Original Research Article

Evaluation of Real-Time Polymerase Chain Reaction in Culture-Negative Cerebrospinal Fluid Samples of Bacterial meningitis Patients

Abstract

Background. Meningitis is a rigorous childhood disease with high morbidity and mortality. It is the main cause of under five mortality in India. Mainly three bacteria *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* are responsible. In low economic set up country like India, documented bacterial meningitis mainly depend on gram staining, cerebrospinal fluid (CSF) culture results or latex agglutination test resulting in less number of positive due to the prior antimicrobial intake which affects culture and latex agglutination test results. This study was taken up rapid and accurate molecular method like RT PCR to diagnose bacterial meningitis in culture-negative CSF samples..

Materials and methods. Fifty culture-negative CSF samples from suspected cases of bacterial meningitis were examined by real-time polymerase chain reaction (real-time PCR) for the presence of lytA, bexA, and ctrA genes specific for *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* respectively.

Results. Positive real-time PCR results for *Streptococcus pneumoniae* were detected in 36 (72%) of culture-negative CSF samples while 10% positive results for *Haemophilus influenzae* type b. Nine (18%) samples were negative by real-time PCR for all tested organisms.

Conclusion. The use of molecular techniques as real-time PCR can provide a valuable addition to the proportion of diagnosed cases of bacterial meningitis especially in settings with high rates of culture-negative results.

Key words: RT PCR, CSF, Bacterial meningitis, Pediatrics

Introduction

Bacterial meningitis is being one of the deadliest childhood communicable diseases having serious neurologic complications[1]. The incidence of meningitis is usually high in developing countries, with poor-socioeconomic status[2]. *Streptococcus pneumoniae, Neisseria meningitidis* and *Haemophilus influenzae* type b account for most encountered bacteria of meningitis[3,4]. Though the implementation immunization strategies using conjugate polysaccharide vaccines changes in the epidemiology of the diseases, but developing countries are lagging behind the universal vaccination program [5,6]. Therefore, prompt diagnosis, treatment, and prevention mandating for better management of bacterial meningitis. Among the different standardized methods for the diagnosis of bacterial meningitis culture and gram staining are still considered the gold standard[7,8]. But it is matter of fact that culture techniques lack sensitivity particularly when the patient is pretreated with antibiotics before lumbar puncture is done and the causative organisms are very fragile making it more difficult to isolate. Similarly sensitivities of gram-staining vary considerably for different microorganisms[2]. Some studies also reported a low sensitivity of latex agglutination test, especially in pretreated patients with antibiotics before lumbar puncture.

With regards to non yield of bacteria in the traditional method used now-a-days molecular methods facilitates prompt diagnosis especially in culture-negative situations[4,8]. This study intended to diagnose bacterial meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* type b (Hib) in culture-negative CSF samples by the aid of real-time PCR.

Materials and Methods

This was a cross sectional study undertaken in a pediatric tertiary care hospital from April 2014 to March 2015 and ethical approval was taken from Institutional Ethical Committee of Regional Medical Research Centre, Odisha, India. The study comprised a total of 50 CSF samples collected from SVPPGIP, Cuttack, Odisha with clinical diagnosed bacterial meningitis.

The purpose of the study was explained to the parent or guardian of the child and informed consent was taken before enrolling them in the study. A written consent was obtained

from the guardian of the patients. Suspected meningitis included children of both sexes below 5 years of age with the history of sudden onset of fever more than 38.5°C rectal or more than 38.0°C axillary and the presence of one or more of the following such as neck stiffness, altered consciousness, meningeal sign[9].

The collected CSF samples were centrifuged at 10000 rpm for 10 min and the supernatant was immediately processed for cytological and biochemical evaluation. Samples were put into aerobic and anaerobic bacterial culture within 1 hour of sample collection. Based on the turbidity of CSF and WBC count more than 100 cell/ml or 10-100 cells with glucose less than 40 mg/dl or protein less than 100mg/dl regarded as the probable case and confirmed case of meningitis was defined as all probable case where CSF demonstrated incriminating pathogen either by Latex test, Culture or RT PCR Test (WHO, 2011).

RT PCR of the CSF Sample

Bacterial DNA was extracted from CSF samples with the aid of Roche Life Science DNA Mini kit (Roche Life Science, India) as per the manufacturer's protocol for DNA purification. The eluted DNA was stored at -20^0C until further processed.

Real-Time PCR with SYBR Green I:

Three runs were sequentially performed for the detection of each organism separately. The ctrA gene of *Neisseria meningitidis*, bexA gene of *Haemophilus influenzae*, and lytA gene of *Streptococcus pneumoniae* [WHO, 2011] were used as species-specific targets (Table 1). The mix for each run included 5 µL of sample DNA, 12.5µL of 2X Quanti Tect SYBR Green PCR master mix (Roche Life Science, India) containing a buffer, dNTP mix, $MgCl_2$ and Hot Stat Taq DNA polymerase, 1 µl of the primer, and RNase-free water for a final volume of 25µl. DNA was amplified with the Step One Real-Time PCR system (Applied Biosystems) by using the following temperature program: an initial Hotstart Taq activation step at 95^0C for 15 min, initial denaturing at 95^0C. for 15 seconds and 40 PCR cycles of denaturing at 95^0C for 30 seconds, annealing at 50^0C for 30 seconds, and extension at 72^0C for 30 seconds followed by melting curve stage of 95^0C and 60^0C. Amplification data were analyzed by instrument software in terms of melting curve graphs of each sample. Positive control strains and negative controls consisting of PCR grade water instead of the target DNA were used in each run.

Results

The present study was carried out in 50 culture-negative CSF samples of suspected meningitis cases in the pediatric age group of 0-5 years. The samples were withdrawn from 35 (70%) males and 15 (30%) females. Demographic data of the patients along with biochemical and cytological findings of the CSF samples are presented in Table 2.

CSF biochemistry revealed elevated protein level (>100 mg/dL) in 82% of the samples and decreased glucose level (<40 mg/dL) in 76% suggesting probable cases of meningitis but on CSF culture there was no growth. White blood cell (WBC) count >1000 cells/mm^3 was found in 42.5 % of the samples whereas 57.5% of the samples showed WBC count between 100 -1000 cells/mm^3. High neutrophil (> 50%) was found in 72.5% of the samples.

36 samples (72%) were positive for *Streptococcus pneumoniae* by real-time PCR and 5 (10%) were positive for *Haemophilus influenzae* type b where as there was no detection of *Nesseria meningitidis* in any of the samples. 9 samples (12%) were negative for all three organisms (Table 3).

Discussion

Prompt and precise diagnostics play crucial role in diseases diagnosis and implementing treatment and preventive measures. Conventional methods such as gram staining and culture though regarded as the gold standard but certain situations demand application of highly sensitive and accuracy in owing to the delay in results availability[10] and in conditions where prior antimicrobial therapy has been implemented. There was evidence of high percentage of culture-negative samples in the pediatric age group previously[11]. This may be because of fastidious nature of the organisms, vaccine implementation against these organisms or antibiotic treatment prior to lumber puncture. The CSF becomes sterilized within 4 hours of parenteral antibiotic treatment in case of pneumococcal meningitis[12]. Males (70%) were more significantly affected with bacterial meningitis than females (30%) though the disease was distributed in all age groups.

Real-time PCR was positive for *Streptococcus pneumoniae* in 36 culture-negative CSF samples (72%) while 10% positive results for *Haemophilus influenzae* type b. The age group of the patients in this study might have contributed to these results as the main causative agents of bacterial meningitis are generally believed to be *Streptococcus pneumoniae*. Real time PCR analysis found more sensitive than culture where the positivity rate is 59%. RT-PCR targets

specific genes and does not require a viable pathogen[13]. Wang et al. also identified bacterial meningitis in five cases (9%) by CSF cultures and 25 (45%) by real-time PCR[14]. They considered real-time PCR much more sensitive than culture for the diagnosis of bacterial meningitis particularly in their study where 68% of patients had received prior antimicrobial treatment and their CSF samples yielded negative culture results. The RT-PCR method offers several other advantages, including the provision of results within hours, high throughput, and high sensitivity and specificity[13]. In contrary its high cost remains a constraint for developing countries which impelling Gram's staining and CSF culture still be the diagnostic method for bacterial meningitis.

This study has its own limitation with the relatively low number of CSF samples analyzed with lack of testing for other common bacteria such as Group B Streptococcus, *E. coli*. According to our findings, we conclude that the use of molecular technique in the diagnosis of bacterial meningitis should be considered in suspected cases with negative culture results before reporting exclusion of the disease.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table 1: Primers used in RT PCR for detection of *S. pneumoniae*, HiB and *Nesseria meningitidis*

Oligonucleotide	**Sequence**
ctrA forward	5′-TGTGTTCCGCTATACGCCATT-3′
ctrA reverse	5′-GCCATATTCACACGATATACC-3′
bexA forward	5′-TGCGGTAGTGTTAGAAAATGGTATTATG-3′
bexA reverse	5′-GGACAAACATCACAAGCGGTTA-3′
lytA forward	5′-ACGCAATCTAGCAGATGAAGCA-3′
lytA reverse	5′-TCGTGCGTTTTAATTCCAGCT-3′

Table 2: Demographic, cytological and biochemical parameters of subject enrolled

Parameters	**Percentage**
Age	22.65 ± 13.0 month
Sex	
Male	35(70%)
Female	15(30%)
Protein (mg/dl)	121.28±3.4
>100	82%
<100	18%
Glucose (mg/dl)	30.46±1.35
>40	34%
<40	76%
WBC (total count/mm^3)	682.±36.16
100-1000	57.5%
>1000	42.5%

Table 3: RT PCR analysis (N=50)

PCR	Number	Percentage
Negative	9	18%
Positive		
S. pneumoniae	36	72%
HiB	5	10%

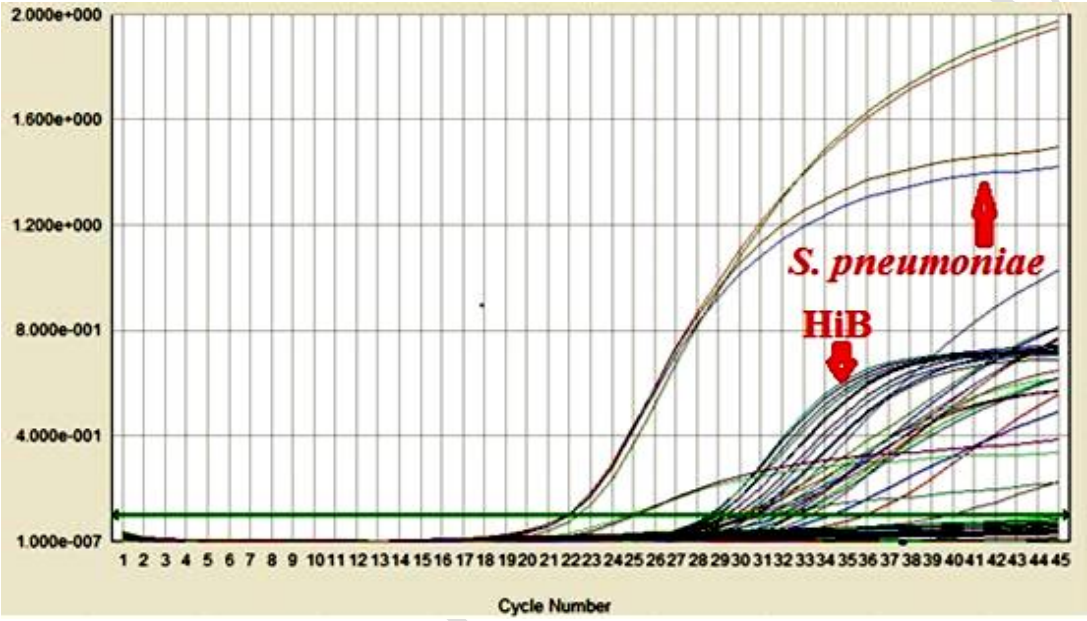

Fig 1: ***Streptococcus pneumoniae*** **and HiB as the causative organism in RT PCR.**