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Short Communication

TaqMan and SYBR Green Real-time PCR-Based Assays for the Detection of Streptococcus pneumoniae and Neisseria meningitidis in Cerebrospinal Fluid

Diawara I[1]*, Katfy K[2], Maaloum M[1], Belabbes H[2], Zerouali K[2] and Elmdaghri N[2]

[1]Department of Microbiology, Hassan II University of Casablanca, Morocco

[2]Department of Microbiology and Bacteriology-Virology and Hospital Hygiene Laboratory, Hassan II University of Casablanca and University Hospital Centre Ibn Rochd of Casablanca, Morocco

***Corresponding author:** Diawara I, Department of Microbiology, Faculty of Medicine and Pharmacy, 19, Hassan II University of Casablanca, Tarik Ibnou Ziad, B.P 9154, Casablanca, Morocco

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Abstract

We proposed here an update for the detection of *S. pneumoniae* and *N. meningitidis* in cerebrospinal fluid using a mutliplex real time PCR TaqMan (RT-PCR) based assay with internal control to the routine diagnosis of meningitis compared to the SYBR Green-based RT-PCR method coupled with melting-curve analysis.

The sensitivity and specificity for the duplex RT-PCR TaqMan as well as SYBR Green-based RT-PCR were 100% (95% confidence limits, 90 –100%) for the two bacteria. The addition of the RT-PCR assays to the gold standard methods increased the rate of pathogen detection from 15% to 40% ($p < 0.0001$) for *N. meningitidis* and from 20% to 35% ($p = 0.01$) for *S. pneumoniae*.

Keywords: *Neisseria meningitidis*; *Streptococcus pneumoniae*; Meningitis; Real-time PCR

Short Communication

Acute community-acquired bacterial meningitis remains a devastating infection that frequently results in severe sequelae and carries a high mortality rate (up to 25%). *Neisseria meningitidis* and *Streptococcus pneumoniae* are now considered the leading causes of bacterial meningitis worldwide since the implementation of the *H. influenzae* type b conjugate vaccine [1,2]. Clinical outcome is influenced dramatically by the quality of treatment and early initiation of appropriate antibiotics is crucial. 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 [3].

Recently, we developed a duplex real time PCR for the detection of *N. meningitidis* and *S. pneumoniae* in cerebrospinal fluid for meningitis diagnosis [4]. This assay was based on SYBR Green real-time PCR (RT-PCR) assay. Although this method is an inexpensive, sensitive, and specific method to rapidly diagnose bacterial meningitis, it requires rigorous validation to be incorporated into the routine diagnostics and it cannot include an internal control. PCR inhibitors have been, for a long time, a major problem for RT-PCR systems. We proposed here an update for the detection of *S. pneumoniae* and *N. meningitidis* in cerebrospinal fluid using a mutliplex RT-PCR TaqMan based assay with internal control to the routine diagnosis of meningitis compared to SYBR Green RT-PCR method.

This study was conducted from January 2014 to August 2015 at the Ibn Rochd University Hospital Centre of Casablanca (Morocco), a tertiary care hospital. We used the previously RT-PCR TaqMan based assay for the detection of *S. pneumoniae* and *N. meningitidis* with some modifications [5,6]. The RT-PCR was performed in multiplex to detect the *lytA* and *ctrA* genes specific to *S. pneumoniae* and *N. meningitidis*, respectively. As an internal control, we used the human *RNaseP* gene to assess the presence of PCR inhibitors [7]. All RT-PCR assays were performed on the CFX96® Real-Time System (Bio-Rad, Hercules, USA). DNA was extracted from 200 µL of CSF using QIAamp DNA Mini Kit (Qiagen) based on manufacturer's recommendations. Each reaction multiplex was carried out in a final volume of 25 µL, consisting of 12 µL of TaqMan Universal Master Mix 2X (Applied Biosystems, Foster City, CA, USA), 5 µL of DNA template. The primers, and probes described are adaptations of those previously described [5,6,8]: forward primer, reverse primer, and probe for each target gene were used in the following concentrations: 0.3, 0.6, and 0.1 µM for *lytA*; 0.3, 0.9, and 0.1 µM for *ctrA*; and 0.3, 0.6, and 0.1 µM for *RnaseP*. The *lytA*, *ctrA*, and *RNaseP* probes were labeled at the 5' end with FAM, Cy5, and TET, respectively. The *ctrA* and *RNaseP* probes were changed from those previously published to allow multiplex amplification. A negative control (molecular-grade water sample) and positive control (DNA preparation) for each bacterial pathogen were included in every run. DNA was amplified using the following cycling parameters: heating at 95°C for 10 min followed by 45 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. An increase of the measured fluorescent signal above a calculated background threshold indicated amplification of the target sequence. A sample was considered negative when no increase in fluorescent signal was observed after 45 cycles.

This multiplex RT-PCR TaqMan and our previously RT-PCR SYBR Green were evaluated on 100 CSF specimens obtained from 100 patients with suspected bacterial meningitis who were admitted at the Ibn Rochd University Hospital Centre of Casablanca during the study period. All CSF samples were referred to the laboratory for routine diagnosis of meningitis. The inclusion criteria were the same as our previous studies [4,6].

The gold standard for bacterial analysis of CSF is direct examination, culture on supplemented chocolate agar and Mueller-

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Table 1: Comparison of CSF diagnosis after microbiological analysis (direct examination, culture and/or latex agglutination) and RT-PCR on the 100 CSF samples.

	Diagnosis result after microbiological analysis	Diagnosis result RT-PCR SYBR Green	Diagnosis result RT-PCR TaqMan	*p*-value
Total diagnosis	**35 (35)**	**75 (75)**	**75 (75)**	**< 0.0001**
***S. pneumoniae* n (%)**	**20 (20)**	**35 (35)**	**35 (35)**	**0.005**
Sensitivity, % (95% CI)	57.14 (39.35 – 73.68)	100 (90 – 100)	100 (90 – 100)	
Specificity, % (95% CI)	100 (94,48 – 100)	100 (94.48 – 100)	100 (94.48 – 100)	
PPV, % (95% CI)	100 (83.16 – 100)	100 (90 – 100)	100 (90 – 100)	
NPV, % (95% CI)	81.25 (70.97 – 89.11)	100 (94.48 – 100)	100 (94.48 – 100)	
***N. meningitidis* n (%)**	**15 (15)**	**40 (40)**	**40 (40)**	**< 0.0001**
Sensitivity, % (95% CI)	37.50 (22.73 – 54.2)	100 (91.19 – 100)	100 (91.19 – 100)	
Specificity, % (95% CI)	100 (94.04 – 100)	100 (94.04 – 100)	100 (94.04 – 100)	
PPV, % (95% CI)	100 (78.20 – 100)	100 (91.19 – 100)	100 (91.19 – 100)	
NPV, % (95% CI)	70.59 (59.71 – 79.98)	100 (94.04 – 100)	100 (94.04 – 100)	
Negative samples	**65 (65)**	**25 (25)**	**25 (25)**	**ND**

ND: Not Determined. Statistical analysis was done by EpiInfo 7 (CDC, Atlanta Georgia, USA); NPV: Negative Predictive Value; PPV: Positive Predictive Value; CI: Confidence Interval

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 [9].

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.

Of the 100 CSF specimens, conventional microbiological methods (direct examination, culture, and/or latex agglutination) detected 15% and 20% of meningitis cases caused by *N. meningitidis* and *S. pneumoniae*, respectively. The addition of the RT-PCR assays to the gold standard methods increased the rate of pathogen detection from 15% to 40% ($p < 0.0001$) for *N. meningitidis* and from 20% to 35% ($p = 0.01$) for *S. pneumoniae* (Table 1). The sensitivity and specificity of RT-PCR and bacterial analysis of CSF (Table 1) showed that the sensitivity of the RT-PCR assays was higher than that of microbiological methods. Interestingly, we found that RT-PCR TaqMan and RT-PCR SYBR Green have allowed to diagnose with the same sensitivity and specificity.

For both RT-PCR, although they have the same detection thresholds, RT-PCR TaqMan is more expensive than RT-PCR-SYBR Green. We estimated the cost of our RT-PCR TaqMan, RT-PCR SYBR Green and bacterial culture at $ 28 U.S., $ 10 U.S., and $ 5 U.S. by test, respectively. However, the evaluation of the cost is an indication. It can vary from laboratory to another because we have not given the depreciation cost of the thermal cycler or the realization of diagnostic service.

DNA extraction for RT-PCR SYBR Green consisted to the boiling and freeze-thaw treatment method [4]. This method is less expensive than commercial kits but cannot be applied to the RT-PCR TaqMan assay due to the configuration and stability of TaqMan probes. This increases the cost of the RT-PCR TaqMan assay because it requires the use of DNA extraction kits. Although our results showed that the RT-PCR TaqMan and RT-PCR SYBR Green assays detected bacterial pathogens with the same diagnostic sensitivities and specificities, it is because the samples were chosen to include the criteria for typical bacterial meningitis.

Moreover, the use of RT-PCR SYBR Green requires rigorous optimization to avoid nonspecific amplification which may optionally overlap with the melting curve of the positive controls. Another disadvantage of the SYBR Green RT-PCR is that it cannot be performed in multiplex. This constitutes a major obstacle for interpreting negative results in lack of internal control. RT-PCR TaqMan easily solves this obstacle. Unlike the RT-PCR SYBR Green assays, the multiplex TaqMan RT-PCR can be performed with an internal control. For this purpose, we used the human *RNaseP* gene 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 [8].

In conclusion, these RT-PCR assays for bacterial meningitis diagnosis for *N. meningitidis* and *S. pneumoniae* was shown to be highly sensitive and specific and could be added to standard routine microbiologic methods, for rapid bacterial meningitis diagnosis and to accurately determine the prevalence of meningitis.

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