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The efficiency of sodC gene / *N. meningitidis* detection in comparison with the classical methods for the diagnosis of meningococcal infection

Evaluarea eficienţei Real Time PCR TaqMan utilizând gena sodC / *N. meningitidis* în comparaţie cu metodele clasice utilizate în diagnosticul infecţiei meningococice

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Abstract

Meningococcal infection requires a fast and accurate diagnostic method in order to correctly initiate the antibiotic therapy. The aim of our study was to assess the efficiency of Real Time PCR –Taq Man using sod C gene / N. meningitidis in comparison with the classical methods for the diagnosis of meningococcal infection – direct microscopy, cultivation, latex agglutination and blood culture. We have detected 24/44 (54.54%) patients with meningococcal infection. In both cases of patients with / without previous antibiotic therapy before admission, the AUC (area under curve) had the highest values for RT PCR in CSF and blood analysis. This sod C RT-PCR assay is a highly sensitive and specific method for detection of Neisseria meningitis and it would be useful to include this method like a multiplex in routine testing of patients with clinical meningococcal infection for other etiological agents also.

***Keywords**: Neisseria meningitidis, meningococcal infection, Real-Time PCR, performance criteria.*

Rezumat

Pentru a iniția corect terapia antibiotică și a institui profilaxia pentru contacți, infecția meningococică necesită un diagnostic cât mai rapid și precis. Scopul studiului nostru a fost evaluarea eficienței Real Time PCR –TaqMan utilizând gena sodC / N. meningitidis în comparație cu metodele clasice de diagnostic utilizate în infecția

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meningococică: examen direct, cultură, latex aglutinare din LCR (lichid cefalorahidian) şi hemocultură. Prin RT-PCR au fost identificaţi 24/44 (54.54%) pacienţi cu infecţie meningococică; atât în cazul pacienţilor trataţi cu antibiotice anterior internării, cât şi a celor fără tratament, cele mai înalte valori ale ariei de sub curbă au fost înregistrate în cazul RT-PCR din LCR şi sânge. În concluzie, testul sodC RT-PCR este o metodă rapidă, cu sensibilitate şi specificitate ridicate pentru detecţia Neisseria meningitidis, motiv pentru care ar fi utilă includerea acestei metode ca variantă multiplex, pentru depistarea şi a altor etiologii, în testarea de rutină a pacienţilor cu suspiciune clinică de infecţie meningococică.

***Cuvinte cheie**: Neisseria meningitidis, infecţie meningococică, Real-Time PCR, criterii de performanţă*

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Introduction

Neisseria meningitidis is the leading cause of bacterial meningitis and septicaemia in children and young adults all over the world. Due to its worldwide distribution, epidemic potential, serious complications that can occur, and increased case fatality and morbidity rate, rapid and accurate diagnosis and early treatment are essential (1). For confirming the aetiology, cerebrospinal fluid (CSF) and/or blood culture was until recently the gold standard samples for the diagnosis of meningococcal infection. However, the culture has some disadvantages in terms of speed. Also, the negative effect of previous antibiotic administration on its sensitivity requires the use of some techniques that do not involve cultivation. Real–time PCR (RT-PCR) can be used for the diagnosis of invasive meningococcal infections when previous antibiotic therapy may inhibit bacterial growth (2). This technique for detecting of meningococcal DNA was used by other authors for both *ctr A* and *sod C* genes; each gene detection has different sensitivities, both of them being superior to the classical methods of diagnosis (3-6).

Objectives: the first objective of our research was to determine the impact of RT-PCR on diagnostic yield in patients clinically suspected of meningococcal disease. Second, we aimed to assess the efficiency of *RTPCR –Taq Man* using *sod C* gene / *N. meningitidis* in comparison with the classical methods for the diagnosis of meningococcal infection.

Material and methods

I. Patients and specimens

A prospective cohort study was conducted at the Iaşi "Sf. Parascheva" University Hospital for Infectious Diseases in the interval January 1, 2012 – May 30, 2013. To assess the performance of RT-PCR in the diagnosis of infections caused by *Neisseria meningitidis* every patient was classified upon admission according to clinical signs as probable or possible case of meningococcal infection (e.g., sepsis and/or meningitis) (7). A probable case was defined by the presence of etiologically unconfirmed typical clinical features of meningococcal infection (abrupt onset of fever, petechial-purpuric/necrotic rash, and symptoms of sepsis/septic shock and/or meningitis) (8) and considered by the clinician as the most likely diagnosis. Possible cases were defined as cases in which the clinician considered diagnoses other than meningococcal infection as likely. These included the cases clinically suspected of primary bacterial meningitis, in which the clinical diagnosis was based on classical features: sudden onset with the triad fever, headache, and central type vomiting, agitation/drowsiness, obnubilation/coma, and syndrome of meningeal contracture (stiff neck, Kernig I/II, Brudzinski I/II, or Lesage signs) (9).

II. The diagnosis of meningococcal infection by classical methods

According to the current protocol for positive and etiologic diagnosis used at Iaşi "Sf. Parascheva" University Hospital for Infectious Diseases, a lumbar puncture for CSF collection and venipuncture for blood culture and complete blood count were performed. Also, *N. meningitidis* was rapidly identified by the classic direct bacteriological examination and latex agglutination (LA). Subsequently, a standard microbiological diagnosis (which included CSF seeding on suitable culture media) and antibiotics susceptibility test were made in the laboratory of hospital. Microscopically examination used Gram and blue methylene staining. CSF was cultivated on blood and chocolate agar and blood samples were inoculated in appropriate aerobic and anaerobic culture media. LA test was performed with *Oxoid* kit **(**10).

III. Inclusion and exclusion criteria of patients in the study

After the primary CSF examination, the patients who met the following criteria were included in the study:

- typical clinical symptoms of meningococcal infection and positive direct microscopy or latex agglutination (LA), or
- clinical symptoms typical of meningococcal infection and no other etiologic agent confirmed, or
- isolation by culture of *Neisseria meningitidis* from a normally sterile site (CSF/blood)
- purulent, turbid, or cloudy CSF (with predominance of neutrophils in the sediment, elevated CSF albumin and low CSF glucose levels) and no other etiologic agent confirmed
- clear CSF and lymphocytic predominance (patients treated with antibiotics prior to admission) and no other etiologic agent confirmed.

The following were excluded from the study:

- patients clinically suspected of bacterial meningitis in which the primary CSF examination (direct bacteriological examination, culture and/or latex agglutination) revealed another etiologic agent than meningococcus;
- patients with secondary meningitis (TB, tumours, space occupying processes).

Blood and CSF samples collected in EDTA tubes were then sent to the Microbiology Laboratory of "Gr. T. Popa" University of Medicine and Pharmacy Iaşi for the diagnosis of meningococcal infection by RT-PCR.

IV. The diagnosis of meningococcal infection by RT-PCR

DNA/*Neisseria meningitidis* was purified using the kit *Primer Design Precision™ Gram Negative Bacterial DNA extraction (Primer Design, UK).*The kit has the advantage of rapidity (a sample of DNA can be purified in 16 minutes) and required the use of *Beckman Coulter* micro centrifuge and of the *Biosan* thermo block. The purity and concentration of DNA were analysed with *Nano Drop Pearl* and RT PCR amplification used *Mx3005P Stratagene* thermo cycler. The RT-PCR method supposed the detection of Superoxide dismutase (*sod C*) gene / *N. meningitidis* by *qualitative end point PCR* method, with the *Primer Design*™ genesis kit *(Primer Design, UK)* for *N. meningitidis Taq Man* principle. The pathogen detection mix contained 10 µl of 2 x *Precision™ Mastermix,* 1 µl *N. meningitidis* Primer/Probe mix, 1 µl internal extraction control primer/probe mix and 3 µl RNAse/DNAse free water for a final volume of 15 µl. Five µl of diluted DNA template was added into each well. To confirm extraction of a valid biological template, endogenous actin beta (ACTB) was quantified in parallel through the FAM channel. The amplification protocol included one cycle for enzyme activation for 10 min at 95°C and 50 cycles of 10 sec denatur-

ation at 95°C, 60 sec annealing and extension at 60°C. The *N. meningitidis* specific primer and probe mix was detected through the FAM channel and the internal control was detected through the VIC channel. *The qualitative end point* supposed the inclusion in the RT-PCR plate of a positive control and of negative controls (between 1 and 3). In the case of qualitative *end point PCR method*, the final calls are based on the value of the final fluorescence; the analysis was made within the experiment of *Quantitative Analysis Plate Read,* which allows the software to mark the positive and negative samples with "+" and "-". This software performs the calculation of a *t test* based on the final values of the fluorescence, the final normalized fluorescence and the fluorescence change pre - and post-experiment (11).

V. Statistical analysis

Data were analysed using SPSS version 20.0 (SPSS, Chicago, IL, USA). The level of statistical significance (p-value, the probability of maximal error) was considered 0.05 (5%), a probability (confidence interval) of 95% showing that the decision was fair. Thus, statistical significance was defined as $p < 0.05$ (95% CI).

To assess the performance of the tests used in the diagnosis of meningococcal infection, their predictive value was calculated. A positive predictive value (PPV) indicates the proportion of patients with meningococcal infection of all patients with a positive test (12). Negative predictive value (NPV) is the probability that patients with a negative test to be free of meningococcal infection (12).

To fully assess the discriminatory power of meningococcal infection diagnostic tests, their sensitivity and specificity were also calculated. The sensitivity of a method indicates the proportion of positive test results in patients who have the disease. Sensitivity, specificity and predictive values of a diagnostic test are closely related. The more sensitive a test, the greater the NPV, and the physician is more sure that the patient with a negative test result does not have the disease; the more specific a test, the more positive a doctor is that the patient with a positive test actually has the disease (12).

Likelihood ratios (LR) are the way to combine the sensitivity and specificity of a test in one unit of measure; these are independent of the disease prevalence in the population. In the present study we calculated the LR of a negative test (LR-) which showed the test performance, by comparing the situation when the disease is absent with when the disease is present (12).

Ideally, the physician should use a diagnostic test that is both sensitive and specific. As this is not always the case in practice, physicians have to compromise between sensitivity and specificity. A means of expressing the relationship between the sensitivity and specificity of a test is the ROC curve (characteristic curve in test performance assessment). This allows describing the accuracy of a test and can be used to compare the different diagnostic tests for the same disease in terms of the trade-off between sensitivity and specificity. A test with a good discriminatory power generates a ROC curve crossing the upper left corner of the graph: thus gradually increasing sensitivity, there is no loss of specificity as long as sensitivity does not reach maximum levels. A less accurate test is when the ROC falls closer to the diagonal, moving from lower left to upper right of the graph. The overall accuracy of the test can be described by the area under the ROC curve (AUC); a good test is when this area is largest. The curve can be used to decide where the best compromise between sensitivity and specificity should be done. (12).

The overall accuracy of the test can be described by the area under the ROC curve (AUC); the larger this area, the better the test. The curve can be used to decide where the best compromise between sensitivity and specificity should be (12).

VI. Research ethics

Written informed consent was obtained from all patients enrolled in the study or a legal guardian, after receiving the approval from the Research Ethics Committee of "Gr. T. Popa" University of Medicine and Pharmacy Iaşi. The study conforms to international recommendations on human studies and respects the ethical standards for laboratory tests performed on pathological products from patients as specified in the Declaration of Helsinki. The Real Time PCR testing was a blind testing, without knowing clinical and microbiological data of the patients.

Results

I. Diagnosis of the meningococcal infection by the RT-PCR technique

During the above mentioned interval 44 patients were eligible for the study. Of these 44 patients, 24 (54.54%) were identified with meningococcal infection, confirmed with *N. meningitidis* infection by RT- PCR on: CSF samples alone - 8 cases, blood samples alone - 2 patients, CSF and blood samples - 14 patients.

Twenty-three of the 44 patients (52.27%) received antibiotic treatment prior to admission.

Through the newly introduced method (RT-PCR), 20.45% of the cases with clinical suspicion of primary bacterial meningitis upon admission were identified with meningococcal infection, and thus, the frequency of cases confirmed by RT-PCR in cases with clinical suspicion of meningococcal infection upon admission (34.09%) reached 54.54% (Table I).

II. The efficiency of RT-PCR-Taq Man using the sod C gene / N.meningitidis compared to the classical methods for the diagnosis of meningococcal infection

The efficiency of RT-PCR diagnostic system compared with the classic methods in CSF (direct examination, culture, LA) and blood (blood culture) was studied. One of the analysed criteria was related to the administration of antibiotics prior to sample collection. It was noticed that the proportion of cases identified by standard laboratory techniques was lower in the patients with previous antibiotic therapy compared to those who did not receive prior treatment. Thus, in CSF samples, the proportion of cases identified by direct examination decreased from 22.22% to 20%, and of those identified by culture significantly decreased from 100% to 20%. The proportion of cases identified by blood culture decreased from 22.22% in non-previously treated to 0% in the pre-treated patients. The proportion of LA from the CSF also decreased significantly, from 44.44% in those without antibiotic treatment prior to admission to 0% in those pre-treated. Alternatively, for RT-PCR, we noted that, the results of the test were not significantly influenced by previously administered antibiotherapy (100% in those without antibiotic treatment prior to admission compared to 86.67% in those pre-treated in the CSF, and 77.78% in non-previously treated compared to 60% in the pre-treated patients in the blood).

PPV estimates the rate of true-positive tests confirmed by RT-PCR of the total positive tests by the classic method. By calculating the PPV of

Table I. Meningococcal infection: frequency of cases confirmed by RT-PCR in relation to the clinical suspicion upon admission.

	RT- PCR Confirmed with *N. meningitidis*		
	absent	present	
Possible cases of MI	20	9	**29**
	45.45%	20.45%	65.9%
Probable cases of MI	0	15	**15**
	0.00%	34.09%	34.09%
Total cases	20	24	44
	45.45%	54.54%	

the used tests, we noted that this was 100% for all diagnostic tests (Table II). This showed that the likelihood of a positive test result in patients with meningococcal infection is 100% that is all patients who tested positive actually had the disease.

Given the high case fatality rate, and in order to avoid the risk of not treating a sick patient, the analysis of cases with negative tests results (NPV) is important in meningococcal infection. NPV estimates the proportion of true-negative results confirmed by RT -PCR of the total negative results by the classic test. Thus, great NPVs indicate the proportion of true-negative results confirmed by RT-PCR. By analysing the NPV of the used tests we found significant test-related NPV variations. Thus, in patients without antibiotic therapy prior to admission the NPV for all diagnostic tests was high, the greatest value corresponding to CSF-culture (100%) and CSF-RT–PCR (100%), followed by blood - RT-PCR (85.71%) and LA-CSF (70.59%). In patients receiving antibiotics prior to admission, the greatest NPVs were recorded for CSF - RT-PCR (80%) and blood - RT -PCR (57.14%) (Table II).

Given the severity of the disease and the high risk of death if the patient is not treated, when assessing the quality of a diagnostic test for meningococcal infection we are interested if the test is highly sensitive. Therefore, we have to measure the sensitivity of a test and always choose a sensitive rather than a specific test. The sensitivity (Se) of the analysed tests showed high sensitivity for CSF-RT-PCR (86.67%) and blood-RT-PCR (60%) in patients with antibiotic treatment prior to admission, and in patients not receiving antibiotics prior to admission for CSF-culture (100%), CSF-RT-PCR (100%) and blood-RT-PCR (77.78%). A slightly lower sensitivity, but which cannot be overlooked, was calculated in patients without antibiotic treatment prior to admission for CSF-LA (Se = 44.4%) (Table2).

Table II. The characteristic of the tests used in MI diagnosis

	Se Sensitivity	**Sp Specificity**	**PPV Positive predictive value**	**NPV Negative Predictive value**	**Accuracy**
With preadmission antibiotic therapy- N=23					
CSF – direct exam.	20%	100%	100%	40%	47.83%
CSF-culture	20%	100%	100%	40%	47.83%
CSF-LA	0%	100%	-	34.78%	34.78%
CSF-RT-PCR	86.67%	100%	100%	80%	91.30%
Blood culture	0%	100%	-	34.78%	34.78%
Blood-RT-PCR	60%	100%	100%	57.14%	73.91%
Without preadmission antibiotic therapy- N=21					
CSF- direct exam.	33.33%	100%	100%	66.67%	71.43%
CSF-culture	100%	100%	100%	100%	100%
CSF-LA	44.4%	100%	100%	70.59%	76.19%
CSF-RT-PCR	100%	100%	100%	100%	100%
Blood culture	22.2%	100%	100%	63.16%	66.67%
Blood-RT-PCR	77.78%	100%	100%	85.71%	90.48%

The best test to rule out a disease after a negative diagnostic test result is the test with the lowest LR value (12). Analysis results of (LR-) indicate that in patients without antibiotic treatment prior to admission the best diagnostic tests were CSF-culture [LR (-) = 0%], CSF-RT-PCR [LR (-) = 0%], blood-RT-PCR [LR (-) = 22.2%] and CSF-LA [LR (-) = 55.6%]**.** In patients treated with antibiotics prior to admission LR (-) values indicate improved performance of CSF-RT-PCR [LR (-) = 13.3%] and blood-RT-PCR [LR (-) = 40%] (Figure 1).

The area under the ROC curve (AUC) describes the overall accuracy of the test. For our study lot, the analysis of ROC curves and AUC values demonstrates that in patients who received antibiotic treatment prior to admission the most effective methods for the diagnosis of meningococcal infection were CSF-RT-PCR(AUC = 0.933, p = 0.001) and blood-RT-PCR(AUC = 0.800, p = 0.020). In patients without antibiotic therapy prior to admission, the diagnostic tests with significant discriminatory power were CSF-RT-PCR (AUC = 1, p << 0.01), CSF-culture (AUC = 1, p << 0.01), blood-RT-PCR (AUC = 0.889, p = 0.003) and CSF-LA (AUC = 0.722, p = 0.038).

Discussion

Invasive disease (sepsis and/or meningitis) caused by *N. meningitidis* continues to be a life-threatening condition. Rapid diagnosis is important for the administration of appropriate treatment. Meningococcal infection is usually suspected on clinical grounds and confirmed by conventional laboratory tests: particularly samples of CSF (direct examination and culture), blood (blood cultures), and less commonly by pharyngeal samples (pharyngeal exudates) (13, 14). However, the clinical diagnosis of meningococcal infection can be difficult due to the variability of symptoms. Moreover, to improve the prognosis in cases of suspected meningococcal infection, current guidelines recommend the administration of an antibiotic as soon as possible, sometimes even before reaching the hospital (15). Thus, the percentage of patients with invasive meningococcal infection in which *N. meningitidis* can be isolated by standard micro-

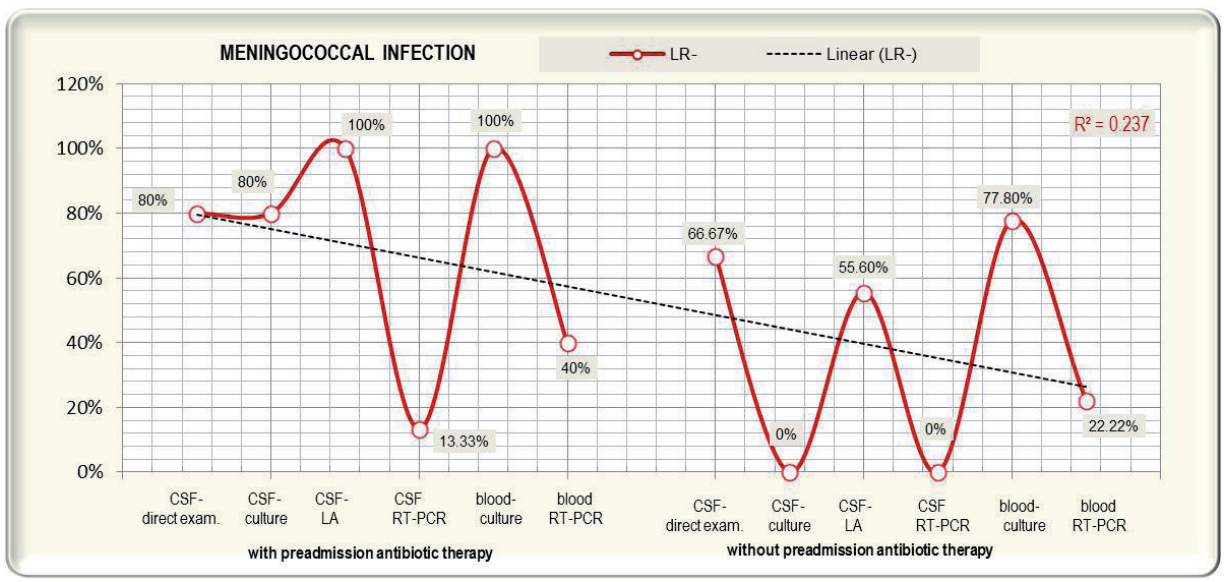

Figure 1. The probability of negative results (LR-) for assessing the diagnostic tests in MI.

biological techniques has decreased. The use of LA for CSF allows the etiologic diagnosis especially in cases pre-treated with antibiotics (16). CSF direct examination and culture are positive in 80-90% of untreated cases of meningococcal infection, significantly reduced rate in case of prior antibiotic administration. Studies show a 50% positivity of blood cultures in patients with untreated meningococcal infection. This rate is reduced to only 5% in case of antibiotic therapy prior to admission (17). This was also found in the cases of meningococcal infection admitted to the Clinic of Infectious Diseases in the last 15 years (1994-2011) (n = 323) in which the antibiotic use prior to admission (39% of cases) resulted in a significant reduction of the proportion of CSF positive results from 82.1% to 56% ($p < 0.001$), positive direct examination from 64.6% to 43.2% ($p < 0.001$), positive CSF culture from 55.9% to 27.2% ($p < 0.001$), positive CSF latex-agglutination from 84.6% to 58.8% ($p = 0.003$), and positive blood culture from 14.7% to 3.5% ($p < 0.01$) (18) and also similar to those from the current study.

Another limitation of conventional methods is the relatively long time required to get the results in case of cultures, as well as the low sensitivity and specificity of LA (19, 20). In regard to the low sensitivity of LA, the results of our study were in accordance with the data from the literature, but contrary to other authors (17), the diagnosis power of this method was lower in patients who received antibiotics prior to admission compared to those non-treated. Therefore, the use of a rapid and more sensitive diagnostic method is required. Thus, RT-PCR is highly sensitive and specific compared to culture (90% in CSF for *N. meningitidis*). Recent studies indicate that in serum, although quite specific, RT-PCR assay is not as sensitive as it contains PCR inhibitors, and the used DNA polymerase is susceptible to this inhibitory activity. For this reason EDTA blood collection is preferred, since it inhibits the degradation activity of extracted DNA (21). The introduction of RT-PCR has significantly improved the confirmation rate, especially in pre-treated patients with negative cultures. Thus, the sensitivity of RT-PCR is clearly superior to that of culture, the meningococcal infection being confirmed in 63% of the patients diagnosed by culture, 88% by PCR, and 96% by RT-PCR (22). The results of our study also proved that by the newly introduced method (RT-PCR), the proportion of cases of identified meningococcal infection increased by **20.45%.** Our results can be compared with the results of other studies (3, 4, 5, 6), which also have found that RT-PCR technique is more sensitive and accurate in detecting DNA/meningococci in comparison with the classical methods. In the present study we found that RT- PCR is a very useful technique for the detection of bacterial DNA in CSF and blood samples from suspected cases of meningococcal infection. In patients who did not receive antibiotic prior to admission, the sensitivity of RT -PCR in CSF samples was 100%, similar to CSF culture, and the sensitivity of RT-PCR in blood samples was 77.79%, compared with only **22.2%** for blood culture. In the patients with antibiotic treatment prior to admission RT-PCR sensitivity was 86.6% in CSF samples compared to classic methods involving the cultivation (Se = 20%), and 60% in blood sample compared to the sensitivity of blood culture which decreased to 0%. Our study also confirms that RT -PCR has a better sensitivity than culture, especially in patients pre-treated with antibiotics. Lumbar puncture is contraindicated in many patients with meningococcal infection, thus reducing the use of conventional diagnosis by culture, direct bacterioscopic examination, and LA from CSF. In these particular cases molecular diagnostic methods using blood samples would be a sensitive and specific alternative that avoids CSF sampling (23). The real advantage of RT -PCR compared to culture is the fast availability of

results, about 2.5 hours after sampling of the samples to the laboratory, as demonstrated by our study. Other studies also show that RT-PCR results are available on the day of admission, compared with culture results requiring a minimum of 24-48 h for confirmation, thus allowing clinicians to initiate targeted therapy, to reduce the tests for other diagnoses, and to institute preventive measures specific for contacts (24, 25).

In conclusion, RT-PCR is a rapid, sensitive and specific test for the diagnosis of meningococcal infection, even for blood samples that are easier to collect than CSF. Since the determination of antibiotic susceptibility of isolates remains a problem for PCR technique, the combination culture and RT-PCR remains essential for antibiotic therapy, although a possible option in the future would be the sequencing of the amplified material in view of detecting the resistance genes. Further studies with larger sample size could be an opportunity to implement this strategy.

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Abbreviations

ACTB - endogenous actin beta
AUC - area under the ROC curve
CSF - cerebrospinal fluid
DNA –desoxy ribonucleic acid
EDTA - ethylenediaminetetraacetic acid
LA – latex-agglutination
LR - likelihood ratios
(LR-) - the likelihood ratios of a negative test
PCR - Polymerase Chain Reaction
RNA - ribonucleic acid
RT-PCR – Real-time PCR
MI – meningococcal infection
NPV - Negative predictive value
PPV - positive predictive value
ROC curve - characteristic curve in test performance assessment
Se - sensitivity,
Sp– specificity
SPSS - Statistical Package for the Social Sciences
TB - tuberculosis

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