Original Article

A duplex real-time PCR for the detection of *Streptococcus pneumoniae* and *Neisseria meningitidis* in cerebrospinal fluid

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Abstract

Introduction: Acute bacterial meningitis is one of the most severe infectious diseases. Rapid, accurate, and inexpensive diagnosis of bacterial meningitis is crucial for patient management. This study describes a duplex real-time (RT) PCR assay for detection of *Neisseria meningitidis* and *Streptococcus pneumoniae* in the cerebrospinal fluid (CSF) for meningitis diagnosis using SYBR Green-based RT-PCR method coupled with melting curve analysis.

Methodology: We used SYBR Green-based RT-PCR method coupled with melting curve analysis to detect *S. pneumoniae* and *N. meningitidis* in CSF samples. The sensitivity, specificity, and limit of detection were determined. The gold standard for routine tests of CSF analysis is direct examination, culture, and/or latex agglutination. The assay was evaluated on 132 CSF samples to measure clinical sensitivity.

Results: A duplex RT-PCR assay for *N. meningitidis* and *S. pneumoniae* detection in CSF was evaluated. Two peaks at different melting temperatures (87.5°C and 85.5°C) for *N. meningitidis* and *S. pneumoniae*, respectively, were obtained. The sensitivity of RT-PCR was 100% (95% confidence limits [CI] = 82.4–100) for *N. meningitidis* and 100% (95% CI = 85.1–100) for *S. pneumoniae*. Specificity was the same (100%) for the bacteria (95% CI = 88.6–100). The percentage of cases accurately diagnosed with meningitis caused by *N. meningitidis* and *S. pneumoniae* increased to 50.7% and 28.6%, respectively, when RT-PCR was added to the standard microbiologic methods.

Conclusions: Duplex RT-PCR and melting curve analysis with SYBR Green is an inexpensive, sensitive, and specific method to rapidly diagnose bacterial meningitis. Accurate identification of the bacterial causative agents will improve patient management and epidemiological investigations.

Introduction

Bacterial meningitis, an infection of the membranes (meninges) and cerebrospinal fluid (CSF) surrounding the brain and spinal cord, is a major cause of deaths due to infectious diseases worldwide. Three organisms are responsible for most cases of bacterial meningitis: *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* [1-4]. *N. meningitidis* and *S. pneumoniae* are now considered the leading causes of bacterial meningitis worldwide since the implementation of the *H. influenzae* type b conjugate vaccine [5-7]. Traditional laboratory culture methods for the isolation and identification of these pathogens are the gold standard, but they can require 24 to 48 hours to obtain results. The etiological diagnosis of bacterial meningitis can be difficult to establish due to an increase in the practice of starting antimicrobial therapy prior to clinical sample collection. This practice has been reported to decrease the ability to culture and identify the pathogens causing bacterial meningitis and septicemia by approximately 30% [8-9].

The rapid progression of symptoms and potentially devastating effect of meningitis necessitate early recognition and treatment. Several rapid methods of diagnosis have been developed, ranging from antigen detection (latex agglutination test) to polymerase chain reaction (PCR)-based methods. Latex agglutination tests, compared to conventional Gram stain and culture, can be subjective and inaccurate [10-11]. Currently, real-time (RT)-PCR as well as conventional PCR have become the most accurate and sensitive

methods for detection of bacterial pathogens, particularly in patients who have previously received antimicrobials [12-14]. RT-PCR assays can be classified into two major groups, fluorescent-probe-based assays and double-stranded DNA binding fluorescent-dye-based assays. TaqMan probes, fluorescent-probe-based assays, are usually expensive and time-consuming to synthesize, and potential false-negative rates have been reported due to sequence variability within the probe-binding site [15-17]. As an alternative to TaqMan probes, simple DNA binding dyes have been used for RT-PCR by detecting the total amount of accumulated DNA during the PCR reaction [18]. A PCR dye is universally applicable to any target sequence and is generally less expensive than an individually synthesized TaqMan probe. The SYBR Green (SG), a highly specific, double-stranded DNA binding dye, detects the PCR product as it accumulates during PCR cycles. Its reliability can be verified semi-qualitatively by analyzing the melting curve of the amplification following the PCR [19]. Although there are currently no multiplex RT-PCR assays that use melting curve analysis for detection of bacterial meningitis, the association of multiplex PCR with melting temperature (Tm) has the potential to greatly expand the power of real-time analysis in diagnostic research.

The aim of this study was to develop and evaluate a duplex single-tube RT-PCR assay coupled with melting curve analysis for the detection of *N. meningitidis* and *S. pneumoniae* in the CSF of suspected meningitis cases.

Methodology

This study was performed from January 2013 to February 2014 at the Ibn Rochd University Hospital Centre of Casablanca (Morocco), a tertiary care hospital. The CSF samples were collected from patients hospitalized for suspected meningitis and referred to the Bacteriology, Virology, and Hospital Hygiene Laboratory for diagnosis. All information concerning the identities of the patients and the results of this study were treated confidentially.

This study and publication of the results were approved by the ethical committee for biomedical research of the University Mohammed V - Soussi, Faculty of Medicine, Pharmacy and Dental Medicine of Rabat, Morocco.

The diagnosis of meningitis was suspected when the patients had the following clinical symptoms upon admission: headache, fever, meningismus, and altered sensorium. Lumbar punctures were performed on suspect meningitis cases, and CSF specimens were submitted for laboratory analysis. A confirmed diagnosis of meningitis due to *N. meningitidis* or *S. pneumoniae* required a positive test result from the CSF in at least one of the following assays: Gram stain, culture, or a pathogen-specific PCR assay [20-21].

The gold standard for bacterial analysis of CSF is direct examination, culture on supplemented chocolate agar and Mueller-Hinton agar plus 5% sheep blood (MHS Kit, bioMerieux, Marcy-l'Etoile, France), and/or latex agglutination test (Slidex Meningite Kit 5, bioMerieux). The primary organisms of interest were identified using recommended techniques [22,23]: morphology on Gram stain, alpha hemolysis, optochin and bile solubility tests for *S. pneumoniae*, and oxidase and carbohydrate utilization test (API NH, bioMerieux) for *N. meningitidis*. Serotype determination of *N. meningitidis* isolates or samples was performed using the Slidex Meningite Kit 5 (bioMerieux), Pastorex Kit (Bio-Rad Laboratories, Marnes-La-Coquette, France), or by PCR.

The duplex RT-PCR assay was developed by using two sets of primers, pneumococcal autolysin gene *lytA* [24], and meningococcal regulator gene *crgA* [25] to identify etiological bacterial meningitis. The assay was first optimized in singleplex PCR reactions; subsequently, duplex RT-PCR was performed. To optimize the RT-PCR, the lowest concentration of DNA that gives the highest normalized reporter fluorescence and the lowest threshold cycle (C_t), was determined. The lower limit of detection (LLD) was determined from extracted DNA, using QIAamp DNA Mini Kit (Qiagen, Valencia, USA) as described by the manufacturer, from *S. pneumoniae* ATCC 49619 strain and a *N. meningitidis* strain identified in the laboratory. The quantity and quality of the purified DNA was determined using a NanoVue plus spectrophotometer (General Electrics Healthcare Limited, Little Chalfont, Buckinghamshire, UK). DNA concentrations were adjusted to 15 ng μL^{-1} from which tenfold serial dilutions (10^{-1} to 10^{-9}) were made to determine the LLD. Genome equivalents were calculated assuming one molecule of *N. meningitidis* and *S. pneumoniae* DNA. Considering a genome size of 2.1 Mb for *S. pneumoniae*, a corresponding 2.2 fg of DNA was calculated, and 2.3 fg was determined for *N. meningitidis* DNA based on a 2.2 Mb genome [26], determined according to the previously published equation [27]. Considering a genome size of 2.1 Mb as determined for *S. pneumoniae*, the number of genomic copies in the nucleic acid extracts from each strain was

determined using the following formula [28]: genome copies = quantity of DNA in extract/2.2 fg or quantity of DNA in extract/2.3 fg. The inter-assay reproducibility was estimated by testing tenfold serial dilutions, with the experiment repeated 10 times. Five replicates of each serial dilution were tested on the same run to assess intra-assay reproducibility.

The laboratory sensitivity, defined as the proportion of cases that are RT-PCR positive (singleplex and duplex) from those positive cases determined by the gold standard (direct examination, culture, and/or latex agglutination), was obtained using 40 culture-positive CSF samples: 18 *N. meningitidis* and 22 *S. pneumoniae*. Laboratory specificity, defined as the proportion of RT-PCR negative cases from negative cases determined by a reference standard, was obtained from 30 bacterial and fungal isolates representing other species used to validate our RT-PCR [26,14]. These species were: *Cryptococcus neoformans*; Gram-negative bacilli (*Acinetobacter baumannii*, *Acinetobacter lwoffi*, *H. influenzae*, *Enterobacter cloaceae*, *Pseudomonas aeruginosa*, *Escherichia coli K1*, *Moraxella catarrhalis*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Providencia stuartii*, and *Enterobacter aerogenes*); Gram-positive bacilli (*Corynebacterium aquaticum* and *Listeria monocytogenes*); Gram-positive cocci (*Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus viridians*, *Staphylococcus aureus*, and *Staphylococcus* spp.); and *Mycobacterium tuberculosis*. These organisms were isolated from CSF specimens during routine laboratory analysis.

DNA extraction was performed using a boiling and freeze-thaw treatment, where 200 µL of CSF fluid was heated at 100°C for 10 minutes and immediately frozen at 0°C for 5 minutes. A volume of 150 µL supernatant was recovered after centrifugation at 14,000 g for 10 minutes and stored at -20°C. A known specimen (positive control) and molecular-grade water sample (negative control) were extracted each time the DNA extraction procedure was performed on unknown samples. This ensured that negative RT-PCR results were not due to DNA extraction failure and that positive PCR results were not due to cross-contamination introduced during the DNA extraction process. To determine the presence of possible inhibitors of the RT-PCR process during DNA extraction, negative samples were purified by the QIAamp DNA Mini Kit (Qiagen) based on manufacturer's recommendations. The purified extracts obtained were retested by RT-PCR.

Each reaction singleplex or multiplex were carried out in a final volume of 20 µL, consisting of 10 µL of SensiFAST SYBR Hi-ROX One-Step mix Kit (Bioline Inc, Taunton, USA), 5 µL of template DNA, and 400 nM of each primer (forward and reverse). A negative control (molecular-grade water sample) and positive control (DNA preparation) for each bacterial pathogen were included in every run. All RT-PCR assays were performed in a CFX96 Real-Time System (Bio-Rad, Hercules, USA). The run consisted of heating at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 5 seconds), annealing (60°C for 10 seconds), and extension (72°C for 15 seconds). Annealing at 51°C for 10 seconds was used for singleplex RT-PCR genogrouping assay for meningococcal serotype B, W135, and Y using previously published primers [25]. The fluorescent signal was registered during the extension step and the data were analyzed using the CFX Manager software version 1.6 (Bio-Rad Hercules, USA). Melting curves were determined to discriminate between specific amplicons and non-specific amplification products from 65°C to 95°C. Melting peaks were automatically calculated using CFX Manager software. The software generates the result after subtracting background fluorescence from a set of water blanks and plots the negative derivative of fluorescence with respect to temperature ($-dFdT^{-1}$ versus T). The Tm value was defined as the peak of the curve. A positive result was defined as a cycle threshold (C_t) value below 39 cycles, and perfect superimposing of the melting curve peak with the Tm of the positive control. A negative result was defined as a C_t value of zero, and no superimposing of the melting curve peak with the Tm of the positive control.

After establishing the specificity and sensitivity, the new duplex RT-PCR was used to assess the incidence of the two leading causes of bacterial meningitis. A total of 132 CSF specimens obtained from 132 patients with suspected bacterial meningitis who were admitted at the Ibn Rochd University Hospital Centre of Casablanca during the study period were evaluated. Patients' ages ranged from 1 month to 77 years, with an average of 24 ± 21 years; 64.4% were males. The inclusion criteria for the samples were based on previous studies [29]. Indeed, it was demonstrated that the PCR positivity correlated with specimens having CSF values of ≥ 100 leukocytes/mm^3 and > 50% neutrophils. As previously described, CSF specimens from meningitis patients typically have bacterial counts in excess of 10^3 to 10^5 colony-forming units (CFU)/mL [30]. In addition to

cell count analysis, the gold standard tests and RT-PCR results were evaluated. The time to results between the gold standard and RT-PCR was compared by measuring time from receipt of specimens to reporting of final test results.

Finally, the percentage of cases accurately diagnosed was calculated using following formula: 100 × ([positive result by RT-PCR or positive result by standard microbiology methods] / [positive diagnosis by standard microbiology methods + positive result by diagnostic RT-PCR]) [26].

To determine the cost of the RT-PCR method, all reagents and expendables such as SensiFAST SYBR Hi-ROX One-Step Mix Kit, primers, PCR plate, optical adhesive film, and molecular grade water involved in the RT-PCR procedure were evaluated. The instruments, such as a thermal cycler or other equipment necessary to perform the RT-PCR, were not included in the cost estimation.

Statistical analyses were done using EpiInfo 7 (Centers for Disease Control, Atlanta, Georgia, USA) and Microsoft Excel as statistical analysis software. The proportions were compared with Chi-square test and 95% confidence intervals (CI) were performed with the recommended Wilson method for small number analysis and the Agresti-Coull method for larger numbers to provide more reliable coverage [31]. A cut-off p value of ≤ 0.05 was considered to be significant.

Results

The sensitivity and specificity of the singleplex and duplex RT-PCR in the species-specific assay targeting the *crgA* and *lytA* genes were 100% for *N. meningitidis* and *S. pneumoniae* (Table 1). Post-PCR melting curve analysis after these RT-PCR assays yielded two distinct Tm peaks corresponding to 87.5°C for *N. meningitidis* and to 85.5°C for *S. pneumoniae*, using positive controls (Figures 1a and

Figure 1. Representative fluorograms (a) and melting curves (b) obtained from positive samples with a simultaneous detection of *S. pneumoniae* (green) and *N. meningitidis* (red). The blue curve represents negative control. The horizontal line (green) is the threshold.
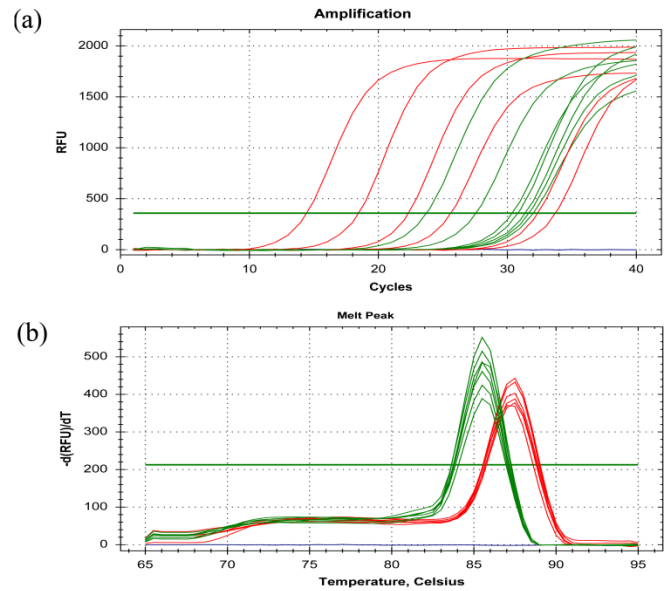
1b). The same melting curve results were obtained when the assays were performed in clinical sample controls (Figures 2a and 2b). In addition, when non-target bacteria or water were used as a template for the duplex RT-PCR, a non-specific Tm peak for the two pathogen templates was observed. Indeed, these nonspecific Tm peaks of primer dimers can be distinguished from the targeted products because they melted at different temperatures (80 ± 1°C and 93°C ± 2), as illustrated in Figures 3a and 3b.
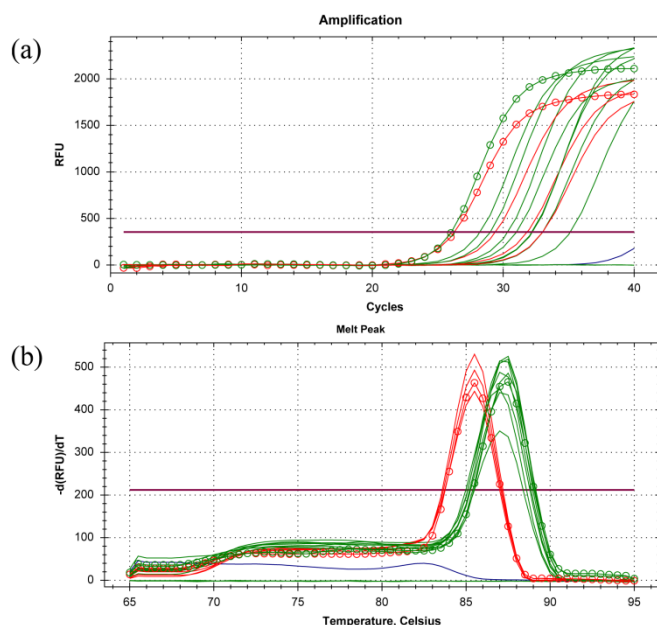
Standard curves were constructed from the C_t values of the duplex RT-PCR assay generated from serial dilution of genomic DNA from positive control of *S. pneumoniae* and *N. meningitidis* (Figures 4a and 4b). The slope (-3.875 and -3.932) of the standard curve indicated a high PCR efficiency (99.9%),

Table 1. Sensitivities and specificities of the multiplex and single-plex RT-PCR assay for detection of *N. meningitidis* and *S. pneumoniae* in cerebrospinal fluid (CSF) with confirmed diagnosis of pneumococcal meningitis and meningococcal meningitis

Assay (type)	No. positive/ no. tested*	Sensitivity %(95% CI)	No. negative/ no. tested**	Specificity %(95% CI)
S. pneumoniae (S)	22/22	100 (85.1–100)	30/30	100 (88.6–100)
S. pneumoniae (D)	22/22	100 (85.1–100)	30/30	100 (88.6–100)
N. meningitidis (S)	18/18	100 (82.4–100)	30/30	100 (88.6–100)
N. meningitidis (D)	18/18	100 (82.4–100)	30/30	100 (88.6–100)

* Number of RT-PCR positive obtained after assay on the positive CSF confirmed by microbiologic standard methods and no. tested were 18 for *N .meningitidis* and 22 for *S. pneumoniae*; ** Number of RT-PCR negative obtained from the 30 bacterial and fungal (no. tested) isolates representing other species; S: singleplex; D: duplex; IC: confidence interval.
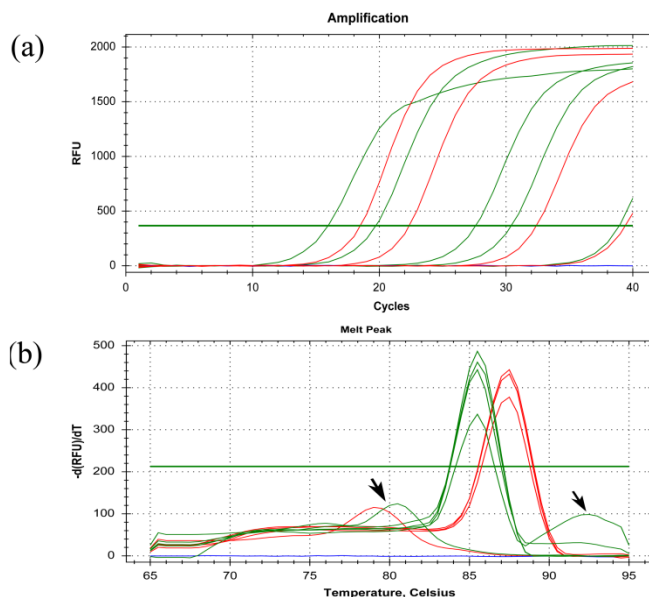
Figure 2. Representative fluorograms (a) and melting curves (b) obtained from clinical samples with a simultaneous detection of *S. pneumoniae* (red) and *N. meningitidis* (green). The curves with circle represent positives control and the blue curve represents negative control. The horizontal line is the threshold.

Figure 3. Representative fluorograms (a) and melting curves (b) of *S. pneumoniae* (green) and *N. meningitidis* (red) showing primer dimers formation (arrow) after SG RT-PCR amplification. The blue curve represents negative control. The horizontal line (green) is the threshold.

indicating a high degree of accuracy and amplification efficiency in the duplex condition. Furthermore, the coefficient of determination (R^2 = 0.79 and 0.81) suggested a good correlation between threshold cycle (C_t) values and DNA concentrations.

The LLD for the individual and duplex RT-PCR assays was 10 fg and 15 fg for *N. meningitidis* and *S. pneumoniae*-positive samples, respectively. Therefore, the minimum limits of detection of 10 fg and 15 fg were equivalent to approximately four and seven *N.*

meningitidis and *S. pneumoniae* genomes per microliter, respectively. The variability tests of the RT-PCR are shown in Table 2. The assays reproducibility ranged from 0.24% to 0.28%, and the variability was 0.25%.

The analysis test results from 132 CSF specimens showed that conventional microbiological methods (direct examination, culture, and/or latex agglutination) detected 13% and 19% of meningitis cases caused by *N. meningitidis* and *S. pneumoniae*, respectively. The RT-PCR assay increased the rate of

Table 2. Inter-specificity and intra-specificity assays of the RT-PCR to determinate the reproducibility between runs

| Bacterial	Intra-specifity assay							Inter-specifity assay											
	Tm values of amplicon					Mean	CV%	Tm values of amplicon										Mean	CV%
S. pneumoniae	85.5	85.5	85.5	85.5	85	85.4	0.25	85	85.5	85.5	85.5	85.5	85.5	85.5	85.5	85	85	85.35	0.28
N. meningitidis	87	87.5	87.5	87.5	87.5	87.4	0.25	87.5	87	87	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.4	0.24

CV% = (standard deviation/mean) ×100; Tm: melting temperature

Table 3. CSF diagnosis after microbiological analysis (direct examination, culture, and/or latex agglutination) and RT-PCR (n = 132)

	Diagnosis result with microbiological standard methods	Diagnosis result by RT-PCR	P value
S. pneumoniae	25 (19%)	45 (34.1%)	0.005
N. meningitidis	17 (13%)	52 (39.4%)	< 0.0001
Other species	7 (5%)	0 (0%)	ND
Negative samples	83 (63%)	35 (26.5%)	< 0.0001
Total diagnosis	49 (37%)	97 (73.5%)	< 0.0001

Microbial standard methods were Gram stain, latex agglutination, and culture; ND: not determined.

Figure 4. Standard curves generated from serial dilution of genomic DNA of positive control from *S. pneumoniae* (a) and *N. meningitidis* (b). Slope, efficacy, and R^2 are reported at the bottom.

(a)

(b)
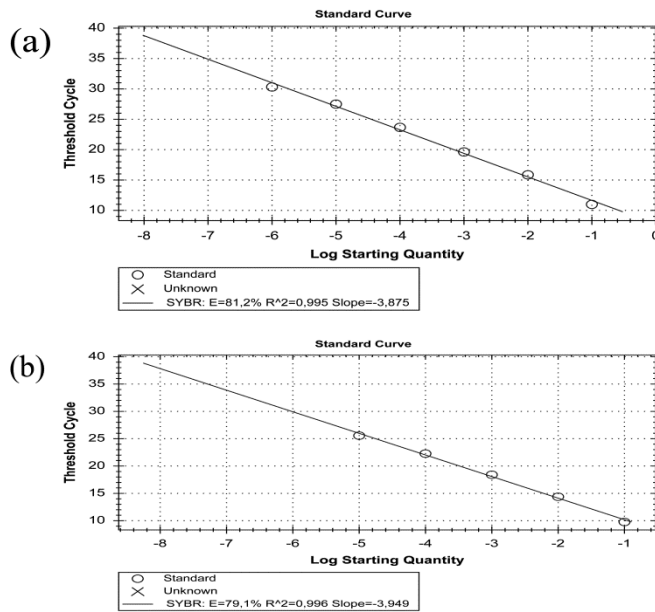
pathogen detection from 13% to 39% ($p = 0.005$) for *N. meningitidis* and from 19% to 34% ($p < 0.0001$) for *S. pneumoniae* (Table 3). Therefore, the addition of the RT-PCR assay to the standard gold standard methods impacted clinical care by increasing accurate diagnosis of meningitis due to *N. meningitidis* and *S. pneumoniae* by 50.7% and 28.6%, respectively. All CSF samples positive for microbial species other than *N. meningitidis* and *S. pneumoniae* were negative by the duplex RT-PCR. The sensitivity and specificity of RT-PCR, Gram stain and/or latex agglutination, and culture of CSF (Table 4) showed that the sensitivity of RT-PCR was higher than that of microbiological methods. The average time to results for the Gram stain and/or latex agglutination was 30 to 40 minutes, and for bacterial culture from 24 to 72 hours. For RT-

PCR, the average time was 3 hours. Analysis of the RT-PCR-negative samples showed no effects by amplification inhibitors. In fact, the results of RT-PCR with DNA extracts by the boiling method were identical with those obtained after purification by the commercial kit. Furthermore, the cost of our duplex SG-based RT-PCR assay coupled with melting curve analysis and bacterial culture is estimated to be USD $10 and $5 per test, respectively.

Among the 17 *N. meningitidis* identified by culture, 94% were serotype B and 6% was serotype Y. For the other *N. meningitidis* identified by RT-PCR, only (35 cases), serotype distribution, determined by molecular serotyping, was 91.4% for serotype B and 8.6% for serotype W135.

Discussion

Identification of the bacteria causing meningitis involves Gram staining, culture, and, when available, PCR. Though Gram staining provides rapid results, sensitivity is poor, requiring approximately 10^5 bacteria/mL for detection [32]. False negative results can occur if the CSF is not centrifuged or if there are sampling errors due to inadequate specimen resuspension. Additional technical errors can occur during the staining process, particularly decolorization or misinterpretation of the bacterial morphology on the slide. The identification of the specific bacteria and determination of antibiotic susceptibility requires bacterial culture. Initiation of antibiotic therapy prior to sample collection and analysis can reduce the accuracy of both Gram stain and culture methods [9]. In this regard, PCR can be a useful adjunctive test, especially in patients who have already received antibiotics.

RT-PCR is a powerful tool for detection of pathogens because of the high sensitivity and throughput capability. Currently, several multiplex

Table 4. Comparison of sensitivity and specificity of RT-PCR, Gram stain and/or latex agglutination, and culture of cerebrospinal fluid (CSF) on the 132 CSF obtained from 132 patients

	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
S. pneumoniae (n = 45)				
Direct examination	48.90 (33.71−64.22)	100 (95.81−100.00)	100 (84.43−100.00)	79.09 (70.30−86.26)
Culture	55.56 (40.00−70.35)	100 (95.81−100.00)	100 (86.16−100.00)	81.31 (72.62−88.19)
SG-RT-PCR	100 (92.05−100.00)	100 (95.81−100.00)	100 (92.05−100.00)	100 (95.81−100.00)
N. meningitidis (n = 52)				
Direct examination	17.31 (8.25−30.33)	100 (95.45−100.00)	100 (66.21−100.00)	65.04 (55.92−73.41)
Culture	34.62 (21.97−49.09)	100 (95.45−100.00)	100 (81.32−100.00)	70.18 (60.89−78.38)
SG-RT-PCR	100 (93.08−100.00)	100 (95.45−100.00)	100 (93.08−100.00)	100 (95.45−100.00)

Direct examination was Gram stain and/or latex agglutination; SG-RT-PCR: SYBR Green Dye-based RT-PCR assays; NPV: negative predictive value; PPV: positive predictive value; CI: confidence interval; On the 132 patients, 45 had a confirmed diagnosis of pneumococcal meningitis, 52 patients with a confirmed diagnosis of meningococcal meningitis and 35 patients were negative for the two bacteria.

RT-PCR assays have been developed to target *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. These multiplex real-time PCR methods are based on fluorescent probes such as TaqMan probes. One of the drawbacks of using multiplex systems with TaqMan is the high cost. In contrast, the SG Dye-based RT-PCR assays are less expensive and may be an excellent alternative for the detection of individual genes; however, they have limited multiplexing capabilities.

In the present study, a duplex SG-based RT-PCR assay coupled with melting curve analysis was optimized and evaluated for detection of *N. meningitidis* and *S. pneumoniae* in CSF samples. Results indicate that this method could be an efficient, highly sensitive tool for the rapid diagnosis of these two important pathogens causing bacterial meningitis compared to the previously conventional PCR method [14] and standard bacterial culture. The primers used in this study were previously validated for the specific detection of *N. meningitidis* and *S. pneumoniae*. These primers were used in RT-PCR TaqMan-based and conventional PCR methods. Here, we described the use of these primers in SG duplex RT-PCR assays.

PCR inhibitors have been, for a long time, a major problem for RT-PCR systems. Here, we adopted a simple and inexpensive DNA extraction method consisting of sample boiling that requires less time than do commercial kits for DNA purification. The fluorogram analysis associated with the melting curve peak allows for more precise RT-PCR results. The inter-assay and intra-assay coefficients of variation of this study demonstrate excellent assay reproducibility. However, unlike the multiplex TaqMan RT-PCR, the SG multiplex RT-PCR cannot simultaneously quantify more than two targets in a single tube. Because only one amplification plot is generated, the assay is suitable for pneumococcal and meningococcal diagnosis, based on the assumption that there will be only one of these two pathogens present in the specimen and not both.

In this study, Gram staining, culture, and RT-PCR were used for testing, and antigen agglutination was rarely used. The LLDs of the multiplex RT-PCR assays described in the present study ranged from four to seven genome equivalents per milliliter (1,000 to 1,500 CFU equivalents per milliliter), which is within the range of bacterial counts in a meningitis CSF specimen. Different LLDs of multiplex RT-PCR assays were described. There was a range of 1 to 210 genome equivalents per RT-PCR (250 to 52,500 CFU equivalents per milliliter) [26]. The evaluation of nucleic acid amplification tests by comparison with less sensitive reference methods such as culture or Gram stain is problematic. The LLD of our RT-PCR is lower than the previously reported LLD for Gram stain (10^5 bacteria/mL). However, CFU/mL does not automatically correspond to the number of DNA copies/mL since several bacteria may aggregate and generate one colony though they constitute several genome equivalents [33]. Gram stain examination of CSF permits a rapid, inexpensive, and accurate identification of the causative bacterium, but it is less sensitive than culture and our RT-PCR. In addition, the diagnosis of meningitis by Gram stain and culture of CSF may be 20% and 30% lower, respectively, in patients who have received prior antimicrobial therapy [9].

The increased sensitivity and specificity of our RT-PCR assay when compared to culture methods is in agreement with published reports for other RT-PCR assays for detection of *N. meningitidis* and *S. pneumoniae* [34]. The sensitivity and specificity of the RT-PCR were 100% (95% CI = 95.45–100) comparable to several multiplex RT-PCR assays recently developed [26,29]. This study demonstrated that the single-tube SG-based RT-PCR described provided a higher sensitivity compared to previously reported conventional single-tube PCR assays for the simultaneous detection of *N. meningitidis* and *S. pneumoniae*. Indeed, with a LLD from 5 to 10 pg, the sensitivity of the conventional PCR tests ranges from 92.3% to 93.9% [14], which is lower than that of our new RT-PCR (from 93.08% to 100%).

Furthermore, our RT-PCR assay provided a detection time (from specimen collection to result) of three hours, which is significantly faster than the one to three days typically required for culture results. The cost of the new RT-PCR is more expensive compared to that of bacterial culture, but RT-PCR is highly sensitive and significantly saves time.

In addition, our SG-based RT-PCR assay can determine the meningococcal serogroup of cases that had negative and positive cultures. The serotype data can impact policy decisions regarding selection of appropriate vaccination programs and can improve epidemiological data. Similar assays are very difficult to perform with pneumococcal serotypes, because *S. pneumoniae* includes more than 94 serotypes. The distribution of serotypes that cause disease varies by age, disease syndrome, disease severity, geographic region, and time [35].

Future improvements of the assay described in this study would include expanding the list of pathogens that could be detected using multiplex RT-PCR assays

coupled with melting curve analysis in one reaction tube.

Regarding the limitations of this study, the overall sensitivity of our new RT-PCR is not high enough to replace culture methods. However, molecular diagnostic assays are currently unable to replace culture due to their limitations in multiplexing capabilities for pathogen identification and antimicrobial resistance detection. The goal of our study was to design an assay that could be used as an adjunct to culture to increase detection of two major bacterial pathogens that cause meningitis and also provide meningococcal serotyping results. Another limitation of our study is that the sample size of CSFs positive for the pathogens in this study was small.

Internal controls were not used in our assays, but it is possible to perform a human *RNaseP* assay to determine if extensive DNA degradation or PCR inhibition occurred. The *RNaseP* gene can serve as an internal control by separately amplifying along with the samples in the same run, as previously reported for fluorescence probe assays [36].

Conclusions

We have designed and tested a duplex single-tube RT-PCR that is rapid, has a very low limit of detection, and is capable of etiologic characterization of bacterial meningitis in suspected meningitis patients. The SG-based RT-PCR for bacterial meningitis diagnosis was shown to be highly sensitive and specific. It can be added to standard microbiologic methods for rapid bacterial meningitis diagnosis and can accurately determine the prevalence of meningitis.

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