False-negative results in Real-Time PCR (RT-PCR) for meningococcal disease diagnosis

Ocorrência de resultados falso-negativos na reação de PCR em Tempo Real (PCR-TR) no diagnóstico da doença meningocócica

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ABSTRACT

Factors responsible for false-negative results (F-N) in RT-PCR assay for detecting *N. meningitidis* in serum and CSF samples were investigated. As the meningococcal disease should be rapidly treated because of its high mortality and epidemic potential, the F-N in diagnostic testing may cause treatment failures and/or on disease restraint in community. Thus, it is crucial to learn the factors which cause F-N in RT-PCR assays. The F-N were induced by inhibition, low quantity of target DNA in extracted samples, and inadequate temperature employed at PCR annealing procedure. As bacterial DNA concentration in samples might be highly variable, the ideal sample volume to be extracted could not be defined. As previously recommended for *N. meningitidis* gene-grouping by RT-PCR assay, the annealing temperature at 60 °C was not suitable for B and W_{135} genogroups. Altogether, these factors induced F-N in 31 samples; therefore, 30 % of *N. meningitidis* detected by RT-PCR were classified as non-genogrouped. The inhibitors and/or the low amount of target DNA induced F-N on RT-PCR**,** independently of the specimen volume used for extracting DNA. However, adjustments on the PCR annealing temperature and amount of extracted DNA added into the reaction might avoid the occurrence of the majority of F-N.
Keywords. real-time polymerase chain reaction, *N. meningitidis*, *N. meningitidis* serogroup, melting temperature, cerebrospinal fluid.

RESUMO

Foram investigados os fatores que causam resultados falso-negativos (RF-N) nas reações de PCR-TR no diagnóstico de *N. meningitidis* em amostras de soro e LCR. Considerando-se que a doença meningocócica deva ser rapidamente tratada pela alta letalidade e potencial epidêmico, o RF-N no ensaio diagnóstico pode ocasionar falhas no tratamento e/ou na contenção da doença na comunidade. Portanto, é importante elucidar os fatores que induzem RF-N na PCR-TR. Os RF-N ocorreram em virtude de inibição, baixa quantidade de DNA-alvo nas preparações de DNA e inadequada temperatura no procedimento de anelamento. Por ser altamente variável a concentração de DNA bacteriano em amostras clínicas, não foi possível definir o volume ideal de amostra a ser extraído. A temperatura a 60 °C para anelamento, recomendada para genogrupagem de *N. meningitidis* por PCR-TR, não foi adequada para genogrupos B e W_{135}. Estes fatores ocasionaram a indução de RF-N em 31 amostras; consequentemente, 30 % das bactérias detectadas pelo PCR-TR foram classificadas como não genogrupáveis. Inibidores e/ou baixa quantidade de DNA-alvo induzem RF-N, independentemente do volume de amostra utilizada na extração de DNA; entretanto, ajustes na temperatura de anelamento e na quantidade de amostra extraída adicionada na reação podem evitar a maioria destes RF-N.
Palavras-chave. reação em cadeia da polimerase em tempo real, *N. meningitidis*, *N. meningitidis* sorogrupo, temperatura de anelamento, líquido cefalorraquidiano.

INTRODUCTION

Bacterial meningitis is a serious disease that requires laboratory confirmation for optimal management. Most bacterial meningitis is still caused by *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* serotype b, although the latter has declined in incidence substantially in many countries since the introduction of *H. influenzae* b vaccine[1,2]. Although bacterial culture is considered the gold standard method for laboratory confirmation, many cases are diagnosed using non-culture approaches, including latex agglutination, counterimmunoelectrophoresis (CIE), conventional PCR and, more recently, real-time PCR (RT-PCR)[3,4].

In April 2007, we begun using multiplex RT-PCR for the diagnosis of bacterial meningitis caused by *N. meningitidis*[4], *S. pneumoniae*[5], and *H. influenzae*[3] on cerebrospinal fluid (CSF) and serum samples in Greater São Paulo, Brazil[6]. For samples that are RT-PCR positive for *N. meningitidis*, genogrouping is also performed using specific singleplex RT-PCR assays (for serogroups A, B, C, Y, W_{135} and X)[4].

Since the introduction of multiplex RT-PCR for diagnosis of bacterial meningitis in Greater São Paulo, two unexpected situations have arisen: (i) we have identified 6 serum samples with false negative RT-PCR results for *N. meningitidis*, and (ii) 25 CSF samples with false negative serogroup-specific RT-PCR results for serogroups B (n=15) and W_{135} (n=10). As RT-PCR is more sensitive and specific than most non-culture diagnostic methods including CIE, the possibility of false negative RT-PCR results is of great interest. The objective of this study was to investigate the reasons for these 31 false negative RT-PCR results.

MATERIAL AND METHODS

Institutional review board (IRB) approvals

The Instituto Adolfo Lutz (IAL), as well as the Comissão Nacional de Ética em Pesquisa, the Brazilian national IRB, approved the study.

Clinical samples

From April to May 2007, we received 534 clinical samples suspected of bacterial meningitis collected in Greater São Paulo, State of São Paulo, Brazil. Six serum and 25 CSF samples with discordant CIE and RT-PCR results were selected to this study (Table 1). Fifteen additional non-discordant sera samples were included in the study for comparison reasons.

Culture, CIE, and latex agglutination

As a retrospective study, culture, CIE, and latex agglutination results for the 25 CSF and 21 sera samples were accessed on our database (Table 2). All 6 serum samples were serogroup C by CIE and latex agglutination, one was *N. meningitidis* C by culture and slide agglutination test using polyclonal antiserum. All 6 sera samples were *N. meningitidis* negative by RT-PCR, but genogrouped as C by RT-PCR genogrouping. Fifteen CSF samples were serogroup B by CIE and latex agglutination, 8 of them were culture positive for *N. meningitidis* and serogroup B by slide agglutination; by RT-PCR all 15 samples were *N. meningitidis* but none of them was genogrouped. The remaining 10 CSF samples were serogroup W_{135} by CIE, latex agglutination, 2 of them were culture positive for *N. meningitidis* and serogroup W_{135} by slide agglutination; by RT-PCR all 10 CSF were *N. meningitidis*, but none of them were genogrouped.

DNA extraction

DNA extraction was performed using QIAGEN QIAamp DNA Blood mini kit (QIAGEN, Valencia, CA) as described by the manufacturer except that an aliquot of 500 µL of sera and CSF were processed, and the DNA was eluted in 50 µL of TE buffer. A second DNA extraction was also performed using 200 µL for all sera and CSF samples with available volume.

Real-time PCR assay

Two different RT-PCR assays were used in this study. The first was a multiplex RT-PCR including primers and probe for *ctrA*[4], *lytA*[5], and *bexA*[3] genes[6]. The second RT-PCR assay was performed as a singleplex for *N. meningitidis* genogroup determination[4]. All samples were submitted to RT-PCR assays with 5 µL as template. As inhibition was one possible reason for discrepant results between CIE and RT-PCR, we also repeated the genogrouping RT-PCR assay using 2 µL and/or 5 µL of extracted DNA as template, from 200 µL and 500 µL of extracted DNA for comparison. Nine CSF samples were submitted to RT-PCR with 2 µL and 5 µL and the remaining 16 CSF samples with 2 µL only.

DNA was amplified with 7300 Real Time PCR system (Applied Biosystems) by using the following

Table 1. Laboratory results for 31 samples with false negative RT-PCR results

Sample #	Material	CIE	Latex agglutination	Conventional PCR	Serogroup from culture*	RT-PCR	
						Multiplex	Genogrouping
36.766	Serum	MenC	MenC	Negative	ND	Negative	C
43.792	Serum	MenC	MenC	MenC	MenC	Negative	C
44.670	Serum	MenC	MenC	Negative	Negative	Negative	C
55.039	Serum	MenC	MenC	Negative	Negative	Negative	C
60.830	Serum	MenC	MenC	Negative	Negative	Negative	C
61.253	Serum	MenC	MenC	Negative	Negative	Negative	C
2.065	CSF	MenB	MenB	MenB	ND	Men	NG
4.128	CSF	MenB	MenB	MenB	MenB	Men	NG
4.366	CSF	MenB	MenB	MenB	ND	Men	NG
6.301	CSF	MenB	MenB	MenB	MenB	Men	NG
8.914	CSF	MenB	MenB	MenB	MenB	Men	NG
8.915	CSF	MenB	MenB	MenB	MenB	Men	NG
9.105	CSF	MenB	MenB	MenB	MenB	Men	NG
17.339	CSF	MenB	MenB	MenB	ND	Men	NG
24.633	CSF	MenB	MenB	MenB	MenB	Men	NG
32.863	CSF	MenB	MenB	MenB	MenB	Men	NG
33.822	CSF	MenB	MenB	MenB	ND	Men	NG
40.276	CSF	MenB	MenB	MenB	ND	Men	NG
40.722	CSF	MenB	MenB	Negative	ND	Men	NG
44.292	CSF	MenB	MenB	Negative	ND	Men	NG
62.107	CSF	MenB	MenB	MenB	MenB	Men	NG
15.333	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG
37.801	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG
61.573	CSF	MenW135	MenW135/Y	MenW135	MenW135	Men	NG
63.237	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG
67.184	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG
79.261	CSF	MenW135	MenW135/Y	MenW135	MenW135	Men	NG
770.501	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG
770.502	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG
770.503	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG
770.504	CSF	MenW135	MenW135/Y	MenW135	ND	Men	NG

*Serogrouping by slide agglutination. CIE: Counterimmunoelectrophoresis; MenB: *N. meningitidis* serogroup B; MenC: *N. meningitidis* serogroup C; MenW135: *N. meningitidis* serogroup W135; NG: non-groupeable; ND: not-done

CSF: cerebros pinal fluid

Table 2. Multiplex RT-PCR results for 21 sera samples according the extraction volume

Sample		CIE	Multiplex RT-PCR Ct value	
			500 µL extraction	200 µL extraction
Group	#		Men	Men
6 false negative sera samples by RT-PCR	36.766	MenC	NA	36
	43.792	MenC	NA	NA
	44.670	MenC	NA	38
	55.039	MenC	NA	NA
	60.830	MenC	NA	38
	61.253	MenC	NA	24
15 additional sera samples	20.929	MenC	38	NA
	24.733	MenC	36	34
	27.382	MenC	28	31
	28.312	$MenW_{135}$	26	24
	36.520	MenC	38	33
	38.655	MenC	40	40
	40.722	MenB	38	NA
	44.292	MenB	39	37
	46.811	MenC	40	28
	49.874	MenC	29	27
	50.859	MenC	26	24
	50.861	MenC	30	29
	53.254	MenC	34	28
	56.607	MenC	34	33
	62.811	MenC	19	24

CIE: Counterimmunoelectrophoresis; MenB: *N. meningitidis* serogroup B; MenC: *N. meningitidis* serogroup C; $MenW_{135}$; NA: no amplification; Men: *N. meningitidis*

cycling parameters: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 seconds and 60 °C or 55 °C for 1 min. Amplification data were analyzed by instrument software. Negative samples were defined as those with cycle threshold (Ct) values greater than 40. As RT-PCR positive controls, 1 ng of extracted DNA from *N. meningitidis* serogroups B, C and W_{135} was used per reaction.

Conventional PCR

Other than inhibition, false negative genogroups B and W_{135} could be related to the annealing temperature used for the RT-PCR assay. To investigate the need for adjusting the annealing temperature, some samples were submitted to conventional PCR using genogroups B and W_{135} RT-PCR primers and using 60 °C and 55 °C, as annealing temperatures. For this conventional PCR we used DNA extracted from the 8 CSF genogroup B *N. meningitidis* culture isolates, and from all 10 CSF with CIE and latex serogroup W_{135} (figure not shown). For some samples RT-PCR genogrouping was also repeated using both annealing temperatures as mentioned above. All 31 samples with suspected false negative RT-PCR results were also tested on a conventional multiplex PCR for *N. meningitidis* identification (*crgA* gene), and serogroup determination (*siaD* for genogroup B, and *synG* for genogroup W_{135})[7]. The PCR products were visualized after run on 2 % agarose gel.

RESULTS

Conventional PCR

Only one of the 6 serum samples was positive for *N. meningitidis* serogroup C by conventional PCR. Twenty

three CSF samples were positive for *N. meningitidis* by conventional PCR, 13 and 10 were serogroups B and W_{135}, respectively (Table 1).

RT-PCR for *N. meningitidis* on sera samples

Four out of 6 false negative sera samples became positive after reducing the extraction volume from 500 µL to 200 µL (Table 2). The remaining two samples were still negative using 200 µL volume for extraction. However, those two samples were RT-PCR genogrouped confirmed as *N. meningitidis* serogroup C. By testing the additional 15 sera samples, the reduction in volume for DNA extraction affected the positivity of 2 samples previously RT-PCR positive for *N. meningitidis* (Table 2). There was no amplification for the other targets of the RT-PCR multiplex assay (*S. pneumoniae* and *H. influenzae*).

***N. meningitidis* RT-PCR genogrouping**

Experiments were conducted for the 15 and 10 false negative genogroups B and W_{135}, respectively. The first experiment was to verify if the recommended annealing temperature for the genogroups B and W_{135} assays were optimized. To accomplish this we used extracted DNA from 8 isolates out of the 15 CIE serogroup B, and all 10 CIE serogroup W_{135} cases (Table 1).

The second experiment was to verify if 500 µL extraction product had inhibitors that could interfere in the reaction. However, because the limited volume for most of the 25 CSF samples, we were only able to additionally extract 200 µL from 9 samples (Table 3). Genogrouping B and W_{135} *N. meningitidis* was performed for these 9 CSF samples using 2 µL and 5 µL at 55° C and 60 °C (Table 3). For the remaining 16 CSF samples,

Table 3. Comparison of RT-PCR genogrouping results using different template volumes, annealing temperatures and CSF extracted volumes

Sample #	CIE	Culture	RT-PCR Genogrouping Ct values							
			500 µL CSF extracted				200 µL CSF extracted			
			60 °C		55 °C		60 °C		55 °C	
			5 µL	2 µL	5 µL	2 µL	5 µL	2 µL	5 µL	2 µL
4.128	MenB	MenB	NA	33	35	23	41	32	26	26
6.301	MenB	MenB	NA	37	32	28	NA	37	32	30
8.914	MenB	MenB	NA	39	NA	26	NA	31	33	27
8.915	MenB	MenB	NA	NA	NA	39	NA	NA	NA	39
9.105	MenB	MenB	NA	33	29	26	41	30	26	27
24.633	MenB	MenB	NA	36	31	28	32	31	27	27
32.863	MenB	MenB	NA	38	33	29	ND	ND	ND	ND
62.107	MenB	MenB	NA	37	32	29	ND	ND	ND	ND
15.333	$MenW_{135}$	ND	43	32	25	26	41	35	24	35
37.801	$MenW_{135}$	$MenW_{135}$	NA	44	NA	32	NA	30	21	19
61.573	$MenW_{135}$	$MenW_{135}$	NA	36	31	31	NA	40	30	30
63.237	$MenW_{135}$	ND	NA	37	32	29	ND	ND	ND	ND
67.184	$MenW_{135}$	ND	41	34	27	30	ND	ND	ND	ND
79.261	$MenW_{135}$	ND	NA	33	27	25	ND	ND	ND	ND
770.501	$MenW_{135}$	ND	NA	31	32	24	ND	ND	ND	ND
770.502	$MenW_{135}$	ND	44	32	26	24	ND	ND	ND	ND
770.503	$MenW_{135}$	ND	NA	44	38	37	ND	ND	ND	ND
770.504	$MenW_{135}$	ND	NA	NA	38	37	ND	ND	ND	ND

CIE: Counterimmunoelectrophoresis; CSF: cerebrospinal fluid; MenB: N. meningitidis serogroup B; $MenW_{135}$: N. meningitidis serogroup W_{135}; NA: no amplification; ND: not-done

genogrouping was performed from the 500 µL extraction at 55 °C using 2 µL as template.

The results from these two experiments show that all the 25 originally false negative genogroup samples became genogrouped when using 500 µL sample extraction, at 55 °C, and using 2 µL of extracted DNA as template (15 genogroup B and 10 genogroup W_{135}).

DISCUSSION

Bacterial meningitis is a public health problem worldwide, and for appropriated control, early diagnosis is necessary. To overcome the lack of culture results, laboratories have used other methods to detect the presence of *N. meningitidis*, *H. influenzae* and *S. pneumoniae* in clinical samples. Conventional PCR and, more recently, RT-PCR assays, are the most promising approaches because they provide rapid detection of different target genes with high sensitivity and specificity.

Some of these PCR assays have been standardized based on whole cell suspensions or DNA extracted from isolates with little or no clinical samples included[4]. In practice moving from bacterial isolates to clinical samples on PCR assays brings at least two new issues to be addressed. The first is the presence of different source of inhibitors, especially on serum samples. The use and amplification of an internal control for inhibition detection does not guarantee that inhibition is not affecting the reaction, since inhibitors do may not affect all reactions equally. In addition, sometimes it is inappropriate or impossible to introduce one more set of primers and probe on a multiplex RT-PCR assay without disturbing the kinetics of other reactions in the mixture, what may result in reduced sensitivity of the assay. The second issue is the wide range of bacteria DNA present in clinical samples. The amount of bacteria DNA is not only related to clinical disease severity, but also on the sample volume available or chosen for DNA extraction[8].

In reality, some PCR assays are introduced in a clinical laboratory routine with strong evidence of high sensitivity and specificity but they have not always been fully validated on clinical samples. It is using prospective results, that adjustments are introduced to optimize the assays. The objective of this work was to optimize our RT-PCR assay, finding the reasons for its apparently lower sensitivity in comparison with the CIE in detecting *N. meningitidis* and its genogroups B and W_{135} on sera and CSF samples, respectively.

When we first began performing multiplex RT-PCR for detection of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* DNA on clinical samples, 200 µL aliquots of sera or CSF were used for DNA extraction. For comparison reasons, CIE was always performed in parallel. After few weeks of testing we had more CIE than RT-PCR positive results on sera and CSF samples. Additionally, we were observing too many RT-PCR results with high Ct values what makes difficult to differentiate a positive from a negative result (data not shown). Increasing the extraction sample volume to 500 µL, we were able to correct these discrepancies. However, after months of using RT-PCR in our laboratory, we found 6 new false negative RT-PCR results. Why did our multiplex RT-PCR not detected *N. meningitidis* on 6 serum samples despite the fact that RT-PCR has higher sensitivity? PCR of blood and blood fractions has been reported to be highly unpredictable and challenging due to the presence of inhibitors in blood and the low concentration of bacterial DNA[3,4,7,9,10,11]. Drawing of blood for PCR at times different from those at which blood for culture was drawn also compromise analysis of sensitivity for both methods.

The reduction in sera volume for DNA extraction reduces the presence of inhibitors but it also reduces the amount of target DNA in the final purified DNA suspension. This reduction appears to have greater effect on the sensitivity of the *S. pneumoniae* component of the assay than on *N. meningitidis* component among our samples (data not shown). As the concentration of DNA present in a sample can be highly variable, it was not possible to define an ideal sample volume to be extracted.

The reduction on sample volume did not make 2 of the 6 false negatives RT-PCR become positive by multiplex RT-PCR (Table 2). As these two samples were genogrouped as C by RT-PCR, and reactions, *ctrA* and *siaD* have the same low limit of detection of 200 fg, the samples still have inhibitors that selectively interfere on the *ctrA* reaction but not on the genogroup C reaction. These are examples of how difficult is to adjust PCR assays for clinical samples, where there is a wide range of DNA target in it, and different types and amount of sample related inhibitors.

The use of extracted DNA from isolates is a necessary step for standardization of a new PCR assay for diagnosis, and that was how *N. meningitidis* RT-PCR genogrouping was optimized[5]. The best primer and probe concentrations were determined based on RT-PCR: (i)

multicomponent signals and exponential curves, (ii) 60 °C annealing temperature, (iii) using as template extracted DNA from isolates. But there was no attempt to check how the annealing temperature was affecting the efficiency of the reaction on clinical samples. Our results show that 60 °C is not the best annealing temperature for B or W_{135} genogrouping reaction on clinical samples, and that was one of the reasons why 15 serogroup B and 10 serogroup W_{135} *N. meningitidis* positives samples by CIE were not genogrouped confirmed by RT-PCR; the other reason was the presence of inhibitors. After reducing the annealing temperature to 55 °C and the amount of template to 2 µL in the reaction, all 25 samples were successfully genogrouped as B or W_{135} and our rate of non-genogrouped samples dropped from 30 % to 7 %. Additional investigations will focus on the reasons for the 7 % of non-genogrouped samples. Interestingly, serogroup C RT-PCR genogrouping is successfully performed using the 60 °C annealing temperature as we have 73 samples genogrouped by RT-PCR as C using this temperature, and 25 and 10 of them were confirmed by CIE and by slide agglutination serogrouping from culture isolates, respectively. The detection of the 25 false negative genogrouping results was only possible because we run in parallel CIE as an additional test to RT-PCR. No problem with positive controls was ever detected. Our results suggest that the annealing temperature and consequently the efficiency of the reaction are less important when DNA template is not a limiting factor.

In summary, false negative results by RT-PCR assay for *N. meningitidis* may occur independently of the chosen volume for DNA extraction (500 µL or 200 µL); a better DNA extraction process where higher sample volumes are processed with more efficiency in removing inhibitors is desirable; optimization of RT-PCR assays for clinical samples should not be only based on extracted DNA from isolates, but also on clinical samples; and it is important to check how the recommended 60 °C annealing temperature for RT-PCR TaqMan assay may affect each individual reaction.

Based on the results of this study, we adopted the following sample processing algorithm:

1) For the DNA extraction process: employ 500 uL for CSF and 200 µL for serum.

2) For the RT-PCR assays for genogroups B and W_{135}: we employ 2 µL of DNA template and annealing temperature of 55 °C. If no amplification, the sample is tested for the presence of the other genogroups.

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