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VIEWS AND REVIEWS

Evolution of bacterial meningitis diagnosis in São Paulo State-Brazil and future challenges

Evolução do diagnóstico das meningites bacterianas no Estado de São Paulo-Brasil e os futuros desafios

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ABSTRACT

Bacterial meningitis (BM) is a severe disease and still represents a serious public health problem with high rates of morbidity and mortality. The most common cases of BM around the world, mainly in Brazil, have been caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* type b. Bacterial culture is the gold-standard technique for BM confirmation, but approximately 50% of suspected cases are not culture-confirmed, due to problems related to improper transportation and seeding or previous antibiotic treatment. Immunological methods present low sensitivity and have possibility of cross-reactions. Real time PCR (qPCR) is a molecular technique and has been successful used for BM diagnosis at Instituto Adolfo Lutz in São Paulo State, Brazil, since 2007. The incorporation of qPCR in the Public Health surveillance routine in our state resulted in diminishing 50% of undetermined BM cases. Our efforts are focused on qPCR implementation in the BM diagnostic routine throughout Brazil.

Keywords: Bacterial meningitis, molecular diagnosis, real time PCR.

RESUMO

A meningite bacteriana (MB) é uma doença grave e ainda representa um sério problema de saúde pública, com altas taxas de morbidade e mortalidade. Os casos mais comuns de MB em todo o mundo, principalmente no Brasil, tem sido causados por *Neisseria meningitidis*, *Streptococcus pneumoniae* e *Haemophilus influenzae* tipo b. Cultura bacteriana é a técnica padrão-ouro para a confirmação de MB, mas cerca de 50% dos casos suspeitos não são confirmados por cultura, devido a problemas relacionados ao transporte inadequado e semeadura ou antibioticoterapia prévia. Métodos imunológicos apresentam baixa sensibilidade e têm possibilidade de reações cruzadas. PCR em tempo real (qPCR) é uma técnica molecular e tem sido utilizada com êxito para o diagnóstico de MB no Instituto Adolfo Lutz, em São Paulo, Brasil, desde 2007. A incorporação da qPCR na rotina de vigilância em Saúde Pública em nosso estado resultou na diminuição de 50% dos casos de MB indeterminadas. Nossos esforços estão focados na implementação da qPCR na rotina diagnóstica de MB em todo o Brasil.

Palavras-Chave: Meningite bacteriana, diagnóstico molecular, PCR em tempo real.

Bacterial meningitis (BM) is a severe disease and still represents a serious public health problem with high rates of morbidity and mortality[1]. Since 1980´s, the most common cases of BM in the United States, Europe and many other developed countries have been caused by ***Neisseria meningitidis*** (Nm), ***Streptococcus pneumoniae*** (Spn), ***Haemophilus influenzae*** type b (Hib), group B ***Streptococcus***, and ***Listeria monocytogenes***[2].

In Brazil the main pathogens involved in BM are Nm, Spn and Hib, which are responsible for approximately 90% of all BM cases[3]. Other bacteria can also cause BM with a lower frequency when compared with Nm and Spn, but with high lethality, as ***Staphylococcus aureus*** (Sa)[4]. In our laboratory at Instituto Adolfo Lutz (IAL) we detect 1,3% of positivity due to Sa among 1214 analyzed cases, demonstrating that these bacterial specie does not show a high frequency in patients attended by the IAL[5].

After the introduction of the Hib vaccine in the Brazilian official calendar in 1999 there was more than 90% reduction in number of meningitis cases by this agent. Reinforcing this fact, IAL laboratory data show that the national percentage of isolation of Hib strains decreased 88% after the introduction of Hib vaccine in Brazil[3,6]. Additionally, there was a reduction in the number of Hib isolates in the post-vaccination (2000-2008) compared to the pre-vaccination (1990-1999) period, i.e., 98% of the Hi strains serotyped by IAL during pre-vaccine period were serotype b while only 59% were Hib in the post-vaccine. Furthermore, there was an increase from 1% to 19%

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isolation of other Hi serotypes and from 2% to 22% of Hi non-typeable (Hi-nt)[7].

In Brazil, the 10-valent pneumococcal conjugated vaccine was introduced since March 2010. According to PORTAL DA SAÚDE (2013)[8], after the inclusion of pneumococcal 10-valent conjugate vaccine on the Brazilian official vaccination calendar, in 2011, there was a 30% reduction in the number of cases of pneumococcal meningitis in children less than two years-old. After the introduction of the conjugated meningococcal vaccine against serogroup C in the Brazilian official calendar, the number of cases of meningococcal disease among children below two years decreased 29%[8].

These data demonstrate the importance of proper diagnosis and surveillance in the post-vaccine era, in order to observe the real impact of the vaccine, the changing epidemiology of circulating strains and vaccine failures.

Here we review the current status of BM diagnosis in Brazil and the future challenges to introduce the molecular diagnosis throughout the Brazil.

Laboratorial diagnosis

The laboratory diagnosis of BM is accomplished through the cerebrospinal fluid (CSF), blood and petechial lesions scraping. The main tests for the confirmation of suspected cases, in Brazil, are:

- CSF chemocytological examination;
- CSF direct bacterioscopy;
- culture (CSF, blood, or petechiae);
- counterimmunoelectrophoresis (CSF and serum);
- latex agglutination (CSF and serum);
- Polymerase Chain Reaction (PCR).

The appearance of the CSF acts as an indicator. The normal CSF should be clear and colorless, like “rock water”. In infectious processes, there is an increase of formed elements (cells), causing a cloudy appearance, whose intensity varies with the amount and type of these elements[9].

1. CSF chemocytological examination

The test allows for the cell count and serum levels of glucose and protein in CSF, revealing the intensity of the infection process. The test only guides the clinical suspicion, but should not be used for diagnostic conclusion, because its low specificity[9].

2. CSF direct bacterioscopy

The Gram stain technique allows characterizing bacteria presence in the CSF sample based on its morphology and coloring, but with a low degree of specificity. The technique can be performed on CSF and other normally sterile body fluids, and material collected from petechial scraping. The chemocytological and bacterioscopy examination must be conducted in the shortest possible time in order to avoid cell and bacterial deterioration[9].

3. Latex agglutination test (CSF and serum)

The test uses latex particles sensitized with monoclonal or polyclonal antibodies specific for a particular antigen, and can be performed in CSF, serum and other biological fluids. Agglutinated latex particles form agglomerates in the presence of soluble antigen released from bacteria, visible to the naked eye[10]. The sensitivity of latex agglutination test is from 90% to Hi, 94.4% for Spn, and 80% for Nm and specificity of the assay is 97%[9].

4. Culture (CSF, blood, or petechiae)

Culture has a high degree of specificity in relation to identification of the etiologic agent and can be performed with various types of body fluids, most commonly the CSF and blood[11].

The isolation of the etiologic agent by culture is essential for epidemiological surveillance, being considered “gold standard”, and allows the final characterization of the agent (antigenic, genetic, and antimicrobial resistance)[12]. However, approximately 50% of suspected cases are not culture-confirmed, due to problems related to improper transportation and seeding or previous antibiotic treatment. Molecular diagnosis can be useful in situations where the antibiotic treatment has been initiated, because it does not require a viable organism to confirm the diagnosis[13,14].

5. Counterimmunoelectrophoresis (CSF and serum)

Widely used in Brazil, counterimmunoelectrophoresis (CIE) was standardized in the 1970s, for serogroups A, B and C of Nm by IAL due to the occurrence of two major epidemics of meningococcal disease in our country. In the 1980s, the Nm W135 and Hib components were added to technique and since then, this methodology has been employed in the routine diagnosis of BM in the entire network of IAL laboratories (Central and Regional laboratories) and in 20 of 27 Central Public Health Laboratories (LACEN) of Brazil.

CIE is a technique for indirect laboratorial diagnosis of meningitis caused by Nm of serogroups A, B, C, W135 or Hib and is based on the detection of antigens involving the polysaccharide capsule of the bacterium present in the clinical sample by using hyperimmune antisera produced in horses or sheeps. Since this technique employs polyclonal antisera, the possibility of the occurrence of cross-reactivity exists if there is antigenic similarity between capsular polysaccharides of different bacterial species. Specifically, it has been known that the capsular polysaccharide of Hib is immunochemically similar to other encapsulated bacteria, including Spn of serotype six[15]. Fukasawa *et al.* (2010)[16] investigated 46 cases with CIE positive results for Hib. The main goal was to determine the ratio of Hib false positive results by CIE in CSF and sera samples. For comparisons, we used real time PCR method, latex agglutination test and culture when available. Among the 46 CIE Hib positive samples, 26 (57%) were

false positive: 21 (46%) samples were Spn positives and the remaining 5 (11%) samples were negatives for Spn and Hib by both latex and qPCR methods. These results showed a high percentage of false positive results by CIE regarding Hib detection in CSF and sera samples. In recent work Fukasawa ***et al.*** (2012)[17] validated the CIE for meningitis caused by Nm serogroups A, B, C and W135 using CSF and serum samples from 236 patients with suspected bacterial meningitis in the cities of São Paulo and Campinas and demonstrated that this technique had a sensitivity of 62.7% and specificity of 88.9% in CSF specimens and sensitivity and specificity of 35.3% and 90.9% in sera, respectively. They didn't calculate these parameters for the Hib component, due to the unavailability of a significant number of samples with positive culture for this bacterium. These results showed that the CIE assay, although having relatively high specificity, showed low sensitivity, especially in serum samples, indicating that CIE is not recommended as the only diagnostic test for bacterial meningitis.

6. Real time PCR (qPCR)

Due to growing need for more rapid diagnostic methods, the use of DNA approach in diagnostic practices quickly has led the development for new formats of PCR, among which we highlight the real-time PCR (qPCR), which is a modification of the conventional PCR that identifies the target DNA with higher sensitivity and specificity and lower reaction time. The main difference between the two methods is the fact that the amplification and detection are performed simultaneously in a closed system, thereby reducing the risk of contamination. In addition to the primers, the system includes a third oligonucleotide in the reaction, known as probe, increasing specificity. Among the types of probes available, the most commonly used are the TaqMan® linear hydrolysis probes[18,19].

Since last decade many researchers have been publishing about molecular diagnosis of bacterial meningitis using qPCR in different assay formats, all with excellent performance in detecting DNA from different pathogens[20-25]. The traditional microbiological methods, such as the Gram stain or latex agglutination are available for the detection of some agents; however they are not enough sensitive to detect them in small concentrations, a fact usually found in clinical samples from patients undergoing antibiotic therapy[13].

In recent years, PCR has been widely applied in the laboratory procedures and considered the method of choice in the diagnosis and molecular characterization of various bacterial agents. The technique allows the analysis of a larger number of pathogens in a more rapid, precise and safer way, independent on the patient's immune response. The biggest advantage of this methodology in relation to culture is the reduction in releasing time of the results and the detection of microorganisms without prior cultivation. Moreover, the PCR has higher sensitivity and specificity when compared to culture[13,14].

In order to improve the epidemiological surveillance of notifiable diseases in Brazil, in 2004, the Ministry of Health established a Sentinel Hospital Surveillance Subsystem. In São Paulo the Network Sentinel of the Epidemiological Surveillance Program for Meningococcal Disease and Meningitis caused by Nm, Spn and Hi included 13 hospitals. Among other objectives, this Program proposed the improvement of laboratory diagnosis of this disease by implementing the qPCR methodology[24].

Corless ***et al.*** (2001)[20] implemented in diagnostic routine in public health laboratories of England, a qPCR Triplex format, for simultaneous detection of Nm, Spn and Hi from clinical samples of CSF, plasma, serum, and whole blood, with an estimated sensitivity of 90% employing targeted ***ctrA*** gene, responsible for transporting the Nm capsule, ***ply*** gene, responsible for Spn pneumolysin production, and ***bexA*** gene, responsible for the Hi capsular expression.

Following this model Sacchi ***et al.*** (2011)[24] evaluated, validated and introduced a TaqMan® qPCR Triplex format in the BM diagnosis routine at Instituto Adolfo Lutz with a modification, i.e., replacement of the ***ply*** gene to ***lytA***, which encodes the Spn autolysin, to increase the reaction specificity for Spn. Although ***Streptococcus viridans*** also have the ***ply*** gene, this agent is usually found in samples collected from non-sterile sites, such as respiratory secretions, which does not comprise the type of sampling for research/diagnosis of meningitis[22].

In this Program, the use of qPCR for simultaneous detection of DNA of the three major pathogens causing BM, showed a significant increase of Nm, Spn and Hi detection in 85%, 52% and 20%, respectively, and consequent reduction on indeterminate meningitis cases[24].

Figure 1 shows percentage distribution of BM in Sao Paulo State, from 1998 to 2013, according to etiology. We can observe decreasing numbers of undetermined etiology BM after implementation of the Sentinel Program, from about 50% before 2007 to 25% after this period.

After incorporation of triplex qPCR into IAL BM diagnostic routine based on ***ctrA***, ***lytA*** and ***bexA*** genes, a new target to detect Hi cases belonging to any of the six serotypes (a, b, c, d, e, f), including Hi-nt, has been proposed by Wang ***et al.*** (2011; 2012)[26,27]. The new target ***hpd*** gene, is responsible for the Hi protein D synthesis. The replacement of the target gene ***bexA*** by ***hpd*** resulted in an assay with greater sensitivity for Hi detection, because ***bexA*** gene has limited detection to capsule strains of 4 serotypes (a, b, c, d)[20]. We tested the new "modified triplex" format assay (with the ***hpd*** gene) in 1619 clinical samples of CSF and/or blood from patients suspicious of BM in the city of São Paulo and were detected 13 additional Hi cases. Of these 13 additional cases, 12 were Hi-nt and one of the "f" serotype. There was no change in the sensitivity to detect positive samples for Nm or Spn[28].

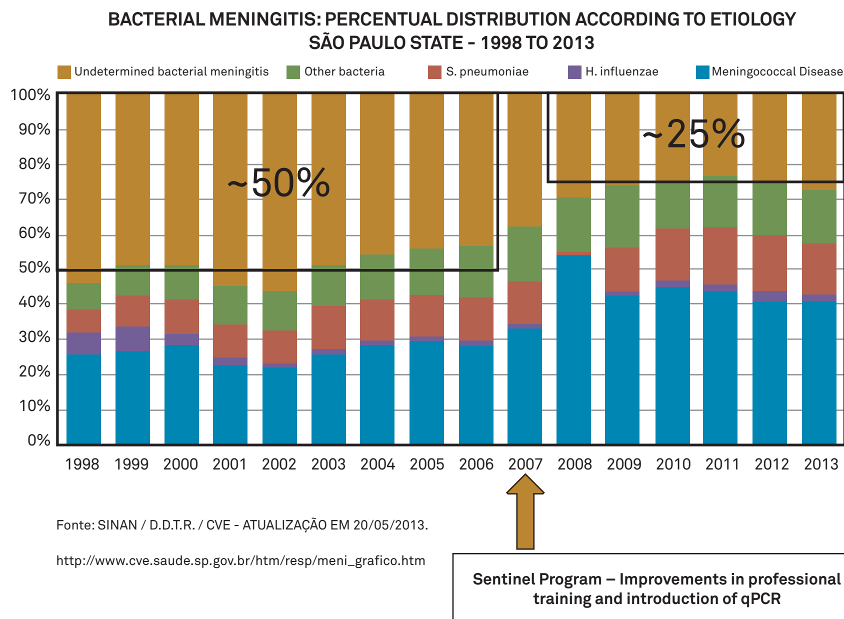

Figure 1. Percentual distribution of bacterial meningitis in São Paulo State-Brazil, according etiology, from 1998 to 2013. (source: Centro de Vigilância Epidemiológica, Secretaria de Estado da Saúde, São Paulo State, Brazil – www.cve.saude.sp.gov.br).

The qPCR assay is also able to identify the Nm genogroups and Hi genotypes. The method relies on the detection of specific genes related to the biosynthesis of capsular polysaccharide responsible for different groups and types of Nm and Hi, respectively, and used in previously positive samples for each agent[21,23,24,27].

Future challenges

IAL is a National Reference Laboratory for BM in Brazil and is responsible for surveillance and constantly improving diagnosis of this severe disease. Based on our encouraging results, the Coordenadoria Geral de Laboratórios (Secretaria de Vigilância em Saúde/Ministério da Saúde – CGLAB/SVS/MS) decided to introduce the qPCR for BM diagnosis to all Brazilian states. Among the goals launched in 2011 by the CGLAB/SVS/MS is to increase to 59% the percentage of BM cases confirmed by specific laboratory diagnosis (culture, latex agglutination test, CIE, and qPCR) until 2015 in our country. Nowadays, this percentage is 51%. Among the essential steps for achieving this goal is the decentralization of qPCR for all State Central Laboratories (LACEN). In this context, training of professionals to work on this technique is the priority. Today, the BM diagnosis by qPCR is performed in two IAL-Regional Laboratories in São Paulo State, and in four Brazilian States. Professionals from 11 other States were recently trained by IAL in this technique and they are going to implement the diagnosis in their home state.

Final considerations

BM diagnosis by qPCR in São Paulo State, Brazil, is a reality, and it has been used to elucidate undetermined BM cases, giving support to surveillance actions in São Paulo and, in some situations, to other Brazilian states. Based on fast results and high sensibility/specificity, the qPCR is a valuable tool for BM diagnosis in combination with microbiological methods.

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