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Comparison of PCR-based methods for the simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in clinical samples

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ABSTRACT

Background: Several in-house PCR-based assays have been described for the detection of bacterial meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* from clinical samples. PCR-based methods targeting different bacterial genes are frequently used by different laboratories worldwide, but no standard method has ever been established. The aim of our study was to compare different in-house and a commercial PCR-based tests for the detection of bacterial pathogens causing meningitis and invasive disease in humans.

Methods: A total of 110 isolates and 134 clinical samples (99 cerebrospinal fluid and 35 blood samples) collected from suspected cases of invasive disease were analyzed. Specific sets of primers frequently used for PCR-diagnosis of the three pathogens were used and compared with the results achieved using the multiplex approach described here. Several different gene targets were used for each microorganism, namely *ctrA*, *crgA* and *nspA* for *N. meningitidis*, *ply* for *S. pneumoniae*, *P6* and *bexA* for *H. influenzae*.

Results: All used methods were fast, specific and sensitive, while some of the targets used for the in-house PCR assay detected lower concentrations of genomic DNA than the commercial method. An additional PCR reaction is described for the differentiation of capsulated and non-capsulated *H. influenzae* strains, the while commercial method only detects capsulated strains.

Conclusions: The in-house PCR methods here compared showed to be rapid, sensitive, highly specific, and cheaper than commercial methods. The in-house PCR methods could be easily adopted by public laboratories of developing countries for diagnostic purposes. The best results were achieved using primers targeting the genes *nspA*, *ply*, and *P6* which were able to detect the lowest DNA concentrations for each specific target.

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Introduction

Infections caused by *Neisseria meningitidis* (Nm), *Streptococcus pneumoniae* (Sp) and *Haemophilus influenzae* (Hi) are responsible for high morbidity and mortality rates among children and adults in many countries each year.[1,2] Despite effective antimicrobial and supportive therapy, mortality rates among children remain high, with significant long-term sequelae in survivors. Although adequate treatment requires rapid detection and identification of the bacteria, traditional laboratory diagnostic methods such as culture for the identification of bacterial meningitis pathogens may take 36 h or more. Because of the similarity of clinical symptoms among invasive infections caused by Nm, Sp and Hi, physicians must frequently request laboratories to test the clinical sample for these three microorganisms to determine the causative bacteria and decide on the management of the case. Rapid diagnosis of the etiological agent is also important for health surveillance, in cases of invasive disease, to avoid transmission to close contacts.[1–3]

To address this problem, nonculture methods like PCR have become available during the last two decades, providing early and accurate diagnosis of bacterial meningitis.[1,4–10] Because of its rapid, sensitive and specific results, PCR is an important tool to improve the diagnostic of such microorganisms. An additional advantage of PCR over conventional laboratory methods is the possibility to detect genomic DNA from clinical samples without the need of previous culture. Recently, the use of a quantitative PCR method called Real Time-PCR, have improved the diagnosis of infectious agents based on molecular methods.[11–17] However, the higher cost of reagents and equipment could be a possible drawback to the implementation of this method in public laboratories of developing countries. The use of a basic PCR method, can be still more appropriate for diagnostic purposes in low-budget laboratories. A number of different target genes have been used in several PCR-based assays to specifically detect the genomic DNA of these agents. For *N. meningitidis nspA*, *ctrA* and *crgA*, for *H. influenzae bexA* and P6, and for *S. pneumoniae ply* and *lytA* genes have been used as targets.[4–10,18,19] In order to determine the best combination of primers, we have developed a simple multiplex PCR for the simultaneous detection of the three main agents of meningitis worldwide, Nm, Sp, and Hi. The multiplex PCR method here described allows the detection of the three microorganisms in a single-tube reaction, saving time and reagents. Target species specific genes used for Nm, Hi, and Sp detection were *nspA*, *P6*, and *ply* respectively. Primers amplifying higher length fragments were chosen for identification because the visualization of bands below 200 bp through electrophoresis may not be clear depending of the agarose gel type and concentration used. Furthermore, we have compared the specificity and sensitivity of our method to other in-house and commercial PCR-based methods previously described for simultaneous or single detection of Nm, Hi, and Sp from clinical samples.[4,5,7–10,18,19]

Materials and methods

Bacterial isolates, clinical samples, and culture conditions

We have analyzed a total of 110 strains isolated from clinical samples (*N. meningitidis* $n = 31$, *H. influenzae* $n = 29$, *S. pneumoniae* $n = 50$) which are part of the Bacterial Culture Collection of the INCQS/FIOCRUZ institute. The strains were isolated during the last five years, from clinical samples of cerebrospinal fluid (CSF) and blood. These samples have been collected from culture-confirmed cases of patients with suspected meningitis in Brazilian public hospitals. The isolates were recovered from clinical samples after inoculation in chocolate agar (blood agar base with 5% sterile defribinated rabbit blood at 56 °C) and incubated at 37 °C in a 5% CO_2 environment. The isolated strains were identified as Nm, Hi, or Sp by colony morphology, latex agglutination of specific antisera (Slidex Meningitis – Biomerieux), Gram staining and optochin susceptibility, the latter only for Sp determination. Each species was confirmed by specific PCR tests[8,18,19] freeze-dried and incorporated into the culture collection.

We have also analyzed 134 clinical samples (99 CSF and 35 blood) collected from suspected cases of invasive disease. Thirty four (25.3%) of these samples were considered positive for one of the three pathogens targeted in this study by conventional laboratory tests (culture, microscopy, and latex agglutination) and 100 samples were considered negative after conventional laboratory tests, but collected from patients with suspected symptoms or epidemiological link to invasive disease caused by one of the three agents here described. The study has been approved by the local research ethics committee.

Twenty nine reference strains were used as positive controls as follows: *N. meningitidis* serogroup A (ATCC 13077), *N. meningitidis* serogroup B (ATCC 13090), *N. meningitidis* serogroup C (ATCC 13102), *N. meningitidis* serogroup W135 (ATCC 35559), *H. influenzae* (ATCC 33391), *H. influenzae aegyptius* (ATCC 11116), *H. influenzae* NT (ATCC 49247), *H. influenzae* serotype a (ATCC 9006), *H. influenzae* serotype b (ATCC 33533) *H. influenzae* serotype c (ATCC 9007), *H. influenzae* serotype d (ATCC 9008), *H. influenzae* serotype e (ATCC 8142), *H. influenzae* serotype f (ATCC 9833), *S. pneumoniae* (ATCC 33400), *S. pneumoniae* serotype 14 (ATCC 6314), *S. pneumoniae* serotype 3 (ATCC 6303), *S. pneumoniae* serotype 33 (ATCC 8333), *S. pneumoniae* serotype 41 (ATCC 10341), *S. pneumoniae* serotype 51 (ATCC 10351), *S. pneumoniae* serotype 19F (ATCC 49619), *S. pneumoniae* serotype 61 (ATCC 10361), *S. pneumoniae* serotype 19A (ATCC 700673), *S. pneumoniae* serotype 14 (ATCC 700672), *S. pneumoniae* serotype 9V (ATCC 700671), *S. pneumoniae* serotype 6B (ATCC 700670), *S. pneumoniae* serotype 4 (ATCC BAA-334), *S. pneumoniae* serotype 6A (ATCC BAA-659), *S. pneumoniae* serotype 5 (ATCC BAA-341). Other 9 reference strains were also used as negative controls as they can also cause bacterial meningitis in humans: *Neisseria lactamica* (ATCC 23970), *Neisseria subflava* (ATCC 11076), *Moraxella catarrhalis* (ATCC 25238), *Streptococcus agalactiae* (ATCC 13813), *Streptococcus pyogenes* (ATCC 19615), *Klebsiella pneumoniae* (ATCC 13883), *Listeria*

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