, Gram stain, culture and PCR were performed on all samples. CSF antibiotic activity was tested in 29 (36.25%) and CSF antigen detection was performed on 50 (62.5 %) samples.

Forty three (53.7%) patients could be classified as having probable bacterial meningitis and 11 (13.7%) patients could be classified as having probable viral meningitis according to the case definitions used (Table 2).

Table 1. Clinical features and comorbid conditions of the study population

Clinical features	*< 12 years (n=23) (28.7%)*	*> 13 years (n=57) (71.3%)*	*All patients (n=80)*
Fever	23 (100%)	52 (91.2%)	75 (93.7%)
Vomiting	20 (86.9%)	39 (68.4%)	59 (73.7%)
Irritability	20 (86.9%)	28 (49.1%)	48 (60%)
Drowsiness	17 (73.9%)	36 (63.1%)	53 (66.2%)
Photophobia	01 (4.3%)	12 (21%)	13 (16.2%)
Muscular pain	05 (21.7%)	35 (61.4%)	40 (50%)
Headache	07 (30.4%)	49 (85.9%)	56 (70%)
Neck stiffness	03 (13%)	27 (47.3%)	30 (37.5%)
Any comorbid condition	00	35 (61.4%)	35(43.7%)
Diabetes mellitus	00	30 (52.6%)	30 (37.5%)
Hypertension	00	31 (54.3%)	31 (38.7%)
Lung diseases	00	01 (1.7%)	01 (1.25%)
Chronic liver diseases (CLD)	00	00	00
Carcinoma	00	00	00
Immunosuppression	00	00	00
Steroid use	00	11 (19.2%)	11 (13.7)
Organ transplant	00	00	00
Connective tissue disorder (CTD)	00	00	00
Peptic ulcer disease (PUD)	00	21 (36.8%)	21 (26.2%)
Hemiplegia	00	10 (17.5%)	10 (12.5%)
Congestive cardiac failure (CCF)	00	00	00

Table 2. Comparison of selected parameters between probable bacterial meningitis and others

Characteristic	*Patients with probable bacterial meningitis (n=43)*	*Others (n=37)*	*P value*
Age	Mean-43.7 (SD 27) Median-49	Mean-31.8 (SD 28) Median-28	
Males	22 (51.2%)	20 (54%)	0.826
Females	21 (48.8%)	17 (46%)	0.826
Delay in CSF sample collection	32 (74.4%)	23 (62.1)	0.334
Antibiotic before LP	30 (69.7%)	18 (48.6%)	0.069
Antibiotic before ad	14 (32.5%),	7 (18.9%)	0.207
Any co morbid	20 (46.5%)	15 (40.5%)	0.655
CSF volume<1mL	37 (86%)	29 (78.3%)	0.394
Hib vaccine	8 (18.6%)	11 (29.7%)	0.297
Antibiotic activity	17 (39.5%)	12 (32.4%)	0.642

Microbiological analysis

Gram stain

None of the CSF specimens demonstrated organisms on Gram stain. Pus cells were seen in 10 (12.5%) samples.

Culture

None of the CSF specimens were culture positive.

Antigen

CSF antigen testing was performed on 50 (62.5%) of the samples. None of the samples gave a positive result for any of the antigens included in the test.

PCR results

Eight of the 80 (10%) samples gave positive results with PCR. Five cases of *Haemophilus influenzae* (6.25%) and 3 of *Streptococcus pneumoniae* (3.75%)were identified. None of the cases were positive for *Neisseria meningitidis.* All positive cases had fever and vomiting (Table 3).

Six of the eight PCR positive samples were from adult medical wards and two were from the paediatric ward. All five positive cases of *H.influenzae* were from the adult age group and two of three cases of *S.pneumoniae* were from the paediatric age group. Out of eight positive cases 6 (75%) were males (Table 4).

Table 3. Summary of investigational data of the 8 positive cases

Clinical and laboratory findings	*Case 1*	*Case 2*	*Case 3*	*Case 4*	*Case 5*	*Case 6*	*Case 7*	*Case 8*
Fever	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Vomiting	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Irritability	Yes	Yes	No	No	Yes	No	Yes	Yes
Drowsiness	Yes	Yes	Yes	No	Yes	Yes	No	Yes
Photophobia	No	No	No	No	No	No	No	Yes
Muscle pain	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Rash	No	No	No	No	No	No	No	No
Neck stiffness	Yes	No	No	No	No	Yes	Yes	Yes
Headache	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CSF Appearance	Clear	Clear	Turbid, Yellow	Clear	Clear	Clear	Clear	Clear
CSF Volume	>1mL	<1mL	>1mL	<1mL	<1mL	>1mL	>1mL	<1mL
Protein	High	Normal	Normal	Normal	Normal	high	high	high
WBC	Normal	Normal	High	Normal	Normal	High	Normal	High
Lymphocyte	100%	100%	75%	100%	100%	80%	100%	32%
PMN	No	No	25%	No	No	20%	No	68%
CSF, blood sugar ratio	0.42	0.49	0.51	0.51	0.51	0.52	0.56	0.63
Culture	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Gram stain	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Antigen	ND	Neg	Neg	ND	Neg	Neg	Neg	Neg
Bacterial PCR	POS-HI	POS-SP	POS-HI	POS-HI	POS-SP	POS-HI	POS-HI	POS-SP

PMNL – Polymorphonuclear leukocytes, Neg – Negative, N.D – Not done, POS – Positive, SP – *Streptococcus pneumoniae* HI – *Haemophilus influenzae.*

Table 4. Comparison between patients with positive PCR results and negative results

Demographic data	*PCR positive (n=8)*	*PCR negative (n=72)*	*P value*
Mean Age	27.0 (SD 20)	39.3 (SD 28)	0.238
Male	6 (75%)	36 (50%)	0.269 (FET)
Female	2 (25%)	36 (50%)	0.269 (FET)
Delay	5 (62.5%)	50 (69.4%)	0.700 (FET)
Time between admission and sample collection (Mean)	2.25 (SD 1.16)	5.04 (SD 2.56)	0.001
Antibiotics given	5 (62.5%)	43 (59.7%)	1.000 (FET)
Hib vaccine given	2 (25%)	17 (23.6%)	1.000 (FET)
CSF apperance Turbid	1 (12.5%)	10 (13.8%)	1.000 (FET)
Protein level>60mg/dl	4 (50%)	28 (38.8%)	0.707 (FET)
WBC count>5	3 (37.5%)	15 (20.8%)	0.370 (FET)
Sugar difference <0.5	2 (25%)	28 (38.8%)	0.703 (FET)
Volume <1mL	4 (50%)	62 (86.1%)	0.028 (FET)
Antibiotic activity	3 (37.5%)	26 (36.1%)	1.000 (FET)

*Delay was defined as CSF sample collected later than 48 hours from admission time. FET – Fisher's Exact Test

Comparison of PCR positive and PCR negative samples

The PCR positive and negative groups were compared in the study parameters assessed. The number of samples with a volume <1mL in the PCR positive group was 4 (50%) while that was 62 (86.1%) in the PCR negative group. The difference was statistically significant (p=0.028, Fisher's Exact test).

The number of samples obtained with a significant delay in the PCR positive group was 5 (62.5%) and mean delay in days is 2.25 while that was 50 (69.4%) in the PCR negative group and mean delay in sample collection was 5.04 days. This difference in mean also was statistically significant (p=0.001).

Number of patients was treated with antibiotic prior to LP in the PCR positive group was 5(62.5%) while that was 43 (59.7%) in the PCR negative group. According to the statistical evaluation, prior antibiotic use did not affect the PCR results significantly (p=1.000, Fisher's Extract test). Likewise antibiotic activity of the CSF also had not influenced the PCR result significantly (p=1.000, Fisher's Extract test).

Discussion

Delay in performing lumbar puncture, antibiotic usage before sample collection, traumatic tap, improper storage delay in transporting the CSF sample, delay in performing the tests and low volume of CSF are known factors affecting conventional methods such as Gram stain and culture [9]. Almost all the patients included in the study were treated based on the CSF full report findings as conventional microbiological tests were negative. Similar findings were observed in a study done by Ranawaka *et al* (2013) and the importance of the molecular methods has been clearly mentioned in this paper [10].

CSF full report-based categorization

Among the 43 samples which had CSF full reports compatible with probable bacterial meningitis, the aetiology was not identified in any of them by conventional testing while four were found to be positive for *H. influenzae* and one was positive for *S.pneumoniae* by PCR. Accordingly, even with PCR aetiological agents responsible could not be identified for the majority of the specimens with CSF full reports indicative of probable bacterial meningitis. A study conducted by Welinder *et al* (2007) using a conventional multiplex PCR was able to identify the responsible agent in 51 CSF specimens out of total of 74 specimens which had features of probable bacterial aetiology. In a study done by Khater and Elabd (2016) in Egypt using 40 culture negative CSF specimens which had a probable bacterial aetiology according to CSF full report, the aetiology of 90% of the samples was identified as *S.pneumoniae* using a Real-Time PCR [11]. When compared with the above studies, PCR positivity in this study is much lower. Among 11 CSF specimens which had CSF full reports suggestive of probable viral

aetiologies, one was positive for *H. influenzae.* According to IDSA 2004 guideline CSF full report shoud be used neither diagnosis nor treatment [12]. Two CSF specimens with normal CSF findings were also positive for *S. pneumoniae.* Uchihara *et al.*, (1996) has reported a culture positive *S.pneumoniae* meningitis case with normal CSF findings [13]. A case report has been published in 2011 by Montassier *et al* also demonstrating the possibility of having a normal initial CSF full report while having *S. pneumoniae* meningitis [14]. A study done by Thomas *et al.*, in 2008 has shown that only 7% of CSF studies were abnormal in paediatric cases of bacterial meningitis [15].

Conventional diagnostics

Though the CSF full report in 53.7% was suggestive of a bacterial aetiology, all conventional methods were negative for an aetiology in these samples. In our study 21 (26.2%) patients were given antibiotics prior to admission and 48 (60%) were given antibiotics prior to sample collection while in the ward. We also found antibiotic activity in 29 (36.2%) samples of CSF. These findings highlight the main contributing factor for the negative culture reports. Reasons for the administration of antibiotics prior to sample collection need to be identified and addressed where feasible. Other studies conducted in Sri Lanka in 2009, 2013 and 2017 were also not able to find the aetiological agent in suspected cases of acute bacterial meningitis [16, 10, 17]. Though there is conflicting evidence about the use of CSF antigen detection tests, multiple studies conducted recently have revealed that CSF antigen detection offers no advantage over existing routine diagnostics [18, 19]. We found that LP was delayed in 68.75% of the sample while 60.0% has been pretreated with antibiotics prior to LP. More importantly, in 82.5%, the CSF sample was highly inadequate. In our study the time taken for transport of the sample to the laboratory after LP was not documented. Delay and transport conditions can also significantly affect the results of CSF tests [20].

PCR results

In our study, the only laboratory test which was able to identify aetiological agents in at least some cases of meningitis was the multiplex PCR which targeted three main pathogens. Bacterial nucleic acid was identified in 10% (8/80) of specimens. The sensitivity and specificity of the bacterial PCR was not calculated due to the lack of a comparator. In 53.7% of CSF, the full report was suggestive of bacterial aetiology, but the aetiology was identified only in 5 specimens even by PCR. In most previous studies a higher PCR positivity rate has been identified [21,22]. It is likely that lower volumes of CSF and delay in LP are related to the negative PCR results. We have measured the antibiotic activity in the CSF. Twenty nine (36.2%) samples had in vitro antibiotic activity. Out of that 3 (10%) had positive PCR. There was no association between the presence of CSF antibacterial activity and PCR results. Further, three (10.3%) patients in whom antibiotic activity was noted in the CSF did not give a history of taking antibiotics at the hospital or before admission. Therefore, this test may be detecting the presence of other antibacterial substances in the CSF, not only antibiotics. The method used to detect antibacterial activity was not a standardized method and needs to be standardized. We failed to identify any PCR positive cases of *N.meningitidis.* The commonest bacterial aetiology identified in our study was *H.influenzae*. Due to vaccination, invasive *H.influenzae* infection is currently mainly seen in adults and is predominantly caused by non typable *H.influenzae* strains [23]. Furthermore, the primers used for *H.influenzae* does not differentiate Hib and similar sequences are seen in closely related organisms (*Aggregatibacter aphrophilus, Haemophilus haemolyticus* and *Bibersteinia trehalosi*) as demonstrated by a Primer BLAST search. This is a limitation of the study and the results need to be confirmed with specific primers. In our study only 10% of the total population had confirmed meningitis. Actual situation of meningitis with regard to its aetiological agent can only be tested in a multicenter study using adequate CSF specimens which are collected early in the disease. So developing a conventional multiplex PCR including all these primers or developing a real time PCR may be useful.

Limitations

Our study had some limitations. We were not able to control the confounders in the CSF study such as delay, prior antibiotic use and volume. Thus, all conventional results were negative and failed to calculate the sensitivity and specificity of multiplex PCR. Due to the limited time frame, unavailability of nearby laboratories with PCR facilities and high cost we did not perform external validation of the PCR. These steps need to be conducted before validation of this method for routine use.

Virus agents had been identified as a common cause for encephalitis/meningoencephalitis [16, 24]. However, identification of the viral etiology had not been done because of limited funding. In addition, this is a single centered study; therefore, epidemiologically it cannot be applied to the other part of the country.

Conclusion and Recommendations

Finding of the study highlights the usefulness and recommendation of multiplex PCR in the diagnosis of pathogens causing acute meningitis. Formulating protocols for timing of lumbar puncture and sample collection and implementing strict antibiotic policies may improve the aetiological diagnosis of meningitis. Our study showed the importance of timing of LP and volume of CSF in diagnosis. Collection of a proper volume of CSF (minimum of 1mL and preferably 3mL) as soon as possible prior to antibiotics needs to be highlighted. Identification

of five *H.influenzae* and three *S.pneumoniae* isolates may have implications on the vaccination policy. However, we need to re-confirm and type the *H.influenzae* positives prior to making a concrete conclusion.

Author contributions

DB – Conception of study, acquisition of funding, sample collection, data base maintenance, data analysis, drafting and finalizing paper

VL – Study design, design and trouble-shooting of molecular lab work, data curation, data analysis, finalizing of the paper

ND – Overall study supervision, conception of study, study design, data analysis, approval of final paper

PH – Sample collection, molecular lab work, molecular biology data interpretation, approval of final paper

JP – Sample collection, non-molecular biology lab work coordination, data collection and approval of final paper

Conflicts of interest approval (for original articles)

VL has received research funding from industry for a different study. Others declare no conflicts of interest.

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NA

Ethics approval

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Patient consent (for case reports where the material is identifiable)

Informed written consent was taken.

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Abbreviations (where relevant)

CDC – Center for disease control and prevention

CSF – Cerebro-spinal fluid

EPI – Expanded programme of immunization

FET – Fisher's Exact test

HiB – *Haemophilus influenzae* type B

HI – *Haemophilus influenzae*

LP – Lumbar puncture

ND – Not done

Neg – Negative

PCR – Polymerase Chain reaction

PMLN – Polymorphonuclear leukocytes

POS – Positive

SD – Standard deviation

SP – *Streptococcus pneumoniae*

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