SIMULTANEOUS DETECTION OF *NEISSERIA MENINGITIDIS, HAEMOPHILUS INFLUENZAE* AND *STREPTOCOCCUS* SP. BY POLYMERASE CHAIN REACTION FOR THE DIAGNOSIS OF BACTERIAL MENINGITIS

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ABSTRACT - The simultaneous detection of *Neisseria meningitidis, Haemophilus influenzae*, and *Streptococcus sp.* was assessed by polymerase chain reaction (PCR) for the diagnosis of bacterial meningitis, as well as the applicability of PCR as a routine test. A cohort study was carried out with 182 children (2 months to 12 years of age) with suspicion of bacterial meningitis. Routine tests identified the etiologic agent in 65/84 children whose clinical status and laboratory findings suggested the presence of bacterial meningitis. Bacterial meningitis was ruled out in 98 children. In 19 children, the etiologic diagnosis was not possible using standard methods; in 14 of these patients, the etiologic agent was identified by PCR (*N. meningitidis*=12; *H. influenzae*=1; *Streptococcus sp.*=1). The sensitivity of PCR was 88.1%; specificity, 99.0%; positive predictive value, 98.7%; and negative predictive, 90.1%. PCR is a useful complementary diagnostic technique, especially when Gram stain, culture, or antigenic detection are negative or inconclusive.

KEY WORDS: polymerase chain reaction (PCR), bacterial meningitis, cerebrospinal fluid (CSF).

Detecção simultânea da *Neisseria meningitidis, Haemophilus influenzae* e *Streptococcus* sp. pela reação em cadeia da polimerase no diagnóstico das meningites bacterianas

RESUMO - Avaliamos o desempenho da reação em cadeia da polimerase (PCR) para detecção simultânea da *Neisseria meningitidis, Haemophilus influenzae* e *Streptococcus sp.* no diagnóstico das meningites bacterianas e sua aplicabilidade na rotina diagnóstica. Foi realizado um estudo de coorte com 182 crianças apresentando suspeita de meningite bacteriana. Em 84, havia alterações clínicas e laboratoriais sugestivas de meningite bacteriana. Destas, 65 tiveram o agente etiológico identificado pelos métodos laboratoriais de rotina e 19 ficaram sem diagnóstico etiológico. Em 98 pacientes foi excluído o diagnóstico de meningite bacteriana. Analisando o desempenho da PCR encontramos sensibilidade de 88,1%, especificidade de 99,0% e valores preditivos positivo e negativo de 98,7% e 90,1% respectivamente. Nos 19 pacientes com meningite bacteriana mas sem diagnóstico etiológico a PCR detectou microrganismos em 14, sendo 12 *N. meningitidis,* um *H. influenzae* e um *Streptococcus sp*. A PCR possui o potencial de poder aumentar os índices de identificação das técnicas tradicionais, principalmente nas situações onde a microscopia direta, cultura ou identificação antigênica são negativos ou inconclusivos.

PALAVRAS-CHAVE: reação em cadeia da polimerase (PCR), meningite bacteriana, líquido cefalorraquidiano (LCR).

Acute bacterial meningitis is responsible for most infections affecting the central nervous system (CNS). Close to two-thirds of all cases occur among children, and Haemophilus influenzae, Neisseria meningitidis, and Streptococcus pneumoniae are the pathogens responsible for 80 to 90% of the cases[1,2]. A rapid and precise etiologic diagnosis of bacterial meningitis is essential to determine adequate treatment, and it can also significantly reduce mortality and the risk for long-term sequelae[3]. In epidemiological terms, the diagnosis of bacterial meningitis may indicate the need for selective immu-

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nization of a community, such as by chemoprevention and vaccination of the contacts of patients carrying meningococcal disease[4].

Among the routine tests used for the etiologic diagnosis of bacterial meningitis, bacterial culture is considered the gold standard. However, this is a time-consuming technique, which requires viable microorganisms for cultivation, and its sensitivity is directly affected by the start of antibiotic treatment before spinal tap[5]. Other faster tests, such as latex agglutination, have a lower sensitivity and present reliable results only with samples containing more than 105 CFU/mL[6]. Currently, molecular biology techniques, such as PCR, are being widely used for the etiologic diagnosis of CNS infections[7]. In 1991, Kristiansen et al. detected the presence of N. meningitidis DNA in the culture-negative cerebrospinal fluid (CSF) specimen of a patient with meningococcal disease after she had received antibiotic treatment[8]. In 1992, Ni et al. studied the use of PCR in diagnosing meningococcal meningitis; results indicated specificity and sensitivity of 91%. Neither sensitivity nor specificity were affected by previous antibiotic treatment[9]. In 1994, Radstrom et al. described a nested PCR strategy for the simultaneous detection of *Neisseria meningitidis, Haemophilus influenzae, and Streptococcus sp.*[10] in CSF. That study showed that it is possible to simultaneously and efficiently amplify the three main pathogens that cause bacterial meningitis. However, it also revealed certain difficulties associated with the technique, such as false-positive results due to contamination, and false-negative results due to the presence of PCR inhibitors in CSF, which still compromise diagnostic reliability[10-13]. Additional studies correlating clinical and laboratory findings to PCR results are essential to confirm the diagnostic value of PCR.

The present study was carried out in order to assess the simultaneous detection of *Neisseria meningitidis, Haemophilus influenzae,* and *Streptococcus sp.* by PCR for the diagnosis of bacterial meningitis. The study also evaluated the applicability of PCR as a routine laboratory test.

METHOD

A cohort study was carried out with children between 2 months and 12 years of age, with suspicion of bacterial meningitis at three public hospitals in the city of Porto Alegre, State of Rio Grande do Sul, Brazil: Hospital de Clínicas de Porto Alegre, Hospital da Criança Santo Antônio, and Hospital da Criança Conceição. Most pediatric cases in the region are treated at these hospitals.

The study was submitted to and approved by the Research Ethics Committees of the participating hospitals. Children with suspected bacterial meningitis were included in our study after an informed consent form was signed by a parent or guardian. A 0.5 mL aliquot of CSF was separated during spinal tap or after the CSF was received in the laboratory. Specimens were stored at - 20° C until PCR was performed.

The signs and symptoms of bacterial meningitis vary depending on the age group. In preschool and school age children, the predominant symptoms include meningeal irritation and increase of intracranial pressure expressed as neck stiffness (Kernig's and Brudzinski's signs). In younger children, these symptoms may be absent, with predominance of unspecific signs of infection. In these cases, meningitis was considered in the presence of fever, lethargy, irritability and sleepiness, especially if associated with signs of meningeal irritation or increased intracranial pressure.

Alterations in CSF, such as leukocytosis with predominance of polymorphonuclears, CSF glucose concentration below 40 mg/dL and increased CSF protein (100-500 mg/dL) are considered to be suggestive of bacterial meningitis. Diagnostic confirmation is based on the identification of the etiological agent through direct microscopy (Gram), culture, latex agglutination or counterimmunoelectrophoresis. Leukocytosis with predominance of lymphocytes, normal or increased CSF glucose and increased proteins suggest viral meningitis.

Clinical history and results of physical examination were reviewed. Patients were followed until discharge from the hospital. The results of routine CSF analysis (differential cell count; glucose; protein; Gram stain; bacterial culture; and latex agglutination) were obtained from the medical chart during hospitalization, or directly from the laboratory. Counterimmunoelectrophoresis was carried out at a state-run laboratory (Laboratório Central do Estado do Rio Grande do Sul); these results were obtained from the State Department of Health.

In the present study, the gold standard for diagnosing bacterial meningitis included clinical signs and symptoms (as previously described), course of disease (antibiotic therapy, length of admission, neurological sequelae), and routine laboratory results (CSF analysis: differential cell count; glucose; protein; Gram stain; bacterial culture; latex agglutination and counterimmunoelectrophoresis). According to these criteria, patients were divided into three groups:

Group I – Clinical indication of bacterial meningitis, positive laboratory results (leukocytosis with predominance of polymorphonuclears, low CSF glucose concentration and increased CSF protein), and etiologic agent diagnosed by routine methods (i.e. Gram stain, bacterial culture, latex agglutination, and counterimmunoelectrophoresis);
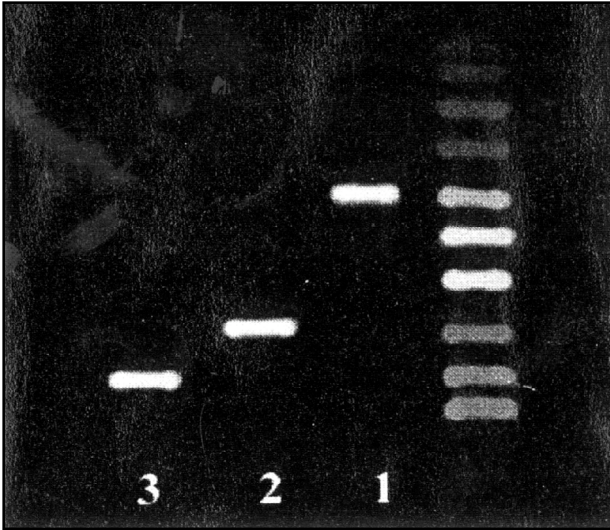
Fig. Electrophoresis of PCR amplification products in agarose gel (2%). Results are compared to molecular weight markers and DNA sequences of Streptococcus sp. (1), H. influenzae (2), and N. meningitidis (3).

Group II – Clinical indication of bacterial meningitis, positive laboratory results, and unsuccessful diagnosis of etiologic agent by routine methods;

Group III – Clinical indication of bacterial meningitis, diagnosis of bacterial meningitis ruled out by course of disease, and negative laboratory results.

PCR sensitivity, specificity, and predictive values were calculated by comparing the results for groups I+II versus group III. It is important to stress that the diagnosis of meningitis and the distribution in the groups was independent, i.e., blinded for the result of PCR.

We employed the seminested multiplex PCR technique for the simultaneous detection of N. meningitidis, H. influenzae, and Streptococcus sp. A region of bacterial gene 16S rRNA was amplified. Species-specific primers were used along with primers complementary to universal regions U3 and U8 of gene 16S rRNA, in a two-stage PCR assay. In the first stage, amplification was performed using external universal primers (u3, ru8). A generic bacterial amplicon was generated, not specific to the type of bacterium present in the specimen. The second stage combined three specific primers - one for each type of bacterium - with the ru8 universal primer, generating species-specific amplicons with different molecular weights.

In the first stage, the PCR reaction contained $(NH_4)_2SO_4$ (16 mM); Tris-HCl (67 mM; pH 8.8 at 25° C); 0.01 % Tween-20 (w/v); MgCl2 (1.5 mM); triphosphate deoxyribonucleoside mixture (0.25 mM); external primers (at 0.1 μM) synthesized according to previously published sequences[10]; and polymerase DNA enzyme (0.625 U). During this first step, 10 μL of CSF were added. DNA extraction from hemorrhagic or xanthochromic CSF specimens, and from specimens with high leukocyte concentration (causing turbidi-

ty) was carried out using the QIAamp kit (70 μL CSF specimen). Other CSF specimens were submitted to PCR directly, without any treatment. The final reaction volume was 50 μL. Amplification was done on a 9600 Perkin Elmer thermocycler. The initial denaturation period was of 1 minute and 40 seconds at 94°C, followed by 33 incubation cycles (20 seconds each) at 94° C, 60° C and 72° C.

The second amplification stage employed species-specific primers and the universal ru8 primer in a mixture identical to that of the first stage. The final reaction volume was 25 μL, and we used 2 μL from the first reaction as specimen. Denaturation was performed for 45 seconds at 94° C, followed by 33 (15-second cycles) at 94° C, 15 seconds of annealing at 55° C, and 20 seconds of polymerization at 72° C. Some procedures were followed to avoid contamination, such as using aerosol-resistant tips, working in four distinct environments, including negative controls, and submitting the reagent mixture to UV radiation (254 nm) for 15 minutes before adding CSF. All positive specimens were retested for confirmation of the results.

A 10 μL specimen from the second PCR reaction was run in an ethidium bromide-stained 2% agarose gel. Amplification products were identified following electrophoresis. Specimens were visualized using UV transillumination. Specific N. meningitidis (700 bp), H. influenzae (500 bp), and Streptococcus sp. (300 bp) bands were compared to the respective controls or to molecular weight markers (Figure).

Measurements of PCR performance were expressed as percentages with their respective 95% confidence intervals (95% CI). Confidence intervals were calculated by binomial distribution. Due to the asymmetric distribution of the data, the Kruskal-Wallis test was used to analyze the comparisons between groups I, II and III. The nonparametric multiple comparison test proposed by Dunn was used to assess differences between the groups.

RESULTS

During the 18 months of the study, we analyzed 182 patients with suspected bacterial meningitis. In 65 patients, the diagnosis of bacterial meningitis was confirmed by identification of the etiologic agent using routine laboratory tests (group I). Nineteen patients presented clinical and laboratory indication of bacterial meningitis without identification of the etiologic agent by standard tests (group II). In the remaining 98 patients, the course of the disease and laboratory results ruled out a diagnosis of bacterial meningitis (group III). The clinical and laboratory characteristics of the three groups are presented in Table 1.

Among the 65 patients from group I, 29 cases (44.6%) were caused by H. influenzae, 21 (32.3%) by N. meningitidis, and 15 (23.1%) by Streptococcus sp. PCR identified the correct pathogen in 60 of the

Table 1. Clinical and laboratory characteristics of groups I, II, and III.

Variable	Group I (n=65)	Group II (n=19)	Group III (n=98)	p
Age (months)	12[a] (5-36)	45[b] (12-84)	48[b] (24-84)	< 0.001
Duration of hospital stay (days)	11[a] (10-16)	10[a] (10-11)	2[b] (2-3)	< 0.001
% of patients receiving antibiotics before spinal tap	14[a,b] (21.5%)	8[a] (42.1%)	13[b] (13.3%)	0.012
CSF leukocytes/mm^3	1320[a] (597-3400)	1200[a] (700-11700)	313[b] (85-604)	<0.001
% neutrophils	88[a] (77-93)	90[a] (81-93)	15[b] (4-64)	<0.001
CSF glucose (mg/dL)	13[a] (2-55)	48[a] (1-62)	59[b] (53-70)	<0.001
CSF protein (mg/dL)	200[a] (120-280)	150[a] (88-190)	43[b] (32-61)	<0.001

All values are expressed as medians (interquartile range), except for percentage of patients taking antibiotics. Superscript letters indicate statistically significant differences.

Table 2. Sensitivity of routine laboratory tests for the etiologic diagnosis of patients with bacterial meningitis.

Method	No. tests	Sensitivity (groups I and III)	CI 95%	No. tests	Sensitivity (groups I, II, and III)	CI 95%
PCR	65	92.3%	82.2-97.1	84	88.1%	78.8–93.8
Gram stain	65	81.5%	70.0-90.1	84	63.3%	52.4–72.9
Culture	65	81.5%	70.0-90.1	84	63.3%	54.4–72.9
Latex agglutination	40	75.0%	58.8-87.3	50	60.0%	46.0–72.8
CI*	36	52.8%	35.5-69.6	46	41.3%	27.8–55.9

*counterimmunoelectrophoresis.

65 cases. However, PCR did not identify the correct pathogen in three cases of N. meningitidis and in two cases of Streptococcus sp. Among the 98 patients from group III, PCR was negative in 97 CSF specimens. There was one false-positive result indicating *N. meningitidis* DNA.

Among the 19 patients from group II, PCR identified pathogens in 14 specimens. Twelve specimens contained *N. meningitidis* DNA, one specimen contained *H. influenzae* DNA, and one specimen contained *Streptococcus sp.* DNA. In group II, 42.1% of the patients had started antibiotic treatment before spinal tap, compared to 21.5% in group I and 13.3% in group III.

The sensitivity of PCR in groups I and III was 92.3% (95% CI: 82.2-97.1), and the specificity was 99.0% (95% CI: 93.6-99.9). The positive predictive value was 98.4% (95% CI: 90.0-99.9), and the negative predictive value was 95.1% (95% CI: 88.4-98.2). When group II patients were included in the assessment of PCR performance, sensitivity dropped to 88.1% (95% CI: 78.8-93.8), and the negative predictive value was reduced to 90.1% (95% CI: 83.1-95.2). Nevertheless, both the specificity and the positive predictive value remained practically the same: 99.0% (95% CI: 93.6-99.0) and 98.7% (95% CI: 91.8-99.9), respectively.

When assessing groups I and III, there was no statistically significant difference between sensitivity of PCR, Gram stain, culture, and latex agglutination. When group II patients were included, the sensitivity of PCR was significantly higher than that of the other tests (Table 2).

DISCUSSION

In general, the number of cases of bacterial meningitis whose etiologic agent is not identified is still significant. Several factors interfere with the recovery of microorganisms from CSF, such as previous antibiotic treatment, inadequate collection and storage of culture specimens, and reduced number of bacteria in CSF. Tests for detecting bacterial antigens, such as latex agglutination and counterimmunoelectrophoresis, may assist in the diagnosis of bacterial meningitis. However, although these tests are fast and not affected by previous antibiotic treatment, their sensitivity is limited. The use of molecular biology techniques, such as PCR, for the etiologic diagnosis of CNS infections is under study. It is expected that such techniques will have a higher sensitivity and specificity for diagnosing meningitis.

We assessed the accuracy of the seminested PCR technique for the simultaneous detection of the three main pathogens which cause bacterial meningitis: *N. meningitidis*, *H. influenzae*, and *Streptococcus sp*. Due to the absence of an absolute gold standard, in the present study clinical status, course of disease, CSF analysis, and identification of etiologic agent by routine tests (Gram stain, culture, latex agglutination, and counterimmunoelectrophoresis) were established as the gold standard for the diagnosis of bacterial meningitis. Since the etiologic agent is not identified in many patients (especially in those receiving antibiotics before spinal tap), we later decided to consider another gold

standard, i.e., the clinical and laboratory diagnosis of bacterial meningitis, regardless of etiologic identification.

When the etiologic diagnosis of bacterial meningitis was used as the gold standard, group II patients (clinical and laboratory indication of bacterial meningitis; unsuccessful etiologic identification by routine tests) were excluded from the calculation of PCR accuracy, and consequently these results may be overestimated. The inclusion of patients with clinical and laboratory indication of bacterial meningitis whose etiologic identification proved unsuccessful through routine laboratory tests reveals a PCR accuracy that is closer to what would be expected if this assay were used routinely for the diagnosis of bacterial meningitis; there is a statistically nonsignificant drop in sensitivity and negative predictive value, whereas specificity and positive predictive value remain practically unchanged.

PCR was the only test that was capable of identifying the etiologic agent in 14 patients from group II. In 63% of these patients, *N. meningitidis* DNA was detected in CSF. The notion that over half of the bacterial meningitis cases without etiologic identification were caused by *N. meningitidis* is important both clinically and epidemiologically, and allows a more adequate management of patients and contacts. The fact that antibiotic use before spinal tap was more frequent in group II underscores the importance of PCR in the identification of the etiologic agent in meningitis.

PCR did not identify pathogens in five cases of bacterial meningitis with etiologic diagnosis by routine tests, a fact that points to the presence of PCR inhibitors in CSF. PCR inhibitors had been detected in some hemorrhagic, xanthochromic, and high leukocyte-concentration CSF specimens, and DNA extraction techniques were used in an attempt to remove the inhibitors. In these five specimens, there had been no CSF alterations indicating the need for extraction, and, therefore, CSF was directly submitted to PCR. At the end of the study, these specimens were submitted to DNA extraction and resubmitted to PCR. After extraction, PCR identified pathogens in three of the five specimens with previously negative results. This suggests that purification and concentration (70 µL) of bacterial DNA in CSF using extraction techniques may improve the sensitivity of the method. Extraction techniques should be applied to all CSF specimens, regardless of their macroscopic aspect. While the detection of PCR inhibitors could indicate the need

for an internal control, the use of a multiplex reaction could lead to difficulties in the interpretation of results.

The high specificity and the positive predictive values found in our study indicate that the probability of false-positive PCR results is low, and that PCR detection of pathogens is a strong indicator of bacterial meningitis. In turn, the sensitivity and negative predictive values found in the present study suggest that a diagnosis of bacterial meningitis should not be ruled out when PCR results are negative. Our results suggest that PCR may enhance the diagnostic accuracy of traditional techniques, especially when Gram stain, culture, and antigenic detection tests are negative or inconclusive.

Molecular biology techniques, such as PCR, have a wide range of applications for diagnosis. However, before incorporating PCR into a diagnostic routine, it is necessary to standardize this procedure, to establish strict laboratory quality control measures, and to carry out further studies aimed at establishing PCR accuracy. Finally, PCR should not replace standard tests, rather, it should contribute to more precise and rapid diagnoses.

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