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Original Article

Molecular diagnosis of bacterial meningitis by multiplex real time PCR in Tunisian children

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Abstract

Introduction: Bacterial meningitis is a medical emergency requiring a fast and reliable diagnosis. Molecular methods such as real-time PCR (rt-PCR) offer an attractive alternative. Thus, this study aims to establish multiplex rt-PCRs detecting *N. meningitidis*, *S. pneumoniae* and *H. influenzae b* from cerebrospinal fluid in Tunisian children beyond neonatal age.

Methodology: Using bioinformatic tools and experimentation, we validated the specificity and optimal criteria of PCRs for primers and probes of *plyA* (*S. pneumoniae*), *ctrA* and *sodC* (*N. meningitidis*) and *bexA* genes (*H. influenzae b*). We performed one multiplex RT-PCR for detection of *S. pneumoniae* and *N.* meningitidis targeting *plyA* and *ctrA, sodC* genes respectively, simultaneously with a singleplex RT-PCR for *H. influenzae* b. The sensitivity and specificity of our methods were assessed. Then, we tested our methods for 122 CSF samples collected from suspected meningitis cases between 2014 and 2016 in Bechir Hamza Children's Hospital of Tunis.

Results: Our results have shown the sensitivity of the designed PCRs was up to 10^{-4} DNA dilution and the specificity was 100%. PCR evaluation has shown 51 positive samples: 38 of pneumococcal cases, 12 meningococcal cases, 1 case of *H. influenzae* b with 8.57% and 50% of supplementary positive cases rates respectively.

Conclusions: Our assay proved to be very sensitive, specific and rapid for bacterial meningitis diagnosis. In the recent context of Hib vaccination, the possibility of detecting *S. pneumoniae* and *N. meningitidis* separately constitute an attractive opportunity. Nevertheless, simultaneous detection of Hib remains relevant in specific clinical context and for epidemiologic study.

Key words: *N. meningitidis; S. pneumoniae; H. influenzae b;* multiplex real-time PCR; bacterial meningitis; children.

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Introduction

Despite the progress recorded during the last two decades, bacterial meningitis remains a medical emergency requiring immediate confirmation and treatment. The main causative agents are *Neisseria meningitidis* (*N. meningitidis*), *Streptococcus pneumoniae (S. pneumoniae)* and *Haemophilus influenzae b* (Hib) [1,2]. Clinical manifestations can be difficult to differentiate from other meningitis aetiology [1]. Thereby, a specific bacteriological diagnosis is essential [1,3,4]. The main diagnosis is the examination of the cerebrospinal fluid (CSF) [2]; it includes CSF culture, the "gold standard" method, CSF count cell and CSF staining and immunological diagnosis such as latex agglutination test and immunochromatography test [5,6]. Nevertheless, these methods have shown limits in assessing bacterial meningitis diagnosis resulting from low bacterial titre in the CSF, long incubation period in bacterial culture, cross reaction in immunological methods and mostly antibiotic pre-treatment prior to clinical sample collection [1,3,6,7].

Currently, nucleic acid amplification methods have proved their effectiveness in detecting DNA in the CSF of patients with suspected bacterial meningitis [5,6]. Conventional PCR has the advantage of being highly sensitive, detecting dead bacteria. However, this method does not allow the detection of small amounts of DNA. Moreover, there are risks of contamination in the post processing step, which could cause false positive results [8]. Therefore, the only alternative is real-time PCR (rt-PCR), which can overcome low sensitivity especially in cases where patients receive

antibiotic pre-treatment before lumbar puncture [6,8]. Additionally, this method requires less time than CSF's culture and conventional PCR [8,9]. Furthermore, it offers high specificity thanks to the use of specific probes. Several -rt-PCR protocols have been developed in recent years. However, no universal standard protocol is yet recommended [9]. Different genes were targeted for the same bacterium with different specificity rate [10-12]. False positive detection was reported for some assays using a *plyA* (pneumolysine gene) and *lytA* (autolysin gene) *S. pneumoniae* primers and probe sets [13-16]. Thus, it was recommended to target specific genomic sequences, if ever these genes were targeted [1]. For other assays using *ctrA* (capsular transport gene) and *sodC* (superoxide dismutase gene) *N. meningitidis* primers and probe sets, a lack of sensibility was reported [17-19].

Therefore, this study aims to establish a simultaneous DNA detection of the three main bacteria causing meningitis, using a methodology based on one multiplex rt-PCR for *N. meningitidis* and *S. pneumoniae* detection and a singleplex rt-PCR for Hib detection, both employing the same amplification program. We have evaluated this method using a representative sampling of CSF from 2014 to 2016 in two laboratories and compared rt-PCR's results with the conventional methods (bacterial culture, Gram staining and latex agglutination test).

Methodology

Ethics statement

Ethical Committee of Béchir Hamza Children's Hospital in Tunis, Tunisia approved this study. All information concerning the identity of the patients of this study (beyond their age and gender) was treated confidentially.

Bacterial strains and CSF samples

The reference strain of *S. pneumoniae* (ATTC 49619) and field strains of *N. meningitidis* serogroup B and Hib were used as positive controls for PCRs design and later PCRs evaluation. The field strains were obtained from bacterial culture of meningitis case and blood culture respectively, during 2014, in the Laboratory of Microbiology of Béchir Hamza children's hospital of Tunis (Tunisia). Strains, stored at -80°C, were cultivated in chocolate agar plate for Hib and in blood agar plate for *S. pneumoniae* and *N. meningitidis.* Then, they were incubated overnight at 37° C in 5% CO_2 atmosphere. To study the sensitivity of rt-PCRs, a range of dilutions (undiluted to 10^{-6}) of quantified DNA strains, corresponding to the *S. pneumoniae* reference strain and the *N. meningitidis* and Hib strains, were tested in individual PCR test and in combination to verify the ability of the assay to co-amplify multiple gene targets.

Furthermore, to evaluate the specificity of the assays, positive confirmed samples of viruses (1 CSF sample of Herpes Simplex Virus 1 or HSV1 and 2 sera samples of Cytomegalovirus or CMV, and Epstein Barr Virus or EBV) and other of bacteria (2 CSF samples of *E. coli* and *S. agalactiae*), collected in the laboratory, during 2014, were used. We also tested bacterial strains of *S. oralis* and *S. mitis* obtained from other clinical samples in 2013.

To evaluate rt-PCRs, 122 CSF samples obtained between 2014 and 2016 were collected in the Laboratory of Microbiology in Béchir Hamza Children's Hospital (Tunis, Tunisia). They were obtained from patients aged from 1 month to 15 years old suspected for bacterial meningitis. These samples were included regarding the presence of clinical signs such as: high fever, neck stiffness, photophobia, hypotonia and seizure, the cell counting result and also CSF glucose and protein levels. First, CSF samples with blood staining were excluded because of the risk of PCR inhibition. Then, samples with at least 5 leucocytes per mm^3 (n=94) were recorded as suspected positive bacterial meningitis cases. They also presented a median of 0.0095 g/L for CSF glucose level and 1.915 g/L for CSF protein level. Moreover, as control samples, we have included a random sampling of 28 CSF presenting less than 5 leucocytes per mm^3 and negative culture result.

According to the conventional methods, our sampling was divided in four groups:

- Group A corresponds to culture positive bacterial meningitis cases (n=39 CSF samples) for *S. pneumoniae* (n=31), *N. meningitidis* (n=7) and Hib (n=1).
- Group B corresponds to suspected cases (n=5 CSF samples) characterized by culture negative result and positive Gram staining and /or latex agglutination test results: *S. pneumoniae* (n=4), *N. meningitidis* (n=1).
- Group C corresponds to suspected cases (n=50 cases) characterised by more than 5 leucocytes per mm^3 and revealed negative by conventional methods.
- Group D corresponds to our previous identified negative control group (n=28 cases).

Among these samples, 10 were obtained after antibiotic pre-treatment including 6 control lumbar punctures, from patients presenting irregular evolution and complication of neurologic signs, and 4 from

patients receiving antibiotic treatment previous to the bacterial diagnosis.

Standard protocol

CSF samples were studied by conventional methods used in the laboratory: First, CSF culture was assessed on specific media: chocolate and blood agar plates and incubated at 37°C with 5% CO_2 during 48 hours. Second, Gram staining was realised by preparing one smear using a cytocentrifuge to detect bacteria presence microscopically. Finally, bacteria's antigens were detected by passive agglutination of sensitized latex particles (Pastorex meningitis, BIO-RAD, Marnes-la-Coquette, France,). It revealed *S. pneumoniae* and *Hib* and differentiates meningococcal serogroups (A, B, C and W/Y).

DNA extraction

DNA was extracted by heat-shock protocol in the case of bacterial culture and by using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in the case of CSF sample. DNA purification was obtained by following QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) protocol.

The extraction and the purification of DNA virus were done with High Pure PCR Template Preparation Kit (Roche Life Sciences, Mannheim, Germany).

DNA quantification

Bacterial DNA concentrations were determined using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Oakwood, USA).

Primers and probes' choice

Primers and probes of the three pathogens were chosen after validation by bioinformatics tools (Blast and Primer Express software) in order to assess their nucleotide identity and to evaluate specific criteria for rt-PCR primer and probe design. For multiplex RT-PCR detecting pneumococcal DNA, we used previously published [10] oligonucleotide primers and dye-labeled probe specific for *plyA* gene of *S. pneumoniae* whereas primers and probe detecting *N. meningitidis* were designed by using Primer Express 3.0 (Applied Biosystems, Drive Foster City, USA). They were chosen based on conserved regions of *ctrA* gene (NC_003112) (Table 1). In the aim to increase the assay sensitivity, we have also used a *sodC* gene set of primers and probe for *N. meningitidis* detection [20,21] (Table 1).

Furthermore, a simplex PCR using oligonucleotide primers and dye-labeled probe of Hib (*bexA* or capsulation gene) [10] was applied (Table 1).

Singleplex and Multiplex real time PCR design

Real-time PCRs designed in this study were performed in Lightcycler 480 Instrument II (Roche Life Science, Salt Lake City, USA).

Singleplex PCR was first performed for each pathogen to determine primers and probes optimal concentrations. Then, multiplex rt-PCR for *S. pneumoniae* and *N. meningitidis* detection and singleplex rt-PCR for Hib were developed. The sensitivity and specificity were then assessed for these assays.

Based on a 25µL reaction volume, the mixture contains 2µL extracted DNA sample, 12.5µL master mix (TaqMan Universal Master Mix, Applied Biosystems, Drive Foster City, USA), all primers and probes (volume and concentration determined after optimization) and adding water if necessary. A no template control, an extracted water negative control

Table 1. Primers and probes used in this study.

Oligonucleotide	Sequence	Position (pb)	Target gene	Bacterium	Reference
PlyA forward	5'TGCAGAGCGTCCTTTGGTCTAT3'	894-915			
PlyA reverse	5'CTCTTACTCGTGGTTTCCAACTTGA3'	954-970	*plyA*	*S. pneumoniae*	Corless *et al.* 2001 [10]
PlyA probe	FAM-5'TGGCGCCCATAAGCAACACTCGAA3'-BHQ1	941-918			
CtrA forward	5'GGTATATCGTGTGAATATGGCTGATG3'	998-1023			
CtrA reverse	5'GGCGCATTCGACACATACAA3'	1093-1074	*ctrA*	*N. meningitidis*	This study
CtrA probe	VIC-5'CGCTATTTTCTATGCAGCGCTTTCCTGTG3'-BHQ1	1030-1058			
SodC forward	5'GCACACTTAGGTGATTTACCTGCAT3'	299-323			
SodC reverse	5'CCACCCGTGTGGATCATAATAGA3'	426-404	*sodC*	*N. meningitidis*	Dolan *et al.* 2011 [21]
SodC probe	Cy5-5'CATGATGGCACAGCAACAAATCCTGT3'-BHQ1	335-360			
BexA forward	5' GGCGAAATGGTGCTGGTAA 3'	142-160			
BexA reverse	5'GGCCAAGAGATACTCATAGAACGTT 3'	241-217	*bexA*	*H. influenzae type b*	Corless *et al.* 2001 [10]
BexA probe	Cy5-5'CACCACTCATCAAACGAATGAGCGTGG3'-BHQ1	189-163			

Table 2. Results of rt-PCR s assays to determine sensitivity value for each target.

	Cp values						
	Concentration						
PCR Target	Undiluted*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
plyA (*S. pneumoniae*)	27.44	24.24	32.49	36.57	39.16		
ctrA (*N. meningitidis*)	18.58	21.94	25.65	29.04	31.85	32.8	33.77
sodC (*N. meningitidis*)	20.73	24.08	28.09	30.17			
bexA (*H. influenzae b*)	20.81	25.63	30.35	33.79	38.22		

(*): Concentrations of undiluted DNA were 67ng/µL for *S. pneumoniae*, 38ng/µL for *N. meningitidis* and 97ng/µL for *Hib*.

and a positive control (DNA extracted from strains) for each pathogen were required in every run.

DNA amplification using the TaqMan system followed the steps below: UNG (uracil-N-glycosylase) incubation at 50°C for 2 minutes, Taq activation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds and of a combined annealing and elongation step at 60°C for 1 minute. All the samples were run in duplicate.

Amplification data was analysed by instrument software (Lightcycler 480. CSF sample was considered as PCR-positive when its DNA amplification occurs at a crossing point (Cp) value less than or equal to 35 [1]. If, the crossing point value was between 35 and 40, it was considered as doubtful result and then was retested once again with a diluted well. If the difference between crossing point values of undiluted and diluted wells were up to 3, the CSF sample is considered as positive by PCR.

Evaluation of the developed methods

The samples collected in this study were tested by standard method, multiplex rt-PCR detecting *S. pneumoniae* and *N. meningitidis* and singleplex rt-PCR detecting *H. influenzae b*. These rt-PCR reactions were evaluated in Lightcycler 480 Instrument II, (Roche Life Science, Salt Lake *City*, USA) instrument in Laboratory of Microbiology (Béchir Hamza Children's Hospital) and in 7500 Applied Biosystems (Drive Foster City, USA) in the Research Platform of the Faculty of Medicine in Tunis according to the previously described methods.

The sensitivity of rt-PCR was calculated as the proportion of confirmed CSF culture that was detected positive by rt-PCR for each pathogen [11]. Furthermore, statistical analyses were done using EpiInfo 7.2 (Centers for Disease Control, Atlanta, Georgia, USA) software. The proportions were compared with Fisher exact test and 95% confidence intervals (CI) were performed. A cut-off p value of ≤ 0.05 was considered to be significant.

The cost of the rt-PCR method was also evaluated by summing all reagents and expendables involved in our experiment such as TaqMan Universal Master Mix (Roche Life Sciences, Mannheim, Germany), primers, probes, and molecular grade water. The instruments like thermal cyclers (Lightcycler 480 Instrument II, and laminar flow cabinets and expendables such as PCR plate, optical adhesive film and PCR tubes essential to perform the rt-PCR were excluded in the cost estimation process.

Results

Sensitivity and specificity

To determine the sensitivity of the rt-PCR assay, serial dilutions of the three bacteria strains have been performed. Dilutions of extracted DNA from bacterial strains were amplified up to 10^{-4} dilutions corresponding to 67.10^{-4}ng/µL for *S. pneumoniae*, 38.10^{-6}ng/µL and 38.10^{-3}ng/µL for *N. meningitidis ctrA* gene and *sodC* gene respectively and 97.10^{-4}ng/µL for Hib (Table 2).

Table 3. Results of CSF samples investigated in this study by classical methods and rt-PCR.

Category	number of samples	Bacterium	Culture		Gram staining		Latex agglutination			Cell count		PCR	
			-	+	-	+	-	+	nd$^+$	> 5 leuco/mm^3	< 5 leuco/mm^3	-	+
A	39	*N. meningitides* (n = 7)		7	2	5	2	5		7			7
		S. pneumoniae (n = 31)		31	2	29	4	23	4	31			31
		H. influenzae b (n = 1)		1		1		1		1			1
B	5	*N. meningitides* (n = 1)	1			1	1			1			1
		S. pneumoniae (n = 4)	4			4		3	1	4			4
C	50	Undetermined *	50		50		25		25	50		43	7
D	28	Undetermined *	28		28				28		28	28	28
Total	**122**		**83**	**39**	**82**	**40**	**32**	**32**	**58**	**94**	**28**	**71**	**51**

(*): undetermined by conventional methods; (nd$^+$): not determined refers to non-realised test or insufficient quantity required for the testing.

Furthermore, no amplification of HSV1 and 2, CMV and EBV and also *E. coli*, *S. agalactiae*, *S. oralis* and *S. mitis* was observed in those developed assays.

PCR evaluation

Among the 122 tested samples by multiplex rt-PCR and singleplex rt-PCR, we have found 51 (41.8%) bacterial meningitis positives cases with 38 (74.5%), 12 (23.52%) and 1 (1.72%) caused by *S. pneumoniae*, *N. meningitidis* and Hib respectively (Table 3).

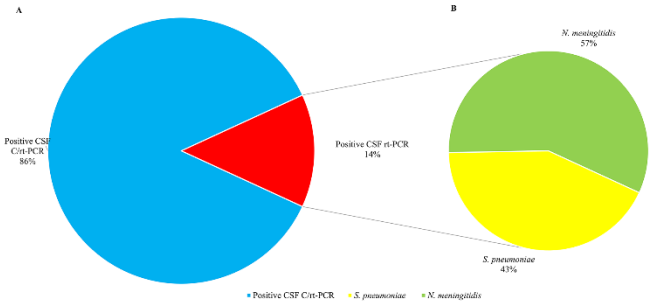
Real-time assays confirmed positive results for cases constituting Group A and B samples

and 7 supplementary cases for Group C corresponding to an additional yield of 15.91% compared to the standard methods (Figure 1). Indeed, as expected, all positive samples by conventional methods were also positive by rt-PCR and effectives of meningococcal, pneumococcal and Hib strains were in accordance. The supplementary positive cases from group C were characterized by a low level of CSF glucose with an average of 0.34 g/L (minimum 0.12 g/L and maximum 0.52g/L), a high level of CSF protein with an average of 2.17 g/L (minimum 1.04 g/L and maximum 2.93 g/L), they also presented typical meningitis clinical signs such as high fever, neck stiffness, photophobia, hypotonia.

The analysis results showed that conventional microbiological methods (direct examination, culture, and/or latex agglutination) detected among the 122 CSF samples, 31.15% of *S. pneumoniae*, 9.02% of *N. meningitidis* and 0.82% of Hib meningitis cases, whereas the designed RT-PCR assays increased the rate of detection for pneumococcal by 8.57% and meningococcal cases by 50%. The differences were highly significant (Table 4). *ctrA* primers and probe detected all meningococcal meningitis cases and *sodC* primers and probe revealed only 5 cases among 12.

The same results were confirmed in the two laboratories (Laboratory of Microbiology of Béchir Hamza Children Hospital in Tunis and Research platform of Faculty of Medicine in Tunis). Crossing point values varied between 16.58 and 35.61 for *S. pneumoniae,* 21.51 and 35.44 for *N. meningitidis*, and 21.81 *H. influenzae b*, when detection was done in

Figure 1. Prevalence of positive bacterial meningitis cases by rt-PCR in comparison with conventional methods (A) and distribution of supplementary cases by rt-PCR.



C: conventional methods.

Lightcycler 480 instrument. In addition, in 7500 Applied Biosystem instrument, crossing point values were slightly different: the differences don't exceed 3 values for the three pathogens (Table 1-S).

Furthermore, the cost of the multiplex and singleplex assays are estimated to be USD $10.97 per test and USD $9.29 per test respectively.

Antibiotic treatment

The ten CSF obtained after antibiotic treatment, are detailed in table 5. Seven (70%) were positive by rt-PCR and only two of them were positive by conventional methods. These two samples were collected after antibiotic pre-treatment and the five others were collected during treatment: two after 3 days and one after respectively 4, 6 and 7 days of treatment. The Cp values varied between 25.7 and 35.6 (Table 5).

Discussion

This study has shown the advantages of rt-PCR in detecting bacterial meningitis cases. The developed PCR assays were rapid, sensitive, specific and reproducible methods. They have an added value for meningococcal detection, although the number of meningococcal meningitis cases is low, our results remains statistically significant (Table 4).

Furthermore, in the recent context of Hib vaccination, the possibility of detecting *S. pneumoniae*

Table 4. Comparative results of positive cases by standard methods and rt-PCRs

Bacterium	positive by conventional methods (n/total effective %)	positive by rt-PCR (n/total effective %)	P value
N. meningitidis	8 (7.51%)	12 (9.84%)	P < 0.05
S. pneumoniae	35 (28.69%)	38 (31.15%)	P < 0.005
H. influenzae b	1 (0.82%)	1 (0.82%)	P < 0.9
Total	**44 (36.07%)**	**51 (43.61%)**	**P < 0.001**

Table 5. Results of rt-PCR analysis for LCS collected during or after antibiotic treatment.

Bacteria	Culture	Sample (n°)	Cp value	Lumbar puncture characterisation
S. pneumoniae	+	23	26.46	Antibiotic pre-treatment
		33	35.61	
	-	12	31.11	3 days*
		26	33.10	
	-	21	35.05	4 days*
	-	1	25.67	6 days*
N. meningitidis	-	44	34.17	7 days*
Undetermined bacteria	-	52	-	Antibiotic pre-treatment
		53	-	
	-	54	-	3 days*

(*): Number of days after treatment initiation.

and *N. meningitidis* in the same reaction constitute an attractive opportunity more reliable and inexpensive.

In Tunisia, since the introduction of Hib vaccine in 2011, *S. pneumoniae* followed by *N. meningitidis* become the main causative agents in bacterial meningitis for children [22,23]. In the same time, in other regions of the world, such as Sub Saharan regions, *N. meningitidis* constitutes the main bacterial meningitidis agent [7]. Also, in Europe, *N. meningitidis* is predominant since the introduction of Hib and pneumococcal vaccines [4]. In accordance with the current Tunisian epidemiological context, we proposed to develop, in first-line, a multiplex rt-PCR detecting *S. pneumoniae* and *N. meningitidis* using three gene targets and, in second-line, a singleplex rt-PCR detecting Hib, if needed. In fact, it has been previously documented that the use of several targets combined in the same assay may be laborious and decrease sensitivity, especially when using TaqMan chemistry [12]. Thus, we advantaged sensitive detection of *N. meningitidis* by using two gene targets (*ctrA* and *sodC* genes) and *S. pneumonia* in first-line. Furthermore, the single use of multiplex PCR may be less expensive and useful. In specific clinical context or for epidemiological study, *H. influenzae* b detection could be assessed simultaneously during the same PCR reaction or in second line by a supplementary singleplex rt-PCR. Other bacteria were reported in neonate meningitis (*Escherichia coli, Streptococcus agalactiae* and *Listeria monocytogenes*) and adults (*Listeria monocytogenes*) worldwide [4]. In paediatric hospital such as Béchir Hamza Children Hospital, we are also concerned by neonate meningitis [24,25]. Thus, and in the global concern of insuring high sensitivity detection these bacteria may be targeted by another specific multiplex rt-PCR assay. In the case of adults 'meningitis an adapted singleplex assay may be used.

For pneumococcal meningitis cases, we used *ply*A primers and probe set [10]. The efficiency of targeting this gene was previously proved in other studies [14,26,27]. Nevertheless, some strains of *Streptococcus mitis* and *Streptococcus oralis* present a pneumolysine coding gene [15,1] which may lead to false positive detection especially in the case of respiratory specimens [1,16,17]. For CSF samples, these have not been reported [1,14]. Furthermore, in our specificity test, we tested DNA amplification of both bacteria and we have not detected any amplification, which confirms the high specificity of the nucleic segment targeted in our assay as previously recommended [1].

For suspected meningococcal meningitis cases, capsule transport to cell surface (*ctrA*) and superoxide dismutase (*sodC*) genes were employed. *ctrA* gene is highly conserved among the *N. meningitidis* strains responsible for invasive infections [1]. It was successfully used by Corless *et al.* [10]. However, it has been proved by Cavrini *et al.* [18] and Jaton *et al.* [19] that this set of primers and probe could not detect two *N. meningitidis* serogroup C strains and one strain of *N. meningitidis* B which were also confirmed with our bioinformatics' analysis. Accordingly, we designed another set of primers and probe of *ctrA* detecting those three reported strains. In the other hand, it was reported that at least 16% meningococcal non-capsulated strains lack of this gene [1,21], which may underestimate meningococcal meningitis cases. The use of *sodC* gene primers and probe permit to overcome this limit. Nevertheless, Higa *et al.* [20] who compared the meningococcal amplification reactions using primers and probe of the two genes showed that *sodC* primers and probe were not able to hybridize 24 among 319 (7.5%) positive samples for *ctrA* primers and probe. Similar results were found in our study; only 5 cases were detected among 12 meningococcal confirmed cases. Thus, *sodC* gene could not be used alone; we

highlight the need to employ *sodC* and *ctrA* genes simultaneously to improve the sensitive detection of all meningococcal strains.

For *Hib* meningitis cases, the gene encoding proteins involved in the transport of the capsule (*bexA*) was used. This gene has the advantage of being very sensitive for the serotype b, more than for serotypes a, c and d and does not detect other serotypes, (e and f) or nonencapsulated strains [1]. In the current context of *Hib* vaccination and depletion of related meningitis case [6], detection of Hib is no longer needed. Nevertheless, it is recommended for monitoring vaccination proper approach as well for epidemiological surveillance [1].

The developed method in our work was able to confirm all the positive cases revealed by the conventional methods (Group A and B) and to detect 7 other supplementary cases initially diagnosed negative by standard methods with high sensitivity performance. Previous studies such as Corless *et al.* [10], Brouwer *et al.* [6], Diawara *et al.* [28] and Kahraman *et al.* [29] have shown that rt-PCR was characterised by high performance sensitivity than conventional methods even by using Sybr Green or TaqMan technologies. The reported sensitivities varied between 72-92%, 61-100% and 88-94% for *H. influenzae, S. pneumoniae* and *N. meningitidis* respectively [6]. However, the use of TaqMan chemistry offers more specific results [6,10]. Furthermore, our assay offers high sensitive detection of *N. meningitidis* by using two gene targets. Cost estimation of our assays shows, like Diawara *et al.* [28], that "in-house" method is more expensive than bacterial culture, which is estimated about 5 USD per test, but it is faster in obtaining a final result (less than 4 hours).

By comparing conventional methods to rt-PCR results, we found that difference in detection was significant especially for the *N. meningitidis*. This may be the consequence of the fragility of this bacterium. In fact, it is a facultative intracellular pathogen that can be easily altered (autolysis) at temperatures less than 37°C. So it is recommended to transport it quickly at 37°C and to cultivate the CSF less than one hour after collection of the sample [30]. Unfortunately, these recommendations are not continually respected. Several studies have shown similar results [31,32], particularly in England, where a study carried out by the reference laboratory for *N. meningitidis* in 2009-2010 showed that out of 1924 cases of invasive meningococcal disease collected from 22 039 suspected meningitis cases, 1099 cases (57.1%) were only confirmed by PCR [32].

Our study has shown the advantage of rt-PCR in detecting bacterial meningitis cases when antibiotic pre-treatment was administered which is very important for treatment monitoring in the case of irregular clinical evolution. This same report is found by Sacchi *et al.* [13] who analysed 460 samples of patients suspected of bacterial meningitis (CSF and serum) from twelve hospitals in São Paulo and surrounding areas (Brazil) [13]. One-third of these samples were collected after antibiotic therapy. Among them, 162 were confirmed positive by culture, while 246 cases were positive by rt-PCR. On the other hand, Brink *et al.* [33] investigated the ability of PCR to detect positive cases of bacterial meningitis after antibiotic therapy over time and showed that this molecular technique can confirm positive cases up to seven days of antibiotic pre-treatment for two pneumococcal meningitis cases [33]. Other study has shown detection of pneumococcal DNA in meningitis cases up to 20 days after treatment onset [34]. The high sensitivity of molecular methods is for great interest for clinical evolution and treatment monitoring.

Nevertheless, in the general concern of the raise of antibiotic resistance, molecular detection methods don't provide information about antibiotic susceptibility [35]. This methodology should be fulfilled by assays detecting the major resistance genes. PCRs developed in this aim are for great interest [36,37]: they should be useful and rapid for delivering a reliable result. However, we cannot exclude emergence of new resistance mutations, which may be only revealed by phenotypic analysis. Thus, methods based on bacterial culture constitute important characterization tools. Nevertheless, rapid identification of bacterial agent by molecular methods is relevant to initiate an early safe antibiotic treatment [33]. Such monitoring may avoid clinical sequelae after meningitis [35].

Conclusion

The developed rt-PCR assays are rapid, sensitive, specific and reproducible methods. They have an added value for *N. meningitidis* and *S. pneumoniae* detection and can overcome the disadvantage of antibiotic pre-treatment. Furthermore, in the recent context of Hib vaccination, the possibility of detecting *S. pneumoniae* and *N. meningitidis* in the first line constitute an attractive opportunity more reliable and inexpensive. Nevertheless, simultaneous detection of Hib remains relevant in specific clinical context and for epidemiologic study.

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Annex – Supplementary Items

Table 1-S. Crossing point values of the positive bacterial meningitis cases obtained by Lightcycler 480 and 7500 Applied Biosystems.

Gene target	Samples	Crossing point values in 480 Lightcycler Instrument	Crossing point values in 7500 Applied Biosystems instrument
plyA gene (*Streptococcus pneumoniae*)	Sample 1	25,67	23.55
	Sample 2	21,00	22.39
	Sample 3	30,00	29.78
	Sample 4	20,00	21.96
	Sample 5	20,69	18
	Sample 6	35,00	34
	Sample 7	23,46	22
	Sample 8	17,43	20
	Sample 9	30,62	33
	Sample 10	28,93	29.17
	Sample 11	23,91	25
	Sample 12	28,93	28.79
	Sample 13	31,11	29.96
	Sample 14	26,61	25
	Sample 15	32,10	32.13
	Sample 16	28,13	28.75
	Sample 17	28,05	28.29
	Sample 18	30,72	33.25
	Sample 19	23,12	22.54
	Sample 20	23,43	24.64
	Sample 21	16,58	19.09
	Sample 22	26,41	25.97
	Sample 23	27,24	28.29
	Sample 24	35,05	33.76
	Sample 25	31,07	29.98
	Sample 26	26,46	26.19
	Sample 27	31,56	33.26
	Sample 28	30,95	28.63
	Sample 29	33,10	33.76
	Sample 30	31,12	33
	Sample 31	24,97	25.20
	Sample 32	24,74	24.67
	Sample 33	26,66	26.19
	Sample 34	22,77	24.64
	Sample 35	27,85	28.63
	Sample 36	24,93	25.08
	Sample 37	35,61	34.47
	Sample 38	16,24	17.83
	Sample 39	20,52	21.03
	Sample 40	25,22	24.66
	Sample 41	17,70	20.52
	Sample 42	28,38	30.16
ctrA gene (*Neisseria meningitidis*)	Sample 43	35,44	34.96
	Sample 44	34,43	35.82
	Sample 45	33,92	31.02
	Sample 46	21,51	23.65
	Sample 47	21,81	22.07
	Sample 48	26,24	28.65
ctrA gene (*Neisseria meningitidis*)	Sample 49	34,17	35.05
	Sample 50	31,46	32.98
	Sample 51	31,77	32.68
	Sample 52	32,82	31.44
	Sample 53	35,17	34.94
	Sample 54	24,75	26.59
	Sample 55	32,75	34.52
	Sample 56	26,71	25.42
	Sample 57	23,63	21.96
sodC gene (*Neisseria meningitidis*)	Sample 43	24.20	26.83
	Sample 46	23.67	25.52
	Sample 47	26.14	29.06
	Sample 48	31.62	33.07
	Sample 55	35.28	35.72
	Sample 57	30.69	33.52
bexA gene (*Haemophilus influenzae b*)	Sample 58	22.81	20.04