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Simultaneous Detection of Bacterial Meningitis in Suspected Cases of Meningitis in Children Using PCR Assay, In Taif, Saudi Arabia

Nour M and Alaidarous A

1 Department of Biology, High Institute of Biotechnology, Monastir University, Tunisia
2 Department of Biology, Faculty of Science, Taif University, Saudi Arabia

Corresponding author: Nour M

mohamednour2805@yahoo.fr

Department of Biology, Faculty of Science, Taif University, Taif –Al-Haweiah-21974, Saudi Arabia.

Tel: +(966)557115241
Fax: +966(012)7274299

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Abstract

Background: Bacterial meningitis (BM) is a medical emergency in children. Delay in diagnosis and treatment remain major concerns in the management of BM. The aim of this study was the use of multiplex PCR (mPCR) for simultaneous detection of *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, and *S. agalactiae* in suspected patient cerebrospinal fluid (CSF).

Methods and findings: 712 CSF specimens were collected from the patients of age <5 years having clinical features suggestive of meningitis. 300 samples were randomly taken from the set of CSF specimens whose culture was negative and analyzed by mPCR. The positive bacterial growth was obtained from only 2 (0.2%) CSF samples. The pathogens identified were *Salmonella sp* and *Citrobacter freundii*. PCR analysis of the randomly selected 300 CSF specimens revealed the following pathogens: *S. pneumoniae* (n=7), *H. influenzae* (n=3) and *N. meningitidis* (n=2). No *S. agalactiae* isolate was detected in the CSF specimens. Serogrouping of *S. pneumoniae* isolates that 4 strains belong to the serogroup 19A and three strains to the serogroup 23F. For *N. meningitidis*, serogroup prediction showed that the 2 isolates detected belong to serogroup A.

Conclusions: Our study emphasizes the importance of PCR as the most common method that is sufficiently accurate and reliable, and it should be included for bacterial detection in all negative cultures. It also reveals that these four bacterial meningitis agents are still an important cause of children meningitis.

Keywords: Bacterial meningitis; Children; Identification; PCR

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Introduction

Bacterial meningitis (BM) is among the most common neurological disorders in children. It represents a major cause of death and disability worldwide, especially in neonates and young infants [1]. Neurological complications are common in children, who survive BM, with 25% of children in developing countries suffering long-term neurological sequelae [2]. The etiologic agents of meningitis and their antibiotic susceptibility vary from place to place and, therefore, knowledge of the locally predominant organisms in different age groups and their sensitivity pattern is essential to minimize adverse outcomes [3].

Before the introduction of vaccines, 90% of reported cases of BM in infants and children, were caused by *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, or *Neisseria meningitidis* [4,5].

Vaccines have reduced mortality and morbidity due to invasive pneumococcal and Hib diseases. The success of these two vaccines did that *N. meningitidis* take the leader cause of bacterial meningitis [6,7]. The vast majority of *N. meningitidis* invasive disease is related to six meningococcal serogroups: A, B, C, W-135, X and Y for review, [8]. The epidemiology of these serogroups varies by geographic region. The serogroup W-135 has emerged in some parts of the world, primarily in the Saudi Arabian (KSA) and has been associated with small outbreaks in Europe due to pilgrims who returned from Hajj [9].

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Various diagnostic assays exist for diagnosing BM; therefore, in developing countries, the standard methods for the laboratory diagnosis of BM are Gram staining of the cerebrospinal fluid (CSF) and bacterial culture. However, these approaches may have some limitations with regard to the relatively low rapidity and sensitivity and a delay in the diagnosis and treatment of BM could lead to irreversible brain damage. It was confirmed by a recent study, which showed that mortality was increased by 13% for every hour without antibiotics [10].

The clinical signs and symptoms of BM vary according to several factors: age, duration of evolution of symptoms at the time of clinical examination, presence of underlying diseases and microorganism involved [11]. These signs have only 40-50% sensitivity in BM diagnosis [12]. Although, it is recognized that bacterial meningitis can be present in a child with an apparently normal CSF initially [13]. In addition, the most common manifestation of BM in childhood is insidious with nonspecific symptoms that progress over 2-5 days before meningitis is diagnosable [14]. Hence, as soon as there is a clinical suspicion of meningitis, lumbar puncture is imperative.

In regard to Saudi Arabia, there have been some published studies regarding meningitis during the Hajj and Umrah [15] but, to our knowledge, there were no previous studies on the detection of *N. meningitidis*, *S. pneumoniae*, *H. influenzae* type b, and *S. agalactiae* in CSF specimens of children. Consequently, a greater understanding of the current epidemiology of BM in children is needed. For this reason, we investigated the frequencies of these four bacterial pathogens in CSF specimens collected from children, hospitalized in Taïf peadiatric hospital with suspected BM, using mPCR assay.

Materials and Methods

Study sites

This study was conducted from January to December 2016. Samples were collected at Taif children's hospital, KSA. Request of verbal consent to participate in this study, clinical examination, lumbar puncture and the fill of the BM case investigation form were performed by the pediatricians attending the admitted child.

Study design and case definition

In this study, a pediatrician performed 712 lumbar punctures on children under 5 years old who were suspected of having BM. Since the pathogens responsible for neonatal meningitis are different from older patients [16], neonates (patients <1 month of age) were not included in the study. According to WHO guidelines, suspected case of ABM is defined as a child aged <5 years with sudden onset of fever (>38.5°C rectal or 38.0°C axillary) and at least one of the following signs: neck stiffness or flaccid neck, bulging fontanel, convulsion, irritability, or drowsiness.

Sample collection and investigation form

CSF samples were collected in sterile plastic bottles from suspected meningitis patients and submitted to the microbiology laboratory. The macroscopic appearances of the samples were noted and CSF specimen was inoculated in MacConkey agar (MA), blood agar (BA) and chocolate agar (CA) plates. MA and BA plates were incubated overnight at 37°C aerobically and the CA plates were incubated up to 24 hours at 37°C in 5% CO_2 atmosphere. The bacterial growth obtained was examined for colonies as well as Gram staining characteristics and identification was done following standard microbiological methods. Antibiotic susceptibility testing, of the isolates, was conducted by determination of Minimum Inhibitory Concentrations (MICs). The results were interpreted according to Clinical and Laboratory Standards Institute guidelines [17].

DNA Extraction

All CSF samples collected were kept at −80°C and were thawed immediately before each test. Two hundred microliters of CSF samples were used for DNA extraction. Nucleic acid extraction was accomplished by Qiagen-Kit according to the manufacturer's instructions. DNA was purified using Qiagen-Kit and eluted in 50 µl of appropriated buffer and stored at −20°C until use. Five to seven microliter of the purified DNA solution was used as a template for PCR.

Detection of bacterial meningitis by mPCR

For the simultaneous identification of bacterial agents, a conventional multiplex PCR assay was performed. The specific gene targets, the primer sets and mPCR conditions were as previously described [18-20]. The PCR products were visualized on 2% agarose gels stained with ethidium bromide. Negative controls consisting of PCR grade water instead of the target DNA were used in each assay as well as positive controls of reference strains (*S. pneumoniae* ATCC 6305, *H. influenzae* ATCC 10211, *N. meningitidis* ATCC 13090 and *S. agalactiae* ATCC 2759).

Determination of *S. pneumoniae* and *N. meningitidis* serogroup by PCR

S. pneumoniae serotyping was performed using primers and sequential multiplex PCR (SM-PCR) as previously described [21-23]. For *N. meningitides*, serogroup prediction was performed and applied as previously reported [9,24]. All serogroups were distinguished on the sizes of the expected amplicons which were analyzed by electrophoresis on standard 1.5% agarose gels and visualized using UV fluorescence.

Results

During one year (2016) study period, 712 CSFs were collected from suspected meningitis patients: 469 males (65.87%) and 243 females (34.12%). The boy-to-girl ratio was 1.93:1. The positive bacterial growth was obtained from 2 CSF specimens. The pathogens identified were *Salmonella* spp and *Citrobacter freundii*.

The *Salmonella* spp strain was sensitive to almost all antibiotics used in the Taif children's hospital except amikacin, gentamicin, cephalothin, cefoxitin, and ceferoxim; whereas *Citrobacter freundii* was sensitive to all cephalosporins tested and resistant only to aminoglycosides, carbapenems and fluroquinolones.

Multiplex PCR (mPCR) was used to detect and identify, simultaneously in 300 enrolled CSF out of 712, the following bacterial pathogens: *N. meningitidis*, *S. pneumoniae, H. influenzae* type b, and *S. agalactiae*. Of the 300 CSF negative cultures 300 samples were randomly taken from the set of 710 specimens whose culture were was negative and they were analyzed by PCR. Subjected to mPCR, 12 samples yielded a positive signal and 288 samples failed to yield detectable amplification signal. The proportion of CSF samples with etiologic agent identified by mPCR is 4%. In 102 CSF specimens (34%) out of 300, their biochemical analyses of CSF were in favor of meningitis. Among the 102 samples, only 12 (11.76%) had positive PCR and 90 patients (88.24%) had negative PCR. Pathogens identified were: *S. pneumoniae* (n=7), *H. influenzae* (n= 3) and *N meningitidis* (n=2). *S. agalactiae* (group B streptococci) was not detected by PCR assay. Serogrouping for *S. pneumoniae* using SM-PCR revealed that the 7 strains detected were typable and their corresponding serotype was identified: 4 strains belong to the serogroup 19A and 3 strains belong to the serogroup 23F. For *N. meningitides*, serogroup prediction revealed that the 2 *N. meningitidis* strains identifed belong to serogroup A.

Discussion

CSF culture based method is considered the diagnostic reference standard for bacterial meningitis and bacteriological profile of CSF facilitates the choice of antibiotic. In this study, CSF cultures of 712 cases of suspected acute bacterial meningitis were done. The positive results were detected in 0.3% of CSF specimens. This results is much lower than in previously reported data [3,18,25,26]. This failure to isolate bacteria by CSF culture and the risk of false negative result were high because either only a small number of microorganisms may be present in the specimens or the low quality of CSF samples and the use of antibiotics prior to the lumbar puncture. It might be due also to differences in hospital practices with respect to infection control, difference in methodology, patient characteristics and spectrum of bacterial pathogens [20,27]. Hereby, biochemical analyzes support meningitis in 102 out of 300 samples (whose culture was negative) however bacterial meningitis were detected, by PCR, only in 12 cases. Our results are in agreement with those reported by Moayedi and colleagues [28]. In 30% of cases, the biochemical analyses of CSF were in favor of meningitis, only 6% had positive universal PCR. Using real-time PCR (qPCR), Mihrel et al. [29] detected an etiologic agent for the meningitis in 33.1% of CSF samples. In similar study, the diagnosis of BM was detected by PCR in 7.14% patients [18].

In fact, the count of leucocytes is not always indicative because meningitis can occur in the absence of the increased number of polymorphonuclears [30]. Moreover, in aseptic meningitis, there are clinical signs of meningitis and inflammatory changes in cerebrospinal fluid along with negative cultures prior to antibiotics administration [31]. The overall low rates of etiologic agent identification may be explained by interpreting meningitis symptoms and CSF turbidity as well as delay in transporting samples to the laboratory. The usual signs and symptoms do not provide optimal sensitivity and specificity for distinguishing possible BM from viral meningitis. In addition, more and more unreasonable and irregular applications of antibiotics have increased the atypical features of BM, which made it difficult to seek pathogenic evidence [25].

PCR might be necessary to detect the causative organisms and allowed to identify the etiological agent in most cases of BM in culture-negative samples [8]. However, the CSF pathogen detection rate might be low and it is difficult to find evidence of cause. In our study, 288 patients had the clinical manifestations of meningitis. However, no evidence of the bacterial detection was revealed by PCR, or bacterial culture.

The failure of PCR in some CSF specimens could be due to the presence of a substance inhibitory for PCR and/or to the small amount of bacterial pathogens DNA in these specific CSF. Also, this result is likely owing to infection by other BM-causing bacteria such as *Escherichia coli* and other Gram-negative enteric bacilli. These reported data are consistent with our findings in the present investigation. We identified, by culture, *Salmonella sp* and *Citrobacter freundii* in two CSF samples. It appears also that other Gram-positive cocci may cause BM in these patients [31]. In the present study we did not detect *S. agalactiae* in CSF neither by culture nor by PCR. In older studies, *S. agalactiae* was the most frequently identified pathogen from cases of young infant bacterial meningitis, followed in incidence by other microorganisms, including *Escherichia (E.) coli* and *Listeria (L.) monocytogenes* [32]. In a 2014, *S. agalactiae* was the most common cause of meningitis, in UK and Ireland [33]. This result contrasts with a 2014 study in California, where *E. coli* remained the most frequently identified pathogen in meningitis.

The *S. pneumoniae* serogroup 19A identified in the present study is covered by the pneumococcal conjugate vaccine (PCV) 13 and not by PCV7, whereas the 23F was covered by PCV7, PCV10 and by PCV13. In regard to KSA, data on meningitis and serotype distribution of *S. pneumoniae* are scarce, and with the exception of one study accomplished by Shibl et al. [34], no other study has yet been conducted. Taking into account the size and geographical and social diversity of KSA, data from our study are difficult to generalize to the entire country. In this regard, it is likely that serotype distribution and antimicrobial sensitivity of *S. pneumoniae* may vary in different regions of KSA. It is noteworthy that the first introduction of the PCV7, in KSA, was in 2006. Its introduction into the national immunization program was in 2008, and the switch to PCV13 was in 2010.

The efficiency of vaccines has greatly reduced *H. influenzae* and *S. pneumoniae* meningitis. This led *N. meningitidis* to be the first bacterial agent for meningitis in many countries. Vaccines are available against strains of *N. meningitidis* belonging to serogroups A, C, Y, and W135 but not against strains of serogroup B [For review, 8]. Hence, serogrouping is necessary to better apply preventive measures. *N. meningitides* serogroup prediction revealed that the 2 *N. meningitidis* isolates belong to serogroup A. This later serogroup accounts for 80–85% of all outbreaks [35]. Our results were in agreement with previously reported data [36]. In fact, Saudi Arabia has historically been associated with outbreaks of *N. meningitidis* serogroup A. The religious

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seasons of Hajj and Umra have historically been associated with epidemics of meningococcal disease. The epidemiology and distribution of these disease-causing serogroups varies widely by geographic region [8]. Especially, serogroup W-135 has emerged in many countries, primarily in the Middle East and Africa causing large epidemics and has been associated with small outbreaks in Europe due to pilgrims returning from Hajj [9].

PCR assay allows the identification of the pathogens in less than 2 hours. It is the most common method that is sufficiently accurate and reliable, especially when there is a history of antimicrobial drug use before lumbar puncture. PCR should be included for bacterial detection in all negative cultures. It also shows that these four bacterial meningitis agents are still an important cause of children meningitis.

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