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MAJOR ARTICLE

Diagnostic Value of Cross-priming Amplification Combined With CRISPR-Cas12b in Detecting Cell-free DNA in Tuberculous Pleural Effusion

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Background. Diagnosis of tuberculous pleural effusion (TPE) remains challenging. Studies have shown that detecting cell-free *Mycobacterium tuberculosis* (cf-TB) DNA in pleural effusion can improve TPE diagnosis. This study aimed to evaluate the diagnostic value of our recently developed TB One-Pot assay, which combines cross-priming amplification with CRISPR-Cas12b, in detecting cf-TB for TPE.

Methods. Pleural effusion samples were collected from inpatients with suspected TPE at Hangzhou Red Cross Hospital. After centrifugation, the precipitate was used for culture, Xpert, and pleural effusion cytologic testing, while the supernatant was used for biochemical and cf-TB assays, including TB One-Pot and the quantitative polymerase chain reaction method (cf-TB-PCR). Assessment of diagnostic performance was based on a comprehensive reference standard.

Results. A total of 115 patients were included: 88 TPE cases (diagnosed per the comprehensive reference standard) and 27 non-TPE cases. The sensitivity of TB One-Pot in detecting pleural cf-TB for diagnosing TPE was 64.8%, with an area under the curve (AUC) of 0.805, significantly superior to culture and Xpert ($P < .05$). When compared with cf-TB-PCR (sensitivity, 53.4%; AUC, 0.767) and the adenosine deaminase assay (sensitivity, 52.3%; AUC, 0.761), TB One-Pot demonstrated slightly higher sensitivity and AUC, but the differences were not statistically significant ($P > .05$). The specificity of TB One-Pot was 96.3%, while the specificity of the other tests was 100%, with no statistically significant differences ($P > .05$).

Conclusions. cf-TB provides direct evidence of the etiology of TPE. TB One-Pot for detecting cf-TB in diagnosing TPE outperforms existing TB laboratory tests and may represent a more effective approach for TPE diagnosis in resource-limited settings.

Keywords. cell-free *Mycobacterium tuberculosis*; cross-priming amplification; cell-free DNA; CRISPR-Cas12b; tuberculous pleural effusion.

Tuberculous pleural effusion (TPE) is a common extrapulmonary manifestation of tuberculosis (TB) in adults, characterized by the dissemination of *Mycobacterium tuberculosis* (Mtb) or its metabolites into the pleural cavity [1, 2]. In China, TPE accounts for approximately 40% of pleural effusion cases [3]. Timely identification of TPE in patients with undiagnosed pleural effusion is crucial, as delayed diagnosis may lead to severe complications, including pleural thickening, empyema, calcification, and long-term pulmonary function impairment [4].

Given the atypical clinical presentation of the disease and the difficulty in isolating Mtb due to low bacterial burden, the diagnosis of TPE presents challenges. Currently, the gold standard diagnostic methods for TPE include culturing Mtb from pleural effusion, performing Ziehl-Neelsen acid-fast staining, and conducting pleural biopsy. Acid-fast bacilli are detected in <10% of cases in pleural effusion samples, and culture results can take up to 8 to 12 weeks with low sensitivity (2%–32%) [5]. Pleural biopsy is an effective diagnostic tool for TPE, with sensitivity ranging from 45% to 85.5%, but its invasiveness may lead to surgical complications, making it unsuitable for repeated examinations and limiting its clinical utility [3]. Nucleic acid amplification tests (NAATs) may play a role in confirming TPE diagnosis. GeneXpert MTB/RIF (Xpert; Cepheid) is a fully automated seminested real-time polymerase chain reaction (PCR) system targeting the *rpoB* gene, capable of simultaneously detecting Mtb and rifampicin resistance within 2 hours [6]. However, NAATs, represented by Xpert, have a sensitivity of approximately 30% in diagnosing TPE [7–9], limiting their utility in early diagnosis. Additionally, while immunologic

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biomarkers such as the adenosine deaminase assay (ADA) and interferon-γ assay are valuable for TPE diagnosis, they suffer from low specificity and may not sufficiently distinguish TPE from malignant tumors of the hematopoietic system or empyema [5, 10], necessitating cautious interpretation of results alongside clinical findings and other test results. There is an urgent need to develop new, more accurate, and effective methods to improve the diagnosis of TPE, aiming to enhance patient outcomes and standardize TPE management.

As a result of cellular turnover or excessive damage, dying human cells and microbes release cell-free DNA (cfDNA) into the bloodstream. This cfDNA enters various body fluids through blood circulation and can be analyzed via PCR or sequencing [11]. cfDNA has well-established applications in cancer detection [12–14], organ transplant monitoring [15], prenatal genetic screening [16], among others. The detection of cell-free Mtb (cf-TB) DNA in human plasma, pleural effusion, and other body fluids can provide direct evidence of Mtb and serve as a biomarker for TB diagnosis [17].

cf-TB serves as an attractive biomarker for diagnosing TPE. Previous studies have demonstrated that NAATs for detecting cf-TB exhibit superior sensitivity as compared with detecting Mtb genomic DNA [7, 18, 19], with cf-TB outperforming Xpert and culture in TPE diagnosis [7, 18, 19]. Despite NAATs demonstrating good specificity and sensitivity for early diagnosis, they require relatively long PCR processing times (approximately 1 hour) [7], expensive precision instruments, and trained personnel. Moreover, these tests are typically conducted in centralized laboratories, which limits their practical application. The clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein (Cas) system, in combination with isothermal amplification methods, has recently been employed for pathogen detection, showing tremendous potential and garnering widespread attention [20–24]. Our team previously developed a novel TB detection method using cross-priming amplification (CPA) combined with the CRISPR-Cas12b system, termed TB One-Pot. This method integrates nucleic acid isothermal amplification and target detection steps into a single tube, overcoming the drawbacks of traditional PCR and the risk of cross-contamination associated with the CRISPR diagnostic mode based on preamplification before adding to the CRISPR system. TB One-Pot can amplify at 58 °C, deliver results within 32 minutes, and demonstrate TB diagnostic sensitivity and specificity comparable to Xpert in sputum testing [25]. Given the favorable performance of cf-TB in diagnosing TPE in previous studies and the excellent diagnostic performance and ease of operation of TB One-Pot, this study aims to evaluate the diagnostic value of TB One-Pot for detecting cf-TB in TPE.

METHODS

Study Design and Populations

We conducted a retrospective collection of medical records from hospitalized patients aged ≥16 years who visited Hangzhou Red Cross Hospital for the first time between April 2022 and November 2022 with pleural effusion and suspected TPE. All patients underwent standard thoracentesis to collect pleural effusion samples for biochemical and routine analysis, Mtb culture, and Xpert testing. Additional tests were performed on some patients to assist in the final diagnosis: imaging and histologic examinations, such as computed tomography and ultrasound; TB-related pleural effusion tests, including acid-fast bacilli smear microscopy and histopathology; and QuantiFERON-TB Gold In-Tube testing in whole blood samples for further diagnostic assistance. All commercial assays were conducted according to the manufacturers' instructions. This study was approved by the Hangzhou Red Cross Hospital Human Research Ethics Committee, and patient informed consent was waived.

Diagnostic Criteria for TPE

Patients were stratified into 3 groups based on diagnostic criteria [26, 27]:

Definite TPE: Isolation of Mtb from pleural effusion, sputum, or pleural tissue via culture, microscopy examination, Xpert, or histopathologic evidence of caseating granulomas in pleural biopsy.

Probable TPE: Absence of microbiological confirmation in pleural effusion, but all patients met clinical suspicion criteria—including typical clinical symptoms, significant radiographic evidence, elevated pleural effusion adenosine deaminase, positive immunologic results, empirical anti-TB treatment, and marked symptom improvement after 1-month follow-up. Definite TPE and probable TPE are considered the composite reference standard for diagnosing TPE, which integrates clinical, radiologic, cytologic, biochemical, microbiological, histopathologic, and molecular findings.

Non-TPE: Cases with confirmed diagnoses of other diseases, with no indications of TB in any investigations.

Pleural Effusion Collection, Pretreatment, and Process of cf-TB Detection

Given the retrospective nature of this study, we collected surplus pleural effusion samples after routine analysis, ensuring that they were sufficient for subsequent cf-TB testing. We then reviewed medical records to identify samples that had undergone simultaneous Mtb culture, Xpert testing, and pleural effusion biochemical and routine analyses. Notably, these analyses were all conducted within 1 to 4 hours of sample collection. For cf-TB testing purposes, the pleural effusion samples were centrifuged at 3000*g* for 10 minutes within 1 hour of collection. Afterward, the supernatant was stored at −80 °C until

cfDNA extraction and testing. For cf-TB extraction, after thawing on ice, 5 mL of the supernatant was centrifuged again at 16 000*g* for 10 minutes. The supernatant was carefully transferred into a new tube, avoiding disturbance of any pellets. cfDNA was extracted from the supernatant via the TIANamp Magnetic DNA Kit (DP710; Tiangen) following the manufacturer's instructions.

TB One-Pot Detection of cf-TB. Following the procedures outlined in previous studies [25], in brief 5 µL of the template was added to a mixture containing the CPA amplification system and the CRSPIR system, including purified Cas12b protein, trRNA, crRNA, and a fluorescent reporter probe. In the presence of the Mtb-specific IS*6110* fragment, the Cas12b/crRNA complex binds to the target sequence, forming a ternary complex that activates the transcleavage activity of Cas12b. Subsequently, the fluorescent reporter probe is cleaved by Cas12b, generating a fluorescent signal for the detection of pleural effusion cf-TB. Signal monitoring was conducted with the CFX96 deep-well real-time system (Bio-Rad Laboratories).

PCR Detection of cf-TB. For PCR detection of cf-TB (cf-TB-PCR), primers were designed to target the IS*6110* sequence, and PCR detection was performed by a TaqMan probe-based assay. The nucleotide sequences were as follows: forward primer, GCAGACCTCACCTATGTGTCG; reverse primer, CGTAGGCGTCGGTGACAAAG; probe, 56-FAM-ACAAA GGCCACGTAGGCGAACCCTGCC-3BHQ1. The following were added for each PCR reaction: 4 µL of cfDNA, 5 µL of Animal Detection U + Probe qPCR Super PreMix (Nanjing Vazyme Biotech Co, Ltd), 0.3 µL each of forward and reverse primer (at a 10µM concentration), and 0.4 µL of the probe (at a 10µM concentration). PCR amplification and signal monitoring were performed by the CFX96 deep-well real-time system. The PCR amplification reaction conditions were as follows: initial denaturation at 95 °C for 2 minutes, followed by 35 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds, and extension. Fluorescence data were analyzed, and a result was considered positive if the curve displayed or approached an S shape and the cycle threshold value was ≤35.

Mycobacterial Culture and Xpert Testing

The sediment from 10 mL of pleural effusion was collected by centrifugation. Liquid Mtb culture was conducted with the MGIT 960 culture system (Becton and Dickinson) in accordance with the manufacturer's guidelines. Subsequently, confirmation of all positive cultures was performed with the Mycobacterium RT-PCR detection kit (CapitalBio Corporation), which enables simultaneous screening for Mtb and nontuberculous mycobacterial infections [28]. Identification as Mtb signifies a positive culture, whereas identification as nontuberculous mycobacterial or negative indicates a negative culture. The sediment, collected by centrifugation at room temperature at 3000*g* for 10 minutes from 10 mL of pleural effusion, was mixed with twice the volume of the sample processing solution from the detection kit, vortexed for 15 seconds, and allowed to stand at room temperature for 15 minutes. Following this, 2 mL of the liquid was then added to the Xpert reaction cartridge, and the system automatically generated Mtb detection results and assessed rifampicin resistance status within 2 hours.

Statistical Analysis

Participant characteristics are presented by median, IQR, and proportions. Statistical analyses were conducted with MedCalc software to determine the sensitivity, specificity, positive predictive value, negative predictive value, and area under the curve (AUC) for different diagnostic methods. The cutoff value was identified as the point on the receiver operating characteristic curve with the maximum Youden index, representing the optimal balance between sensitivity and specificity. Continuous variables were compared by *t* tests, categorical variables with chi-square tests, and AUC comparisons with *Z* tests. All statistical tests were 2-tailed, with a significance level of $P < .05$ considered statistically significant. Venn diagrams and heat maps were generated with online tools (https://hiplot.com.cn; https://www.chiplot.online), and data visualization was performed by Prism version 9.4.1 (GraphPad).

RESULTS

Participant Characteristics

Among 146 patients with suspected TPE, 8 were excluded due to inconclusive diagnoses, 13 due to duplicate specimens, and 10 due to unavailability of pleural effusion samples (Figure 1). Among the 115 patients included in the study, 88 were diagnosed with TPE according to the composite reference standard, comprising 45 cases of definite TPE and 43 cases of probable TPE, while 27 served as controls without TPE. The control group consisted of 6 cases of nonhematologic malignancies, 2 of lymphoma, 7 of heart failure, 2 of hypoproteinemia, 5 of pleural effusion due to other causes, and 5 of parapneumonic effusion (PPE). Based on previous studies [29], PPE is defined as any exudative effusion associated with bacterial pneumonia, lung abscess, or bronchiectasis. Among the 5 PPE cases, 3 were classified as complicated PPE and 2 as simple PPE. Definite and probable TPE cases were considered TPE and used as the gold standard for calculating diagnostic performance. Table 1 summarizes the demographic and baseline characteristics of the participants included in this study. Overall, the majority of the study population was male (81.7%, 94/115), with a median age of 63 years (range, 16–98), and patients with TPE were younger than patients without TPE. Examination of pleural effusion samples showed significantly higher levels of white blood cells, total protein, lactate

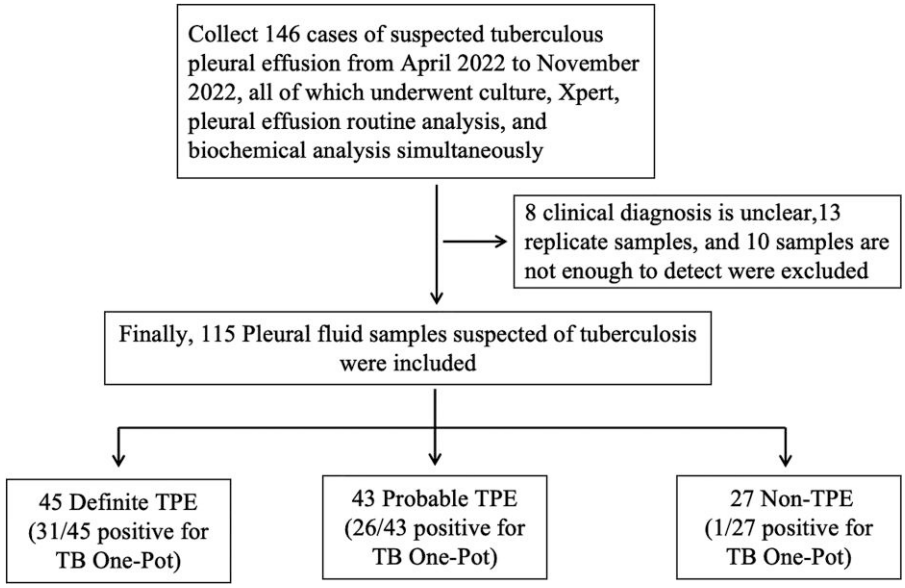

Figure 1. Flowchart of patient enrollment scheme. Abbreviations: TB, tuberculosis; TPE, tuberculous pleural effusion.

Table 1. Baseline Characteristics and Demographics of Participants

	No. (%) or Median (IQR)		
Characteristic	CRS-Diagnosed TPE (n = 88)	Non-TPE (n = 27)	*P* Value
Sex			.042
Male	76 (86.4)	18 (66.7)	
Female	12 (13.6)	9 (33.3)	
Age, y	61.0 (39.3–69.0)	80.0 (59.0–88.0)	<.001
Pleural fluid			
Lymphocyte ratio	82.5 (56.3–96.8)	68.0 (37.0–93.0)	.106
White blood cell, × 10^6	1110.0 (470.0–3250.0)	580.0 (290.0–1040.0)	<.001
Red blood cell, × 10^6	2500.0 (1005.0–5438.0)	800.0 (300.0–7750.0)	.080
Total protein, g/L	47.6 (35.7–52.8)	34.7 (29.0–49.8)	.007
Albumin, g/L	24.6 (18.5–29.0)	19.0 (16.2–26.0)	.062
LDH, U/L	309.5 (175.8–589.3)	147.0 (115.0–319.0)	.002
ADA, U/L	35.6 (13.5–51.9)	7.4 (4.9–12.8)	<.001
Glucose, mmol/L	6.5 (4.8–8.3)	7.1 (5.8–8.1)	.492
Chlorine, mmol/L	102.8 (100.7–105.1)	106.0 (101.2–110.6)	.008
hs-CRP, mg/L	15.1. (8.0–34.1)	6.1 (2.8–15.2)	<.001

Abbreviations: ADA, adenosine deaminase; hs-CRP, high-sensitivity C-reactive protein; LDH, lactate dehydrogenase; TPE, tuberculous pleural effusion.

dehydrogenase, adenosine deaminase, and high-sensitivity C-reactive protein in the TPE group as compared with the non-TPE group ($P < .05$), while chloride levels were significantly lower ($P < .05$).

Comparison of Different Testing Methods

Figure 2*A* presents the clinical and diagnostic data for participants suspected of having TPE, revealing limited overlap between culture and Xpert results from pleural effusion and respiratory samples. The specificity of blood QuantiFERON-TB Gold In-Tube for TPE diagnosis appears to be low. The Venn diagram illustrates the positive samples obtained by Xpert, TB One-Pot, ADA, and mycobacterial culture in patients with TPE, revealing the overlapping distributions of these methods. When positive cases detected by a single method were assessed, TB One-Pot identified 17 cases alone, while Xpert, ADA, and culture detected 2, 10, and 0 cases, respectively (Figure 2*B*). Furthermore, fluorescence values in the TPE group were significantly higher than those in the non-TPE group ($P < .0001$; Figure 2*C*). There was no

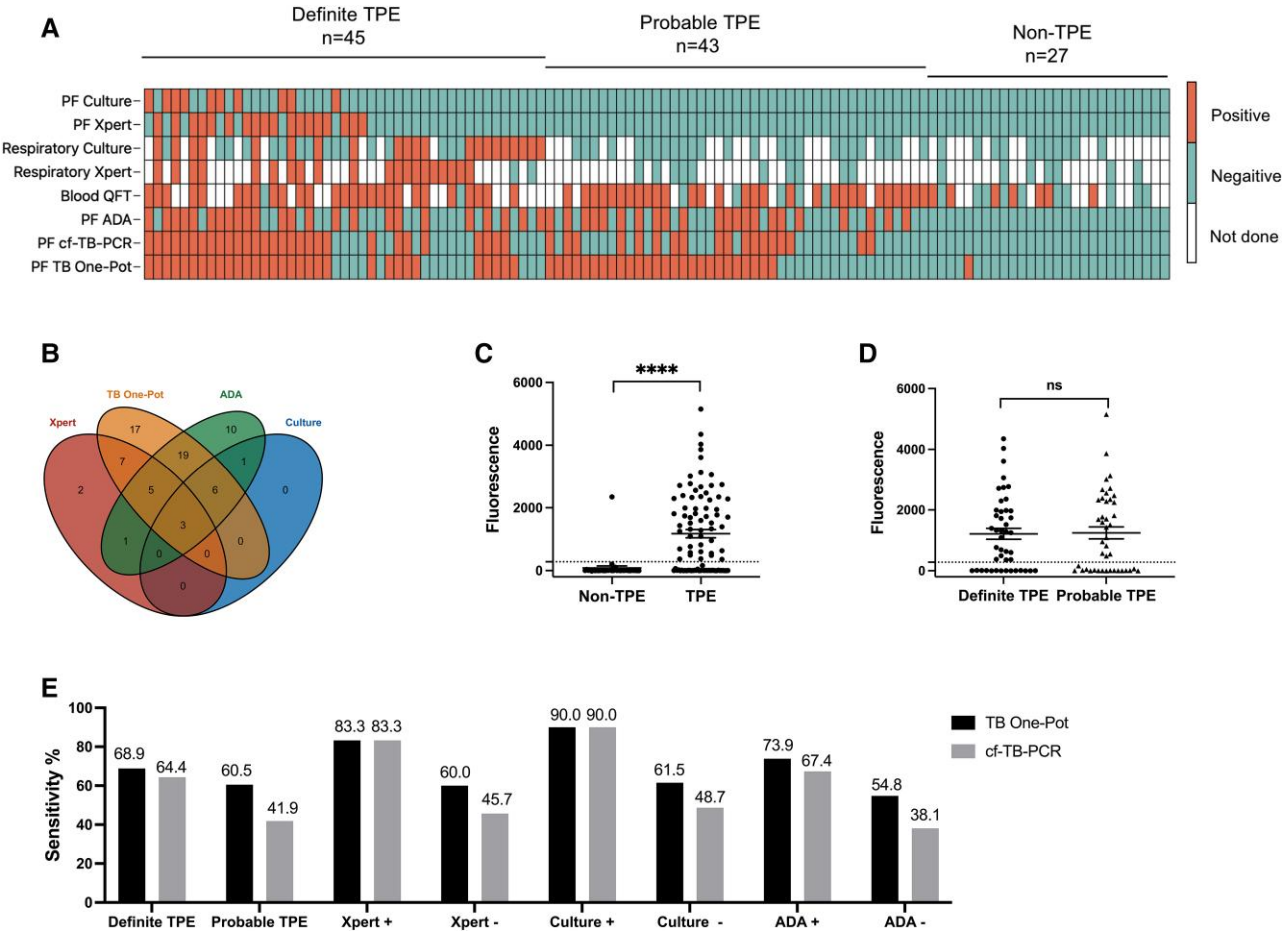

Figure 2. Performance of TB One-Pot in pleural effusion samples. *A*, Clinical and diagnostic data of participants suspected of having TPE, categorized into definite, probable, and non-TPE according to diagnostic criteria. *B*, Venn diagram illustrates the positive results of Xpert, TB One-Pot, ADA, and mycobacterial culture in TPE samples. Scatter plots depict the distribution and comparison of fluorescence values from TB One-Pot testing of pleural effusion samples: *C*, TPE cases (n = 88) and non-TPE cases (n = 27); *D*, definite and probable TPE. The dotted line represents the cutoff value at 280.2, and the black bars represent the mean and SEM. Unpaired *t* test. ****$P < .0001$. *E*, Comparison of the positivity rates of TB One-Pot and cf-TB-PCR detection of pleural effusion cf-TB in different patient groups. Abbreviations: ns, not significant; ADA, adenosine deaminase assay; cf, cell-free; QFT, QuantiFERON-TB Gold In-Tube; PCR, polymerase chain reaction; PF, pleural fluid; TB, tuberculosis; TPE, tuberculous pleural effusion.

statistically significant difference in fluorescence values between the definite TPE and probable TPE groups (Figure 2*D*). A comparison of the sensitivity of TB One-Pot and cf-TB-PCR across different patient groups (definite TPE, probable TPE, positive, and negative groups for Xpert, culture, and ADA) showed that TB One-Pot's sensitivity for detecting TB was comparable to or slightly higher than that of cf-TB-PCR, though the differences were not statistically significant
($P > .05$; Figure 2*E*).

We assessed the performance of cf-TB, Xpert, culture, and ADA in diagnosing TPE in pleural effusion, as outlined in Table 2. The sensitivity and AUC of the assays, ranked from highest to lowest, were as follows: TB One-Pot for cf-TB detection, cf-TB-PCR for cf-TB detection, ADA, Xpert, and culture. TB One-Pot demonstrated higher sensitivity and AUC for cf-TB detection as compared with cf-TB-PCR, although the differences were not statistically significant ($P > .05$). Similarly, TB One-Pot showed higher sensitivity and AUC as compared with ADA, without statistically significant differences ($P > .05$). Notably, the sensitivity of TB One-Pot for detecting cf-TB surpassed that of culture by 5.7 times and Xpert by 3.2 times, indicating significant superiority over both methods ($P < .05$). Moreover, TB One-Pot demonstrated a specificity of 96.3%, while other tests showed a specificity of 100%, with no statistical distinctions ($P > .05$). Considering the rapidity and cost-effectiveness of TPE diagnosis, combining TB One-Pot, the most sensitive single assay, with ADA achieved a sensitivity of 78.4% and an AUC of 0.874.

Table 2. Diagnostic Performance of Several Tests and Their Combinations in Pleural Effusion Samples

Test	CRS-Diagnosed TPE		% (95%)				
	Positive (n = 88)	Negative (n = 27)	Sensitivity	Specificity	PPV	NPV	AUC
TB One-Pot			64.8 (53.9–74.7)	96.3 (81.0–99.9)	98.3 (89.2–99.7)	45.6 (38.5–52.9)	0.805 (.721–.873)
Positive	57	1					
Negative	31	26					
cf-TB-PCR			53.4 (42.5–64.1)[a]	100.0 (87.2–100.0)[a]	100.0 (100.0–100.0)[a]	39.7 (34.5–45.2)[a]	0.767 (.679–.841)[a]
Positive	47	0					
Negative	41	27					
Xpert			20.5 (12.6–30.4)[b]	100.0 (87.2–100.0)[a]	100.0 (100.0–100.0)[a]	27.8 (25.8–30.0)[b]	0.602 (.507–.692)[b]
Positive	18	0					
Negative	70	27					
Culture			11.4 (5.6–19.9)[b]	100.0 (87.2–100.0)[a]	100.0 (100.0–100.0)[a]	25.7 (24.3–27.2)[b]	0.557 (.461–.649)[b]
Positive	10	0					
Negative	78	27					
ADA ≥35 U/L			52.3 (41.4–63.0)[a]	100.0 (87.2–100.0)[a]	100.0 (100.0–100.0)[a]	39.1 (34.1–44.4)[a]	0.761 (.673–.836)[a]
Positive	46	0					
Negative	42	27					
TB One-Pot + ADA			78.4 (68.4–86.5)[b]	96.3 (81.0–99.9)[a]	98.6 (91.0–99.8)[a]	57.8 (47.7–67.2)[a]	0.874 (.799–.928)[b]
Positive	69	1					
Negative	19	26					

Abbreviations: ADA, adenosine deaminase; AUC, area under the curve; cf, cell-free; CRS, composite reference standard; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value; TB, tuberculosis; TPE, tuberculous pleural effusion.

[a]TB One-Pot showed no statistically significant difference ($P > .05$) when compared with cf-TB-PCR, Xpert, culture, ADA, and TB One-Pot + ADA.

[b]TB One-Pot showed a statistically significant difference ($P < .05$) when compared with cf-TB-PCR, Xpert, culture, ADA and TB One-Pot + ADA.

DISCUSSION

Due to the paucibacillary nature of TPE, accurately diagnosing TPE remains a challenge, as centrifugation for acid-fast bacilli smear, genomic DNA detection, and culture often yields unsatisfactory results [7, 18, 19]. In this study, the sensitivity of Xpert and culture in diagnosing TPE was 20.5% and 11.4%, respectively, indicating significant limitations in diagnosing TPE directly from pleural effusion samples. The overlap between positive detections by culture and Xpert in respiratory and pleural effusion samples was minimal in our study, with combined culture and Xpert from such samples achieving a diagnostic sensitivity of 48.9% (43/88) for TPE. Although ADA exhibited a sensitivity of 52.3% in diagnosing TPE in our study, it does not serve as direct evidence of pathogen presence. Given the absence of a single recommended test for TPE diagnosis, any test that aids in the clinical diagnosis of TPE is crucial.

cf-TB provides direct evidence of the presence of Mtb in the body. Previous studies have demonstrated that quantitative PCR detection of cf-TB in pleural effusion with IS*6110* is an effective method for diagnosing TPE. When compared with previous quantitative PCR studies (sensitivity, 63%–75%) [7, 18, 19], the sensitivity of cf-TB-PCR in this study (53.4%) was slightly lower, potentially due to 3 underlying factors. First, there were differences in the study population, as culture can reflect the quantity of viable bacteria in the sample. The sensitivity of culture in this study was 11.4%, which is lower than in previous studies (14.9%–26.7%) [7, 18]. Second, this study employed a different cfDNA extraction method. We utilized the TIANamp Magnetic DNA Kit manufactured by Tiangen, whereas previous studies [7, 18] employed silica membrane–based columns manufactured by Qiagen. Research has suggested [30] that Qiagen extraction kits outperform Tiangen kits in extracting cfDNA from plasma samples. However, considering the suitability of magnetic bead extraction for automated extraction systems, as well as practicality and future translatability, we opted for domestically produced magnetic bead extraction. Third, this study collected residual clinical samples, and even though those with minimal processing delay were chosen, these samples experienced a longer collection interval before storage at −80 °C as compared with previous prospective studies [7, 18].

When compared with Xpert and culture detection, cf-TB is a superior choice for diagnosing TPE. When a composite reference standard is employed, the sensitivity of quantitative PCR for cf-TB detection ranges from 46.8% to 75%, with a specificity of 100% [7, 18, 19, 31], which is significantly higher than the sensitivity of Xpert and culture. Several potential mechanisms can explain why cf-TB exhibits higher sensitivity than Xpert. First, the concentration of cf-TB in pleural effusion may exceed that of genomic DNA. The pathogenesis of TPE is thought to stem from a hypersensitive reaction triggered by direct contact with Mtb or proteins secreted or lysed by Mtb entering the pleural cavity, with low or even absent intact Mtb content [2,

32]. cf-TB in pleural effusion originates from the apoptotic necrosis of Mtb and circulates into the pleural effusion as cf-TB. The enclosed nature of the pleural cavity or other factors may result in a higher concentration of cfDNA. Second, the detection targets differ. Xpert targets an 81–base pair rifampicin resistance–determining region of the *rpoB* gene, which is a single-copy gene, whereas this study's cf-TB detection targets the Mtb-specific multicopy repetitive insertion sequence IS*6110*, present in various mycobacterial strains in 10 to 12 copies, allowing for signal amplification [33]. Xpert cartridges capture only intact Mtb from the filter membrane samples, containing solely genomic DNA, without cf-TB [6]. The sensitivity of TB One-Pot in detecting cf-TB is 3.2 times higher than that of Xpert. Additionally, the sensitivity of detecting TB in cerebrospinal fluid filtrate (cfDNA) from patients with tuberculous meningitis is 1.6 to 2.8 times higher than that from precipitates (genomic DNA) [34], further affirming that detecting cf-TB in closed cavity effusion is a superior target selection as compared with genomic DNA.

Through TB One-Pot detection of cf-TB in pleural effusion, a higher sensitivity (64.8% vs 53.4%) is demonstrated when compared with quantitative PCR, albeit there is no statistical difference between them. Further subgroup analysis of patients with TPE revealed no statistical difference in sensitivity between TB One-Pot and cf-TB-PCR in distinguishing between the TPE and probable TPE groups or between the positive and negative groups for Xpert, culture, and ADA. However, in patients with TPE who were negative for Xpert, culture, and ADA, the sensitivity of TB One-Pot is 60.0%, 61.5%, and 54.8%, respectively, suggesting its potential to address the limitations of current detection methods.

In previous studies, the specificity of ADA ≥30 for diagnosing TPE was 87% [29], while the specificity of ADA ≥40 ranged from 90% to 100% [31, 35]. In our study, ADA ≥35 demonstrated a specificity of 100% for diagnosing TPE. This result may be attributed to the low prevalence of conditions that typically reduce the diagnostic specificity of ADA, such as complicated PPE, nonpneumonic pleural infections, and hematologic malignancies [29]. In our control group of 27 individuals, there were only 2 cases of complicated PPE and 2 cases of lymphoma. A limitation of this study is its design as a single-center, small-sample study comparing the TB One-Pot assay with existing TPE diagnostic methods, conducted at a specialized TB hospital, which may limit the generalizability of our findings. Therefore, we plan to conduct a larger, multicenter prospective study to validate the diagnostic generalizability and robustness of the assay. Despite this limitation, we believe that the results of this study are reliable. It is worth noting that cfDNA is less stable than genomic DNA, and after treatment, pathogen-derived cfDNA in patients is rapidly cleared by immune cells [36]. Recent research by Huang et al [37] indicates a rapid decrease in serum cf-TB concentration after the initiation of TB treatment, with cf-TB approaching or completely cleared after 6 months of anti-TB therapy. This suggests that cf-TB, as a detection marker, can reflect the current disease status to some extent, indicating the potential of quantitative cf-TB detection for monitoring existing treatment regimens or evaluating drug efficacy in future new anti-TB regimens.

In summary, this study is the first to propose the efficacy of the TB One-Pot method, which combines isothermal amplification CPA with CRISPR-Cas12b assistance in detecting cf-TB in pleural effusion specimens. When compared with other Mtb pathogen detection methods, such as Xpert and culture, this approach not only exhibits significantly enhanced sensitivity but also offers the advantages of simplicity and rapidity, with results reportable at 58 °C within 32 minutes. Therefore, utilizing TB One-Pot for cf-TB detection may represent a more effective approach for diagnosing TPE in resource-limited settings.

Notes

Author contributions. J. P. and L. C. conceptualized the study and drafted, reviewed, and edited the full manuscript. T. F. and S. D. performed the experiments and conducted statistical analysis. All authors reviewed and approved the manuscript for submission.

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Potential conflicts of interest. All authors: No reported conflicts.

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