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Analytical Optimization of GeneXpert Ultra for Detection of Tuberculosis in CSF Samples

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Abstract

Detection of *Mycobacterium tuberculosis* complex (MTBC) in extrapulmonary specimens can be challenging due to their paucibacillary nature. This difficulty is especially true for cerebrospinal fluid (CSF), and the low sensitivity and specificity for diagnosis hampers rapid detection and treatment in vulnerable populations. GeneXpert MTB/RIF Ultra has been shown to provide rapid results for detection of MTBC and associated Rifampin resistance, but there is limited data regarding standardized methods for CSF processing on this assay. In this study, we sought to provide guidance regarding the best methods for CSF processing, including optimal volumes to test, length of incubation with sample reagent and finally effects of long-term freezing on detection.

Keywords

GeneXpert Ultra, Tuberculous Meningitis, *Mycobacterium tuberculosis*, CSF

1. Introduction

Tuberculous meningitis is one of the most serious forms of tuberculosis (TB) infection and occurs in 2% - 5% of those with the disease [1]. This form of extrapulmonary TB disproportionally affects young children and individuals with HIV [2] and is associated with higher rates of morbidity and mortality. However, it is likely to be substantially under-reported because the detection of TB from cerebrospinal fluid (CSF) can be challenging due to paucibacillary infections.

The GeneXpert MTB/RIF (Xpert MTB/RIF) was endorsed by the World Health Organization (WHO) in 2015 as the best initial test for the diagnosis of

tuberculous meningitis [3]. However, despite excellent sensitivity in tests of smear-positive sputum samples (97% to 99.2%) [4] [5] [6], results ranged from 18% to 59% in studies of tuberculous meningitis [7] [8] [9] [10] [11]. The Xpert MTB/RIF Ultra was developed as a more sensitive assay, with detection limits approaching that of culture (~10 CFU/mL) at approximately 15.6 CFU/mL vs Xpert MTB/RIF (100 - 120 CFU/mL). This increase in sensitivity is largely due to the "trace call" results, which indicate the detection of minimal bacilli (less than approximately 113 CFU/mL) and do not allow determination of rifampin resistance as the DNA concentration is too low to detect rifampin resistance conferring mutations. Therefore, for tests positive in the trace category, rifampin resistance is reported as indeterminate. Since 2017, WHO recommends Xpert MTB/RIF Ultra as the initial test for extrapulmonary tuberculosis in adults and children [12] [13]. According to this guidance, *Mycobacterium tuberculosis* complex (MTBC) trace results should be considered true-positive results when testing samples from children, extrapulmonary specimens, persons living with HIV, and HIV-negative persons without a prior TB episode or a recent history of TB treatment [14].

The performance of Xpert MTB/RIF Ultra for the diagnosis of tuberculous meningitis has been evaluated in several studies [9] [10] [11] [15] [16] [17] [18] [19]. However, although WHO has put forth guidance regarding optimal processing of CSF using Xpert MTB/RIF [20], neither the manufacturer, Cepheid, nor WHO have provided technical instructions or optimized procedures for extrapulmonary specimens using the Xpert MTB/RIF Ultra. Determining the optimal processing methods for CSF on the Xpert MTB/RIF Ultra can improve the diagnosis of tuberculous meningitis, which can lead to fewer physician consultations and diagnostic tests, and reduced use of antibiotics and visit-cost. Therefore, this study sought to determine the optimal processing methods for CSF on this platform, including specimen volume, incubation time, sample reagent to sample ratio, and the potential impact of freezing/thawing CSF specimens on MTBC detection.

2. Materials and Methods

2.1. CSF Specimens

The CSF specimens (n = 234) used in this study were leftover samples previously submitted to the Johns Hopkins Hospital clinical mycobacteriology laboratory. Briefly, following standard operating procedures, CSF specimens with a volume greater than 5 mL were centrifuged for 17 minutes at 3000 ×g, otherwise, they were processed without prior centrifugation, and inoculated into mycobacterial growth indicator tubes (MGIT 960, Becton Dickinson, Sparks, MD) and Lowenstein Jensen agar slants (BBL™, Becton Dickinson Life Sciences, Sparks, MD). Any leftover CSF specimens were held at 4˚C until a negative result from mycobacterial culture was obtained after 6 weeks of incubation. In addition, prior to spiking with strains of MTBC, all original CSF specimens were tested on

Xpert MTB/RIF Ultra to confirm MTBC negativity.

2.2. CSF Testing

In both phases of the study, CSF specimens were spiked with either a drug-susceptible strain (Strain #2) or a rifampin-resistant MTBC strain (Strain #48) using different concentrations of bacilli ranging from 10^1, 10^2, 10^4 and 10^6 CFU/mL, which includes concentrations above and below the limit of detection for smear. All spiking aliquots were enumerated for CFU/mL by determination of viable counts on Middlebrook 7H11 agar plates (ThermoFisher, Waltham MA).

All testing was performed at the Johns Hopkins Hospital clinical mycobacteriology laboratory after approval by the Johns Hopkins University Institutional Review Board and we followed the manufacturer's assay protocol except where noted as experimental variables. CSF testing occurred in two phases. In phase 1 (n = 144), to determine the optimal parameters needed for testing, spiked CSF specimens with a volume of 0.5 mL were tested at sample reagent to sample ratios (SR) of 3:1 and those with a volume of 1 mL were tested using an SR of 1:1 or 2:1 and mixed by inverting the tubes three times. The tubes were incubated at room temperature for 0, 7.5 or 15 minutes and similarly mixed a second time before being loaded into the Xpert MTB/RIF Ultra cartridge and run on the Cepheid platform.

Additionally, in phase 2, to evaluate the effect of freezing the CSF specimens (n = 90) on MTBC detection, spiked CSF samples (1 mL) were frozen at −20˚C or −80˚C for 35 or 50 days without glycerol being added. After thawing the specimens at room temperature and rigorous vortexing, they were mixed with sample reagent at a 2:1 ratio and allowed to incubate for 15 minutes before being tested on the Cepheid platform.

All experiments were performed using biological replicates to establish a value range. The lowest cycle threshold (Ct) value and the range were recorded for all tests with a positive value (Ct > 0). Trace results were noted as positive only (no Ct value available).

3. Results

Overall, all but one CSF specimen spiked with 10^6 or 10^4 MTBC CFU/mL in phase 1 were detected as positive for MTBC with Ct values in the upper teens to low twenties, respectively, regardless of the sample volume, SR or incubation time (Table 1 and Table 2). CSF specimens inoculated with 10^2 MTBC CFU/mL were detected as positive in 81% of the cases (29/36), including three (10% of positive results) that were trace call results and inoculation of CSF specimens with 10^1 CFU/mL resulted in 58% (21/36) positive tests, including 5/21 (24%) trace calls (Table 1).

In samples inoculated with 10^2 CFU/mL, when not accounting for the positive trace results, the reference incubation time of 15 minutes provided the most positive results, (10/12, 83%), versus 9/12 (75%) and 7/12 (58%) when incubation

time was 7.5 minutes or 0 minutes, respectively (Table 2). However, incubation of CSF specimens with the sample reagent for 15 minutes did not result in better detection of MTBC compared to no incubation time when the trace call results were considered, with both conditions providing 83% positive tests (Table 2).

For samples containing 0.5 mL, increasing the incubation time from 0 to 15 minutes increased the positivity rate from 0% (0/4) to 100% (4/4) and from 75% (3/4) to 100% (4/4) in specimens inoculated with 10^1 CFU/mL and 10^2 CFU/mL, respectively (Table 3). On the other hand, for specimens containing 1 mL of CSF and spiked with 10^1 or 10^2 CFU/mL, the longer incubation time did not increase the detection of MTBC either at a 2:1 or 1:1 SR (Table 4 and Table 5).

Table 1. Phase 1: Xpert MTB/RIF Ultra results on 0.5 and 1 mL CSF specimens. All tests (n = 144).

Positive Xpert MTB/RIF Ultra Tests and Ct values by CSF volume						
Spiked CFU/mL	0.5 mL (n = 48)			1 mL (n = 96)		
	n (%) (# Trace)	Median Ct	Average Ct (range)	n (%) (# Trace)	Median Ct	Average Ct (range)
10^1	5/12 (42%) (1)	23.1	29.7 (22.3 - 39.6)	16/24 (67%) (4)	30.8	31.1 (24.3 - 32.1)
10^2	10/12 (83%) (1)	26.4	25.6 (23.3 - 27.6)	19/24 (79%) (2)	26.4	29.1 (24.3 - 33.4)
10^4	12/12 (100%)	21.9	23.1 (17.6 - 29.7)	23/24 (96%)	18.2	21.9 (16.8 - 26.9)
10^6	12/12 (100%)	18.3	18.3 (17.1 - 20.2)	24/24 (100%)	18.2	18 (16.1 - 18.6)

Table 2. Phase 1: Xpert MTB/RIF Ultra results on CSF specimens incubated with sample reagent for 0, 7.5 or 15 minutes. All tests (n = 144).

Positive Xpert MTB/RIF Ultra Tests and Ct values by incubation time with sample reagent						
Spiked CFU/mL	None (n = 48)		7.5 minutes (n = 48)		15 minutes (REF) (n = 48)	
	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)
10^1	5/12 (42%) (1)	31.45 (30.5 - 32.1)	8/12 (67%) (3)	34.1 (28.1 - 39.6)	8/12 (67%) (1)	29.9 (26.9 - 35.5)
10^2	10/12 (83%) (3)	26 (24.5 - 27)	9/12 (75%)	27.5 (23.7 - 35)	10/12 (83%)	25.3 (22.6 - 27.5)
10^4	12/12 (100%)	22.6 (19.6 - 29.7)	11/12 (92%)	21.8 (16.8 - 27.1)	12/12 (100%)	22.5 (17.6 - 26.9)
10^6	12/12 (100%)	18.2 (16.3 - 20.2)	12/12 (100%)	17.7 (16.1 - 18.3)	12/12 (100%)	18.4 (17.4 - 19.6)

Table 3. Phase 1: Xpert MTB/RIF Ultra results on 0.5 mL CSF specimens by incubation time (n = 48).

Positive Xpert MTB/RIF Ultra Tests and Ct values by SR Incubation Time						
(SR 3:1) Spiked CFU/mL	None (n = 16)		7.5 minutes (n = 16)		15 minutes (REF) (n = 16)	
	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)
10^1	0/4	n/a	1/4 (25%)	39.6	4/4 (100%) (1)	27.2 (26.9 - 27.4)
10^2	3/4 (75%) (1)	26.8 (26.6 - 27)	3/4 (75%)	26.8 (26.6 - 27)	4/4 (100%)	25.3 (22.6 - 27.5)
10^4	4/4 (100%)	24.2 (19.6 - 29.7)	4/4 (100%)	24.2 (19.6 - 29.7)	4/4 (100%)	21.2 (17.6 - 24.3)
10^6	4/4 (100%)	18.3 (17.1 - 20.2)	4/4 (100%)	18.3 (17.1 - 20.2)	4/4 (100%)	18.7 (17.9 - 19.1)

Table 4. Phase 1: Xpert MTB/RIF Ultra results on 1 mL CSF specimens with SR 1:1 by incubation time (n = 48).

Positive Xpert MTB/RIF Ultra Tests by SR Incubation Time						
(SR 1:1) Spiked CFU/mL	None (n = 16)		7.5 minutes (n = 16)		15 minutes (REF) (n = 16)	
	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)
10^1	3/4 (75%) (1)	30.8 (30.5 - 31.1)	3/4 (75%)	35.4 (30.2 - 38.8)	2/4 (50%)	28.7 (21.9 - 35.5)
10^2	4/4 (100%) (1)	25.7 (24.5 - 26.7)	3/4 (75%)	29.2 (23.7 - 34.5)	3/4 (75%)	29.6 (22.5 - 37.7)
10^4	4/4 (100%)	22.1 (19.6 - 25.4)	4/4 (100%)	21.8 (19 - 27.2)	4/4 (100%)	21.8 (17.9 - 24.1)
10^6	4/4 (100%)	18.5 (18.3 - 18.7)	4/4 (100%)	18.1 (17.9 - 18.3)	4/4 (100%)	18.7 (18.3 - 19.6)

Table 5. Phase 1: Xpert MTB/RIF Ultra results on 1 mL CSF specimens with SR 2:1 by incubation time (n = 48).

Positive Xpert MTB/RIF Ultra Tests by SR Incubation Time						
(SR 2:1) Spiked CFU/mL	None (n = 16)		7.5 minutes (n = 16)		15 minutes (REF) (n = 16)	
	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)	n (%) (# Trace)	Avg Ct Value (Range)
10^1	2/4 (50%)	32.1 (32.1)	4/4 (100%) (3)	28.1	2/4 (50%)	27.2 (24.3 - 30.1)

Continued

10^2	3/4 (75%) (1)	26.2 (26.2)	3/4 (75%)	30.8 (26 - 35.3)	3/4 (75%)	28.2 (24.3 - 33.4)
10^4	4/4 (100%)	21.5 (20 - 23.2)	3/4 (75%)	18.1 (16.8 - 19.4)	4/4 (100%)	24.1 (18.8 - 26.9)
10^6	4/4 (100%)	18.1 (16.3 - 18.4)	4/4 (100%)	17.6 (16.1 - 17.9)	4/4 (100%)	17.9 (17.4 - 18.6)

In addition, in phase 1 of this study, there were no differences in the level of detection between MTBC drug-susceptible and rifampin-resistant isolates (data not shown).

For Phase 2, storage of spiked CSF specimens under freezing conditions for up to 50 days did not affect the detection of MTBC when present at concentrations of 10^6 or 10^2 CFU/mL (Table 6). All CSF specimens stored at −20˚C or −80˚C for 35 or 50 days and spiked with 10^6 or 10^2 CFU/mL of a drug-susceptible (n = 54) or rifampin–resistant strain (n = 36) were detected as positive for MTBC, which included 6/18 (33%) trace call results within the specimens inoculated with 10^2 CFU/mL of a drug-susceptible strain. On the other hand, MTBC was detected in 80% (24/30) of the CSF specimens spiked with 10^1 CFU/mL of a drug-susceptible (n = 18) or rifampin-resistant strain (n = 12) before being frozen, including trace call results in 2/18 (11%) specimens spiked with a drug-susceptible strain. All 12 CSF specimens inoculated with 10^1 CFU/mL of an MTBC rifampin-resistant strain and kept frozen for 50 days were detected as positive, while the drug-susceptible strains were detected in 75% and 60% of the specimens when they were frozen at −20˚C or −80˚C, respectively, for 35 days. Overall, Ct values were slightly lower when the CSF specimens were spiked with a rifampin-resistant MTBC than with a drug-susceptible strain.

4. Discussion

Several studies have evaluated the performance of Xpert MTB/RIF Ultra for the diagnosis of tuberculous meningitis, which have shown conflicting results regarding sensitivity. While some studies suggested that Xpert MTB/RIF Ultra was significantly more sensitive than Xpert MTB/RIF for the diagnosis of tuberculous meningitis [10] [11], others proposed that Xpert MTB/RIF Ultra was not statistically superior than Xpert MTB/RIF for diagnosing tuberculous meningitis in HIV-infected and -uninfected adults [9]. These contradictory results could be due to the use of non-standardized procedures to perform the assays and therefore, the type of CSF sample (fresh/frozen), volume of CSF tested, SR ratios and incubation times differed significantly between studies. Given the variability in sample processing protocols, we aimed to evaluate the effect of those variables on the detection of MTBC by Xpert MTB/RIF Ultra with the goal of developing a standardized protocol for processing CSF specimens for use in subsequent studies and in clinical settings.

Table 6. Phase 2: Xpert MTB/RIF Ultra results on 1 mL CSF specimens spiked with a drug-susceptible or a rifampin-resistant MTBC strain and stored under freezing conditions for 35 or 50 days (n = 90).

Positive Xpert MTB/RIF Ultra Tests on CSF specimens stored at −20˚C or −80˚C								
(SR 2:1)/15min Spiked CFU/mL	Drug-susceptible MTBC Stored for 35 days (n = 54)				Rifampin-resistant MTBC Stored for 50 days (n = 36)			
	−20˚C (n = 23)		−80˚C (n = 31)		−20˚C (n = 15)		−80˚C (n = 21)	
	n (%) (Trace)	Avg Ct value (Range)	n (%) (Trace)	Avg Ct value (Range)	n (%) (Trace)	Avg Ct value (Range)	n (%) (Trace)	Avg Ct value (Range)
10^1	6/8 (75%) (1)	31.1 (26.2 - 33.7)	6/10 (60%) (1)	28 (26 - 30.6)	5/5 (100%)	27.4 (24.1 - 31.7)	7/7 (100%)	24.7 (22.5 - 27.4)
10^2	8/8 (100%) (3)	29.2 (29.4 - 31.2)	10/10 (100%) (3)	28.1 (24.8 - 30.4)	5/5 (100%)	23.1 (21.5 - 24.4)	7/7 (100%)	22.4 (21.1 - 24.2)
10^6	7/7 (100%)	20.1 (18.5 - 22)	11/11 (100%)	19.7 (18.4 - 22.1)	5/5 (100%)	18.5 (17.2 - 19)	7/7 (100%)	18.6 (17.4 - 19.9)

The sample reagent supplied by the Xpert MTB/RIF Ultra manufacturer contains NaOH and isopropanol used to liquefy the sample, reduce the inherent biohazard risks, and inactivate PCR inhibitors [21]. Since CSF is a liquid and frequently a paucibacillary specimen, it has been proposed that the addition of sample reagent is not necessary and that it dilutes the bacillary concentration in the specimen potentially reducing the probability of detecting MTBC [22]. However, our results showed that even though the volume of CSF tested, and SR ratio used did not appear to affect the performance of the test, incubating the CSF specimen with the sample reagent for 15 minutes significantly increased the detection of MTBC when compared with no incubation time, particularly at the lower bacillary loads and when specimens containing 0.5 mL of CSF were tested. The better performance of the assay with increased incubation time was demonstrated not only by a higher proportion of positive samples but also by the lower Ct values obtained with the specimens incubated with the sample reagent for 15 minutes (**Tables 2-4**). Therefore, taking into consideration the potential biohazard challenges with either eliminating sample reagent addition or removing the incubation step with it, it is suggested that the standard 15-minute incubation with sample reagent be continued, even when volumes of CSF larger than 0.5 mL are being used for testing.

The two volumes of CSF tested in this study (0.5 and 1 mL) performed similarly for the detection of MTBC and using a volume as low as 0.5 mL did not appear to affect the performance of Xpert MTB/RIF Ultra. Whether the use of a volume lower than 0.5 mL of CSF could affect the sensitivity of the assay warrants further investigation as often, the amount of CSF received in the laboratory is extremely limited and studies evaluating the performance of Xpert MTB/RIF Ultra using 200 µL to perform the assay failed to demonstrate increased sensitivity compared to the Xpert MTB/RIF [9] versus several other studies using higher

volumes of CSF [10] [11] [15] [16] [17].

Consistent with previous studies [11], the storage of CSF specimens under freezing conditions at −20˚C or −80˚C for up to 50 days did not appear to impair or alter the performance of the assay and both drug-susceptible and rifampin-resistance strains were similarly detected. However, storage at −80˚C appears to provide better results than storage at −20˚C since the Ct values from samples stored at the former temperature were lower than those obtained with the specimens stored at −20˚C. This observation may be related to differences in cell wall damage or clumping of bacilli under the two conditions especially considering that the bacilli were frozen in the specimen matrix without glycerol. Addition of glycerol to the CSF is an important consideration as it may help to avoid cell wall damage and excessive clumping of bacteria, both of which could impact assay results. However, currently the impact of glycerol and CSF specimen freezing on the performance of the Xpert MTB/RIF Ultra assay warrants further investigation.

Our study did have some limitations. We did not evaluate the effect of CSF centrifugation on assay performance. While most previously published studies used centrifuged CSF specimens to evaluate the performance of Xpert MTB/RIF Ultra, others have suggested that centrifugation is not strictly necessary, and it offers minimal advantage if any for the detection of MTBC by Xpert MTB/RIF Ultra [22]. In addition, we did not perform parallel testing of fresh and frozen specimens which did not allow us to compare the performance of these two samples under the different environmental conditions.

In conclusion, incubation with the sample reagent is needed for optimal performance of the Xpert MTB/RIF Ultra assay using the standard incubation time of 15 minutes recommended by the manufacturer. This incubation time recommended for processing sputum specimens appears to be optimal for CSF specimens as well, improving detection while minimizing the biosafety risks of exposure to live MTBC. Additionally, using a volume of CSF as low as 0.5 mL with an SR of 3:1, or an SR of 1:1 or 2:1 for volumes of 1 mL, provides similar results. Finally, if storage of the CSF specimens is needed before testing, storage at −80˚C appears to perform slightly better than storing at −20˚C for up to 35 days and does not affect detection of either drug-susceptible or rifampin-resistant strains.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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