Original research

Enhanced diagnosis of tuberculous pleurisy using a multiplex droplet digital PCR assay targeting circulating mycobacterial DNA

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► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/thorax-2025-223553).

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Received 3 May 2025
Accepted 12 November 2025
Published Online First
9 December 2025

To cite: Zhang S, Xu Y, Huang M, *et al*. *Thorax* 2026;**81**:738–748.

ABSTRACT

Background Tuberculous pleurisy (TBP) is a leading aetiology of exudative pleural effusion in tuberculosis (TB)-endemic regions and poses significant diagnostic challenges due to its paucibacillary nature of *Mycobacterium tuberculosis* (MTB) in pleural fluid. This study aims to characterise MTB-derived cell-free DNA (cfDNA) in pleural effusion and to develop an optimised molecular assay to improve TBP diagnostic accuracy.

Methods We quantified MTB cfDNA/genomic DNA (gDNA) concentrations and characterised cfDNA fragment profiles in pleural effusion specimens using ddPCR. A multiplex droplet digital PCR (ddPCR) assay was developed, targeting two insertion sequences (IS6110 and IS1081) using ultra-short amplicons (49–59 bp) to enhance detection efficiency. Diagnostic performance was prospectively evaluated in 356 consecutive adults with radiologically confirmed pleural effusion, using composite microbiological criteria as the reference standard.

Results MTB cfDNA exhibited significantly higher detection rates than gDNA in definite TBP cases (p=0.0006), with dominant fragment lengths of 60–80 bp. The multiplex ddPCR assay demonstrated a limit of detection of 0.2 genome equivalents per reaction. Among microbiologically confirmed TBP cases (n=69), the assay achieved a sensitivity of 94.2%, significantly outperforming Xpert MTB/RIF (52.0%, p<0.0001) and liquid culture (35.3%, p<0.0001). Specificity remained high at 97.0% among cases of non-TB effusion (n=208).

Conclusions Our findings establish MTB cfDNA as the predominant nucleic acid form in tuberculous pleural effusion. The optimised multiplex ddPCR platform, leveraging multicopy targets and fragment-length-adapted amplification, achieves improved diagnostic sensitivity without compromising specificity in a high-prevalence TB setting. This approach may help address key limitations of TBP diagnostics and shows promise for clinical implementation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Diagnosing tuberculous pleurisy (TBP) remains challenging due to the paucibacillary nature of *Mycobacterium tuberculosis* (MTB) in pleural effusion. While detection of cell-free DNA (cfDNA) has shown markedly higher sensitivity than assays targeting genomic DNA (gDNA), most existing molecular assays are not optimised for the short, fragmented MTB cfDNA amid a high background of human DNA in pleural fluid. Moreover, the impact of pre-analytical variables on assay performance remains poorly characterised.

WHAT THIS STUDY ADDS

⇒ This study provides a comprehensive characterisation of MTB cfDNA in pleural effusion and introduces a novel multiplex droplet digital PCR (ddPCR) assay for the diagnosis of TBP. We demonstrate that MTB cfDNA is more abundant than MTB gDNA in pleural fluid, that cfDNA fragment length is critical for detection sensitivity and that high levels of background human DNA substantially reduce molecular assay performance. The newly developed multiplex ddPCR assay, specifically optimised for cfDNA, represents a potential paradigm shift in TBP diagnostics.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings establish MTB cfDNA as a valuable diagnostic biomarker for TBP and highlight the importance of understanding its molecular characteristics and optimising pre-analytical workflows. Implementation of the multiplex ddPCR assay may significantly enhance diagnostic accuracy and inform more timely and appropriate clinical management of TBP.

INTRODUCTION

Pleural effusion is a common yet diagnostically challenging manifestation of diverse conditions, including heart failure, malignancy, infections and autoimmune diseases.[1 2] Clinical presentations are often nonspecific, and definitive diagnosis typically requires invasive procedures such as thoracentesis and pleural fluid analysis.[3 4] However, even with these interventions, diagnostic uncertainty frequently persists, particularly for exudative effusions where conventional tests, such as cytology, culture and biochemical assays, exhibit limited sensitivity.[2 4–6]

Tuberculous pleurisy (TBP) is a common clinical pleural disease and one of the most frequent causes of pleural exudates globally, particularly in regions with a high tuberculosis (TB) burden.[6–8] In South Africa, TBP accounted for up to 82% of undiagnosed pleural effusions in adults undergoing pleural biopsy.[9] In India, TB is responsible for 30%–80% of pleural effusion cases,[10] while in China, the proportion is approximately 40%.[11] The incidence of TBP in China has notably increased from 2005 to 2018, with disproportionately high rates reported in regions such as Tibet, Beijing, Xinjiang and Inner Mongolia.[11] These observations underscore the need for more accurate, accessible and scalable diagnostic strategies for TBP, particularly in endemic settings.

The gold standard for the diagnosis of TBP is detection of *Mycobacterium tuberculosis* (MTB) bacilli or their specific components in pleural specimens.[5 6] However, traditional bacteriologic tests such as acid-fast bacilli (AFB) smear microscopy and MTB culture exhibit poor sensitivity, less than 5% and 24–58%,[4 5] respectively, thus limiting their clinical value. Molecular testing, mainly real-time PCR assays originally designed to detect genomic DNA (gDNA) separated from intact MTB bacilli,[12] has shown improved sensitivity when applied to pleural fluid.[6] Nevertheless, the widely used Xpert MTB/RIF and Xpert MTB/RIF Ultra assays, while improving diagnostic yield, remain suboptimal with pooled sensitivities of 46.7% and 69.5% in pleural fluid, respectively.[13] Droplet digital PCR (ddPCR) enhances rare target detection by partitioning DNA into thousands of droplets, reducing background interference.[14 15] Although ddPCR has demonstrated higher sensitivity than Xpert MTB/RIF (57.6% vs 23.0%),[16] the overall gains remain modest, reflecting intrinsic limitations of targeting gDNA in pleural specimens.

Microbial circulating cell-free DNA (cfDNA) has emerged as a promising alternative diagnostic target. Microbial cfDNA, released into host fluids during cell breakdown by colonising or invasive microbes, is typically short, fragmented and easily degraded—distinct from intact gDNA.[17] Short fragments of MTB cfDNA have been detected in plasma, urine and pleural effusion, and early studies suggest that cfDNA-based assays substantially improve diagnostic sensitivity, reaching approximately 80%.[18 19] More recently, ddPCR targeting MTB cfDNA has achieved sensitivities as high as 90%,[20] the highest reported for TBP to date. Nevertheless, limited understanding of MTB cfDNA characteristics, including its relative abundance compared with gDNA, fragment size distribution, stability under storage conditions and optimal extraction protocols, has constrained further advances. In this study, we systematically characterised MTB cfDNA in pleural effusion, developed a multiplex ddPCR assay optimised for minimal amplicon length and evaluated its performance in a prospective patient cohort, with the goal of establishing a clinically applicable platform for improved TBP diagnosis.

METHODS

Study design overview

This study aimed to develop and validate an enhanced cfDNA-based ddPCR assay for TBP diagnosis in three phases. First, we compared the abundance of cfDNA and gDNA and characterised the fragment sizes of cfDNA in MTB-positive pleural effusion samples to inform assay design. Second, we optimised pre-analytical conditions, including pleural fluid storage and cfDNA extraction. Third, we prospectively assessed the assay's diagnostic performance in 356 patients with radiologically confirmed pleural effusion.

Study participants

A prospective diagnostic accuracy study was conducted at Fuzhou Pulmonary Hospital (Fujian, China), a municipal-level referral centre, from January to September 2022. Most patients admitted to the pulmonary inpatient clinic had already undergone initial evaluation at district-level healthcare facilities and were referred to this hospital because of a high clinical suspicion of TB. Written informed consent was obtained from all participants aged ≥18 years; for minors <18 years, consent was provided by their legal guardians. The study was approved by the Medical Ethics Committees of Xiamen University (approval number XYDD2021016) and Fuzhou Pulmonary Hospital (approval number 2022-001-01).

A total of 356 participants aged ≥15 years with radiologically confirmed pleural effusion were prospectively and consecutively recruited from the pulmonary inpatient clinic. Patients with pleural fluid volume of less than 100 mL were excluded. The enrolled participants were categorised into two groups: those with suspected TBP (n=183) and those with other pulmonary diseases (OPDs) (n=173). Patients with suspected TBP presented with clinical symptoms such as cough, sputum production, haemoptysis, chest pain, fever and night sweats. OPDs, including lung cancer, ventilator-associated pneumonia (VAP) and other pulmonary conditions, were diagnosed according to established clinical criteria.

For patients with suspected TBP, pleural effusion specimens were subjected to routine clinical testing, including smear microscopy, culture, Xpert MTB/RIF, adenosine deaminase (ADA) testing and biochemical analysis (details provided in the online supplemental materials. Patients were classified into three categories on the basis of published TBP diagnostic criteria:[21] *definite TBP (n=69), possible TBP (n=68) and non-TBP (n=208)*.

Definite TBP was defined by positive microbiological evidence of MTB in sputum, pleural effusion or pleural tissue (detected by smear microscopy, culture or Xpert MTB/RIF) or the presence of caseating granulomas with positive AFB staining.

Possible TBP included patients with negative microbiological results but elevated ADA levels in pleural effusion, positive immunological findings (tuberculin skin test, tuberculosis-specific enzyme-linked immunospot test (T-SPOT.TB) or MTB antibodies), and a favourable clinical response to anti-TB chemotherapy.

Non-TBP comprised patients diagnosed with other diseases or those without laboratory evidence of TB who improved without anti-TB treatment.

To minimise bias, blinding was implemented throughout the study. Treating clinicians were blinded to the results of cfDNA ddPCR, gDNA ddPCR and cfDNA quantitative PCR (qPCR) assay, while laboratory personnel were unaware of the clinical data and other diagnostic results.

MTB gDNA extraction and fragmentation

The DNA template used for the ddPCR assay development was extracted from the H37Rv reference strain (ATCC 27294). MTB gDNA was extracted using the AxyPrep

Bacterial Genomic DNA Miniprep Kit (Axygen Scientific, Union City, CA) and stored at −80°C in a restricted-access facility. DNA concentrations were measured using an ND-2000 ultraviolet-visible (UV-VIS) spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and adjusted to 5 ng/µL (~10^6 genomes/µL) in tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5) prior to use. For fragmentation, samples were sonicated using a M220 Focused Ultrasonicator (Covaris, Woburn, MA, USA), and fragment size profiles were assessed using a Qseq100TM DNA Analyzer (BiOptic Inc., Taiwan, China).

Extraction DNA from pleural effusion

For each patient, 10 mL of pleural effusion remaining after clinical examination was collected, centrifuged at 16,000×g for 10 min within 4 hours after collection and separated into supernatant and sediment. The supernatant was stored at −80°C, and MTB cfDNA was extracted from 3 mL supernatant using either the MiniMax High Efficiency Cell-Free DNA isolation kit (Apostle Inc, Suzhou, China) or the Circulating Nucleic Acid Extraction Kit (Zeesan Biotech, Xiamen, China). MTB gDNA was extracted from the sediment using the Lab-Aid 824 Automatic DNA Extraction System (Zeesan Biotech). Further details on pleural effusion preservation and extraction conditions are provided in the online supplemental materials.

ddPCR assay

The ddPCR assays were performed in eight parallel reactions using the TargetingOne Digital System (TargetingOne, Beijing, China). A 30 µL reaction mixture containing 15 µL of Probe ddPCR SuperMix (TargetingOne), 800 nmol/L of each primer pair (online supplemental tables S1 and S2), 250 nmol/L of FAM- and HEX-labelled 5′-hydrolysis probes and 5 µL of template DNA was prepared. The mixture was processed through droplet generation, PCR amplification (10 min at 95°C, 50 cycles of 30 s at 94°C, 1 min at 58°C and a final hold at 12°C) and droplet generation using the Chip Reader R1. Raw data were analysed using a ddPCR Analyzer. MTB genome equivalents were calculated using the *katG* ddPCR assay validated in our previous study,[15] with conversion based on the molecular weight of the MTB genome (2.86×10^9 Daltons). Details on the evaluation of the limit of blank (LOB) and limit of detection (LOD) for the ddPCR assays are provided in the online supplemental materials.

qPCR assay

A real-time qPCR assay was developed using the same primer-probe set as the ddPCR assay for direct comparison. Further details on the qPCR protocol are available in the online supplemental materials and table S3.

Statistical analysis

Sensitivity was assessed in TBP patients, and specificity was evaluated in non-TBP patients. Group differences were analysed using the non-parametric Mann-Whitney U test for continuous variables and Pearson's $\chi 2$ test for categorical variables. Comparisons of sensitivity between diagnostic methods were performed using McNemar's test. Estimates and 95% CIs for paired proportions were calculated using the Newcombe-Wilson method. The Cochran-Mantel-Haenszel test was applied to compare ddPCR sensitivity across different subgroups. A two-sided P value of <0.05 was considered statistically significant. All statistical analyses were conducted using SPSS (V.26.0, IBM) or R (V.4.3.2), and data visualisation was performed using GraphPad Prism (V.10.0).

RESULTS

Relative abundance of MTB cfDNA and gDNA in pleural effusion

Previous studies have demonstrated that MTB cfDNA in pleural effusion offers higher diagnostic sensitivity for TBP.[18 19] However, a direct comparison between cfDNA and gDNA remains lacking. To address this gap, we compared the concentrations of these two nucleic acid species in 30 pleural effusion samples (online supplemental materials). Both MTB cfDNA and MTB gDNA were detectable in 21 samples, with cfDNA levels significantly higher than gDNA (p=0.0006, online supplemental figure S1). Notably, nine samples were positive only for cfDNA. These results demonstrated that MTB cfDNA was more abundant than MTB gDNA in pleural effusion and could thus serve as a more sensitive target for TBP diagnosis.

Apparent size distribution MTB cfDNA in pleural effusion

An efficient PCR assay requires amplicon sizes to be smaller than the length of the template DNA. However, the exact length distribution of MTB cfDNA in pleural effusion samples remains largely unknown. To address this, we designed eight ddPCR assays using a constant forward primer and stepwise backward reverse primers on the IS6110 region, generating amplicons ranging from 40 to 200 bp (figure 1A, online supplemental figure S2). To test the ability of these assays in differentiating templates of varying lengths, we analysed both unfragmented H37Rv gDNA (1–4 kb) and fragmented H37Rv gDNA (mean size ~130 bp) at identical concentrations (figure 1B). The ddPCR assays yielded consistent copy numbers for the unfragmented H37Rv gDNA (mean=22 740.8 copies, SD=3.79%) (figure 1C). However, with fragmented H37Rv gDNA, a gradient decrease in copy number was observed with increasing amplicon length, with only the 40 bp assay yielding copy number consistent with unfragmented DNA. These observations demonstrate that ddPCR assays with varying amplicon sizes can effectively distinguish the length distribution of template DNA.

We then analysed MTB cfDNA in pleural effusion samples from five patients with definite TBP. A decrease in copy number with increasing amplicon size was consistently observed across all samples (figure 1D). Normalised abundance plotted against amplicon length revealed highly similar profiles across all five samples, regardless of their varied MTB cfDNA concentrations (figure 1E). The size distribution analysis indicated that fragments ranging from 60 to 80 bp were the dominant species in all samples (figure 1F, online supplemental materials and table S4).

Design of the ddPCR assay

Based on the observed size distribution of MTB cfDNA, we designed a ddPCR assay with amplicons shorter than 60–80 bp. To maximise cfDNA recovery, we selected three multicopy insertion sequences as targets: one in IS1081 (five to seven copies per MTB genome;[22] six copies in H37Rv) and two in IS6110 (1–20 copies per MTB genome;[23] 16 copies in H37Rv). The shortest amplicon sizes chosen for each target were 59 bp, 57 bp and 49 bp, respectively. Serial dilution tests of the fragmented H37Rv gDNA showed that the triplex ddPCR assay, comprising all three targets, yielded the highest and most stable

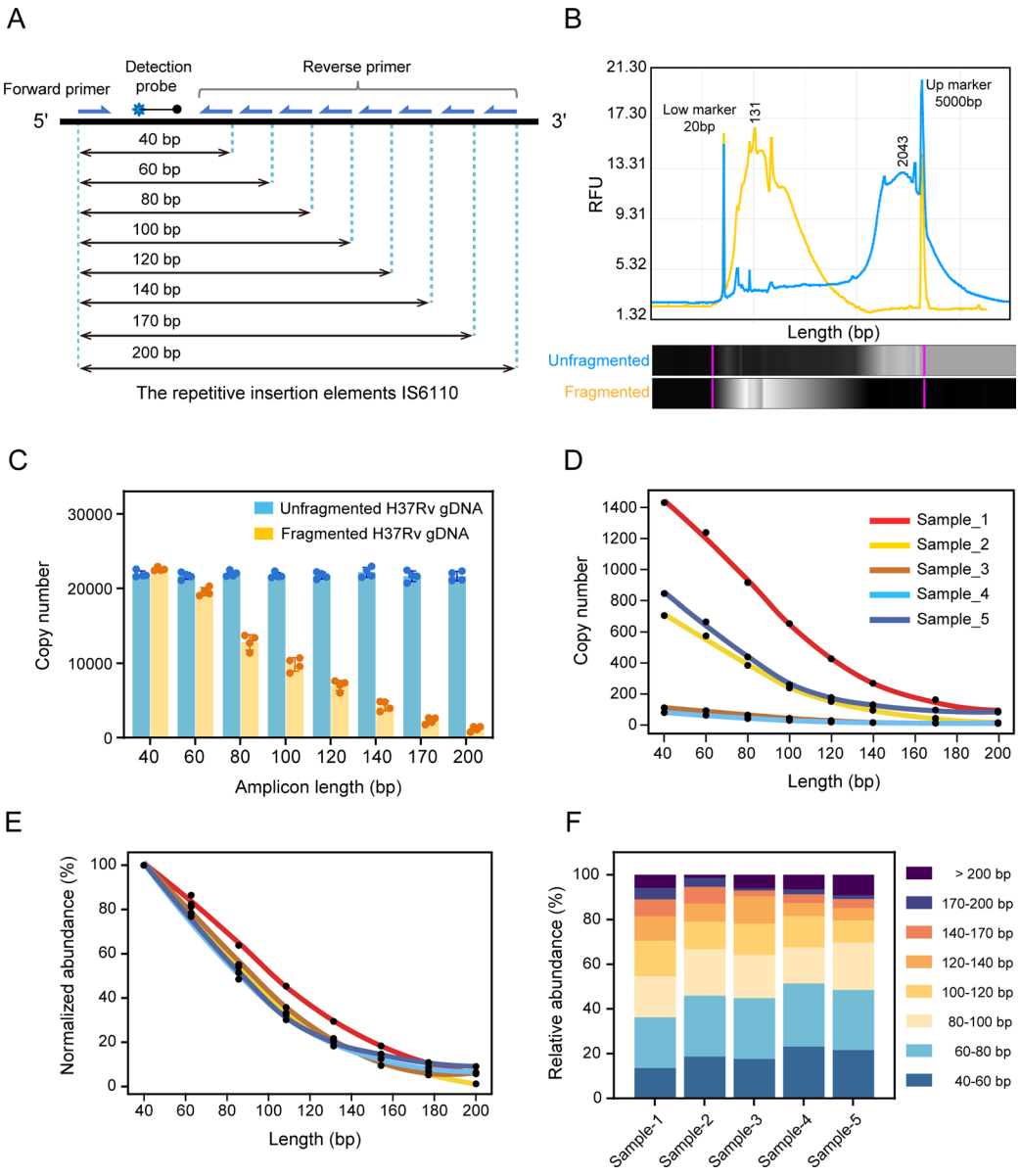

Figure 1 Characteristics of TB cfDNA in pleural effusion. (A) Schematic representation of the 40 bp to 200 bp amplicons within the IS6110 region. The depicted amplicons consist of an upstream primer and progressively downstream primers. (B) Template length assessment using capillary electrophoresis: unfragmented H37Rv gDNA (yellow line) and fragmented H37Rv gDNA (blue line). (C) Detection of unfragmented and fragmented H37Rv gDNA by the eight PCR with amplicon size between 40 and 200 bp. (D) The copy number detected using the eight ddPCR for the pleural effusion samples from five definite TBP patients. (E). Normalisation of the detected copy number from the samples using the eight PCRs. (F) Apparent size distribution of the MTB cfDNA in the patients at different size intervals. cfDNA, cell-free DNA; ddPCR, droplet digital PCR; gDNA, genomic DNA; MTB, *Mycobacterium tuberculosis*; TB, tuberculosis; TBP, tuberculous pleurisy.

copy number, followed by two and one target (figure 2A). Using the human housekeeping gene *RPP30* as an internal control, the quadruplex ddPCR assay exhibited distinct clusters for different target combinations (figure 2B). The copy number obtained from all clusters correlated strongly with that obtained from the single-copy *katG* ddPCR assay[15] ($R^2 > 0.998$, figure 2C, online supplemental figure S3). The ddPCR assay demonstrated stable fluorescence intensity in both intra- and inter-batch experiments (online supplemental figure S4), with an LOB of 1.5 copies/reaction and an LOD of 4 copies/reaction, corresponding to 0.2 copy/reaction for *katG*. Given that *katG* is a single-copy gene in the MTB genome, the ddPCR assay effectively achieved an LOD of 0.2 MTB genome equivalent per reaction. When assessed on pleural effusion samples with varying MTB concentrations (19.8 to 3478.6 copies per reaction), the ddPCR assay consistently yielded positive results, with coefficients of variation ranging from 7.1% to 34.3%, further confirming its high reproducibility in real samples (online supplemental figure S5).

Tolerance of the ddPCR assay to human DNA

Pleural effusion often contains high levels of human DNA due to its high lymphocyte content, which may interfere with the detection of MTB cfDNA. We compared human DNA levels of pleural effusion samples from patients with TBP, lung cancer, VAP and OPDs (n=20 per group). Human DNA concentration was significantly higher in TBP pleural effusion (median=500.2 ng/reaction) compared with cancer, VAP or other diseases (online

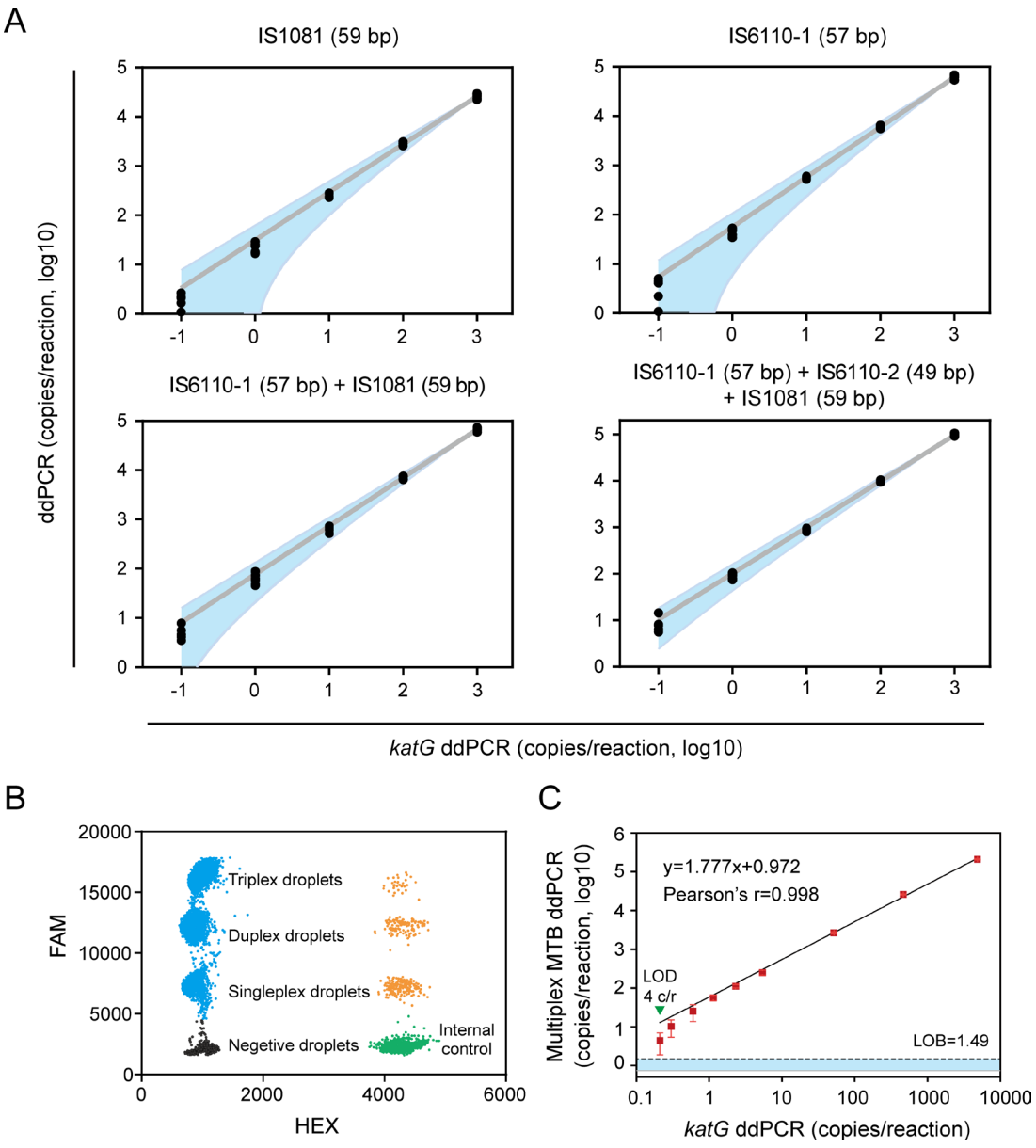

Figure 2 Analytical evaluation of the MTB cfDNA ddPCR assay. (A) Analysis of target number and output stability in the ddPCR assay. The assay includes different targets, including IS1081, IS6110-1, IS6110–1+IS1081 and IS6110–1+IS6110-2 + IS1081. Detection was performed on H37Rv cfDNA templates with concentrations ranging from 10^3 to 10^{-1} copies/µL (n=5). Shaded regions represent 95% CIs. (B) Typical results of the TB ddPCR assay. The FAM channel shows the combined signal for IS6110 and IS1081, while the HEX channel targets the *RPP30* gene. (C) Limit of detection analysis on engineered samples. The grey line represents the mean false-positive rate, the blue shading indicates the 95% CI and the dashed line marks the limit of blank. cfDNA, cell-free DNA; ddPCR, droplet digital PCR; MTB, *Mycobacterium tuberculosis*; TB, tuberculosis.

supplemental figure S6). To evaluate the tolerance of ddPCR assay to human DNA interference, we conducted a titration experiment, analysing MTB gDNA at varying concentrations (0.1 to 100 copies/μL) in the presence of increasing human DNA levels (0 to 500 ng/reaction). The ddPCR assay consistently detected MTB DNA copy number across all human DNA levels, whereas the qPCR assay exhibited marked increases in Ct values and even failed to yield positive results when MTB DNA concentration was one copy/μL or lower (online supplemental figure S7). These findings demonstrate that ddPCR exhibits superior resistance to human DNA interference compared with qPCR, underscoring its suitability for TBP diagnostics using pleural effusion.

Clinical evaluation of the cfDNA ddPCR assay

Prior to initiating the prospective clinical study, we assessed MTB cfDNA's stability in pleural effusion samples. Thirteen samples were divided into three aliquots and stored under distinct conditions: 4°C, 4°C with EDTA and −80°C. Only samples stored at −80°C maintained stable MTB DNA copy numbers over 3, 7 and 21 days compared with baseline (online supplemental figure S8A). Consequently, all pleural effusion samples for the clinical study were processed within 2 hours after collection, centrifuged to separate supernatant and sediment and stored at −80°C until DNA extraction. For cfDNA extraction, we evaluated two commercial kits. The Zeesan kit yielded significantly higher MTB cfDNA copy numbers than the Apostle kit, with an

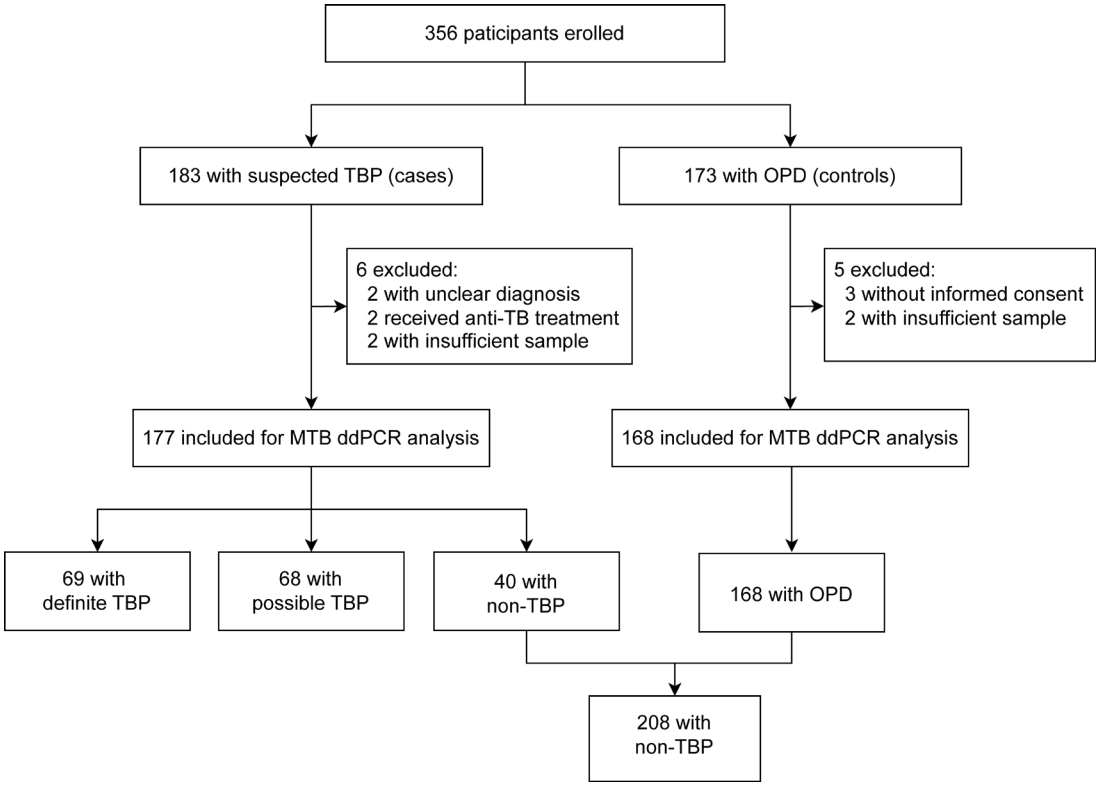

Figure 3 Flow diagram of study participants. ddPCR, droplet digital PCR; MTB, *Mycobacterium tuberculosis;* OPD, other pulmonary disease; TB, tuberculosis; TBP, tuberculous pleurisy.

order of magnitude difference observed in seven samples (online supplemental figure S8B). The Zeesan kit was therefore chosen for subsequent clinical study.

The prospective clinical study enrolled 356 patients with radiological evidence of pleural effusion (figure 3). Among them, 183 (51.4%) were suspected TBP cases, and 173 were OPDs (48.6%). After exclusion, 177 suspected TBP cases and 168 OPDs were eligible for analysis. Among the 177 suspected TBP cases, 69 were classified as definite TBP, 68 as possible TB, and 40 as non-TBP (table 1). A sample was considered positive if the detected copy number by the ddPCR assay exceeded the LOD of four copies/reaction. The ddPCR assay yielded 122 positive results: 65 in 69 definite TBP, 52 in 68 possible TBP, 5 in 69 definite TBP, 52 in 68 possible TBP, five in 208 non-TBP (figure 4A). In definite TBP cases, the cfDNA ddPCR assay detected more positives than gDNA ddPCR (65 vs 50) and cfDNA qPCR (65 vs 47). Among 87 patients tested with culture, Xpert and cfDNA ddPCR, the cfDNA ddPCR assay identified 60 positives not detected by culture or Xpert (figure 4B). The smear method detected 10 positives, all of which were also detected by cfDNA ddPCR, indicating the highest positive rate for cfDNA ddPCR among the tested molecular and microbiological methods.

In pleural effusion samples positive for both cfDNA and gDNA, the cfDNA copy number (median=96.7 copies/reaction) was significantly higher than that of gDNA (median=21.5 copies/reaction) ($p<0.0001$, figure 4C). No significant differences in cfDNA content were observed based on culture results, patient age or ADA levels (figure 4D–F). Nearly all samples from both definite and possible TBP cases exhibited higher MTB cfDNA copy number than MTB gDNA, although both were lower than background human DNA levels (figure 4G).

Performance comparison of cfDNA ddPCR assay with other tests

The cfDNA ddPCR assay demonstrated a sensitivity of 94.2% (95% CI 86.0% to 97.7%) in definite TBP cases, significantly higher than that of gDNA ddPCR (72.5%, 95% CI 61.0% to 81.6%), Xpert (52.0%, 95% CI, 38.5% to 65.2%), culture (35.3%, 95% CI 25.0% to 47.2%) and smear (14.7%, 95% CI 8.2% to 25.0%) (table 2). The cfDNA ddPCR assay also exhibited higher sensitivity than the cfDNA qPCR assay (88.7%, 95% CI 77.4% to 94.7%), although the difference was not statistically significant ($p=0.25$). Compared with immunological methods, the cfDNA ddPCR assay was more sensitive than ADA (76.8%, 95% CI 65.6% to 85.2%) and comparable to T-SPOT.TB (95.3%, 95% CI 87.1% to 98.4%).

In possible TBP cases, the cfDNA ddPCR assay achieved a sensitivity of 76.5% (95% CI 65.1% to 85.0%), which was higher than that of all other methods except T-SPOT.TB. In combined definite and possible TBP cases, the cfDNA ddPCR assay had an overall sensitivity of 85.4% (95% CI 78.5% to 90.4%), surpassing all other methods except T-SPOT.TB. However, T-SPOT.TB exhibited lower specificity than the cfDNA ddPCR assay (59.9% vs 97.6%).

Among different subgroups, cfDNA ddPCR assay demonstrated higher sensitivity in patients aged ≤60 years (90.2%, 95% CI 81.9% to 95.0%) compared with those with ADA ≥40 U/L (88.5%, 95% CI 80.6% to 93.5%) and T-SPOT.TB positive patients (86.8%, 95% CI 79.6% to 91.7%) ($p<0.0001$ for all) (online supplemental table S5). Lower ADA levels (<40 U/L) and negative T-SPOT.TB in pleural effusion were associated with a reduced likelihood of TBP, highlighting the effectiveness of the cfDNA ddPCR assay in ruling out TBP in such cases.

Table 1 Demographics and clinical characteristics of enrolled patients

Characteristic	Definite tuberculous pleurisy (n=69)	Possible tuberculous pleurisy (n=68)	Total tuberculous pleurisy (n=137)	Non-tuberculous pleurisy (n=208)	P Value*
Age, median (range), y	52 (16 to 85)	55 (15 to 89)	54 (15 to 89)	65 (18 to 92)	<0.0001
Sex, no. of males/females	56/13	50/18	106/31	154/54	0.4820
Pleural effusion ingredient test					
White blood cell (10^6/L)	1694.0 (606.8 to 2609.2)	1771 (1020 to 3161)	1755.0 (922.0 to 2911.0)	993.0 (560.8 to 2399.8)	0.0062
Monocyte (%)	90.4 (64.4 to 96.1)	94.8 (85.5 to 97.9)	93.5 (73.5 to 97.6)	80.3 (53.6 to 90.7)	<0.0001
Protein (g/L)	50.1 (44.6 to 53.5)	48.7 (43.1 to 51.8)	48.9 (44.3 to 53.0)	41.8 (32.1 to 49.4)	<0.0001
Glucose (mmol/L)	4.8 (3.0 to 6.7)	5.4 (4.6 to 6.5)	5.0 (3.8 to 6.5)	6.4 (4.9 to 7.8)	<0.0001
Adenosine deaminase (U/L)	50.2 (40.3 to 64.8)	46.7 (34.6 to 56.8)	49.5 (38.8 to 59.5)	11.5 (7.5 to 18.2)	<0.0001
Lactate dehydrogenase (U/L)	499.4 (271.0 to 767.9)	328.0 (232.9 to 518.8)	403.4 (248.3 to 606.3)	306.3 (174.4 to 582.3)	0.0225
CD4+ (cells/μL)	392 (229.0 to 542.0)	478.0 (225.3 to 627–8)	419.0 (230.5 to 594.0)	480.0 (344.0 to 700.0)	0.0392
Inflammatory markers					
C-reactive protein (mg/L)	52.8 (18.4 to 84.2)	33.3 (13.4 to 62.9)	43.1 (16.7 to 73.0)	12.4 (4.3 to 34.9)	<0.0001
Procalcitonin (ng/mL)	0.078 (0.051 to 0.104)	0.064 (0.044 to 0.112)	0.072 (0.047 to 0.105)	0.079 (0.044 to 0.133)	0.5137
Erythrocyte sedimentation rate (mm/h)	60.5 (46.0 to 74.2)	51.5 (34.0 to 68.0)	56.5 (40.2 to 70.0)	38.0 (20.2 to 70.8)	0.0215
Clinical symptoms					
Fever	28 (40.5%)	16 (23.5%)	44 (32.1%)	21 (10.1%)	<0.0001
Cough	60 (86.9%)	55 (80.8%)	115 (83.9%)	165 (79.3%)	0.2835
Chest pain	17 (24.6%)	15 (22%)	32 (23.4%)	39 (18.8%)	0.3003
Dyspnoea	45 (65.2%)	48 (70.5%)	93 (67.9%)	148 (71.2%)	0.5171
Night sweat	2 (2.8%)	0	2 (1.5%)	0	—
Haemoptysis	3 (4.3%)	0	3 (2.2%)	12 (5.8%)	0.0485
Effusion site					
Right	35 (50.7%)	37 (54.4%)	72 (52.9%)	97 (47.8%)	0.3519
Left	22 (31.8%)	23 (33.8%)	45 (33.1%)	49 (24.1%)	0.0712
Bilateral	11 (15.9%)	8 (11.7%)	19 (14.0%)	57 (28.1%)	0.0023

The values are presented as medians (IQRs) or n (%) unless otherwise stated.
*p value indicated the significance level between the total tuberculous pleurisy and non-tuberculous pleurisy groups. Continuous variables were analysed using the nonparametric Mann-Whitney U test, while categorical variables were assessed using Pearson's $\chi 2$ test.

DISCUSSION

This study represents the first comprehensive characterisation of MTB cfDNA in pleural effusion, with the goal of optimising molecular assays for the confirmative diagnosis of TBP. Our findings not only enabled the development of an MTB cfDNA ddPCR assay with the highest diagnostic sensitivity reported to date but also provided critical insights into factors that have contributed to the low sensitivity observed in previous assays. Recent studies have highlighted the potential of MTB cfDNA as a diagnostic biomarker for TBP, with molecular assays such as real-time PCR and ddPCR showing promise, particularly in cases where conventional methods fail to yield definitive results.[18 19] However, challenges remain, including limited understanding of MTB cfDNA abundance relative to MTB gDNA and human DNA, uncharacterised MTB cfDNA size distribution and unclear impact of pre-analytical variables such as sample storage and cfDNA extraction protocols. All of these have hindered the establishment of an optimal TBP diagnosis for routine use. Our study overcomes these obstacles by providing a detailed characterisation of MTB cfDNA in pleural effusion and introducing an optimised ddPCR protocol tailored for TBP diagnosis. This optimised approach substantially improves diagnostic sensitivity without compromising specificity in a high-prevalence TB setting, offering a practical basis for potential clinical application.

A critical advancement in our work is the confirmation that MTB cfDNA is more abundant than MTB gDNA in pleural effusion, and the elucidation of the size distribution profile of MTB cfDNA. These findings address two key aspects of molecular assays: the selection of an appropriate target and the optimisation of amplification parameters. PCR amplification relies on the binding of both primers to the target DNA. However, when the amplicon length exceeds the average size of the target DNA, amplification efficiency declines. Our study revealed that when MTB gDNA was fragmented to approximately 130 bp, only a 40 bp amplicon retained amplification efficiency comparable to that of unfragmented gDNA, longer amplicons exhibited reduced efficiency in an inversely proportional manner. The size distribution analysis indicated that fragments ranging from 60 to 80 bp were the dominant species across all pleural effusion samples. The absence of such data in prior studies may explain the limited sensitivity of existing ddPCR assays, which typically use amplicon lengths optimised for intact bacilli-derived gDNA but not for fragmented cfDNA. While cfDNA fragmentation imposes constraints on amplicon design, it also offers a distinct advantage: by targeting multiple genomic sequences distributed across separate droplets, ddPCR can detect sub-genomic quantities of MTB cfDNA. Leveraging three multicopy insertion sequences, our cfDNA ddPCR assay achieved an LOD of

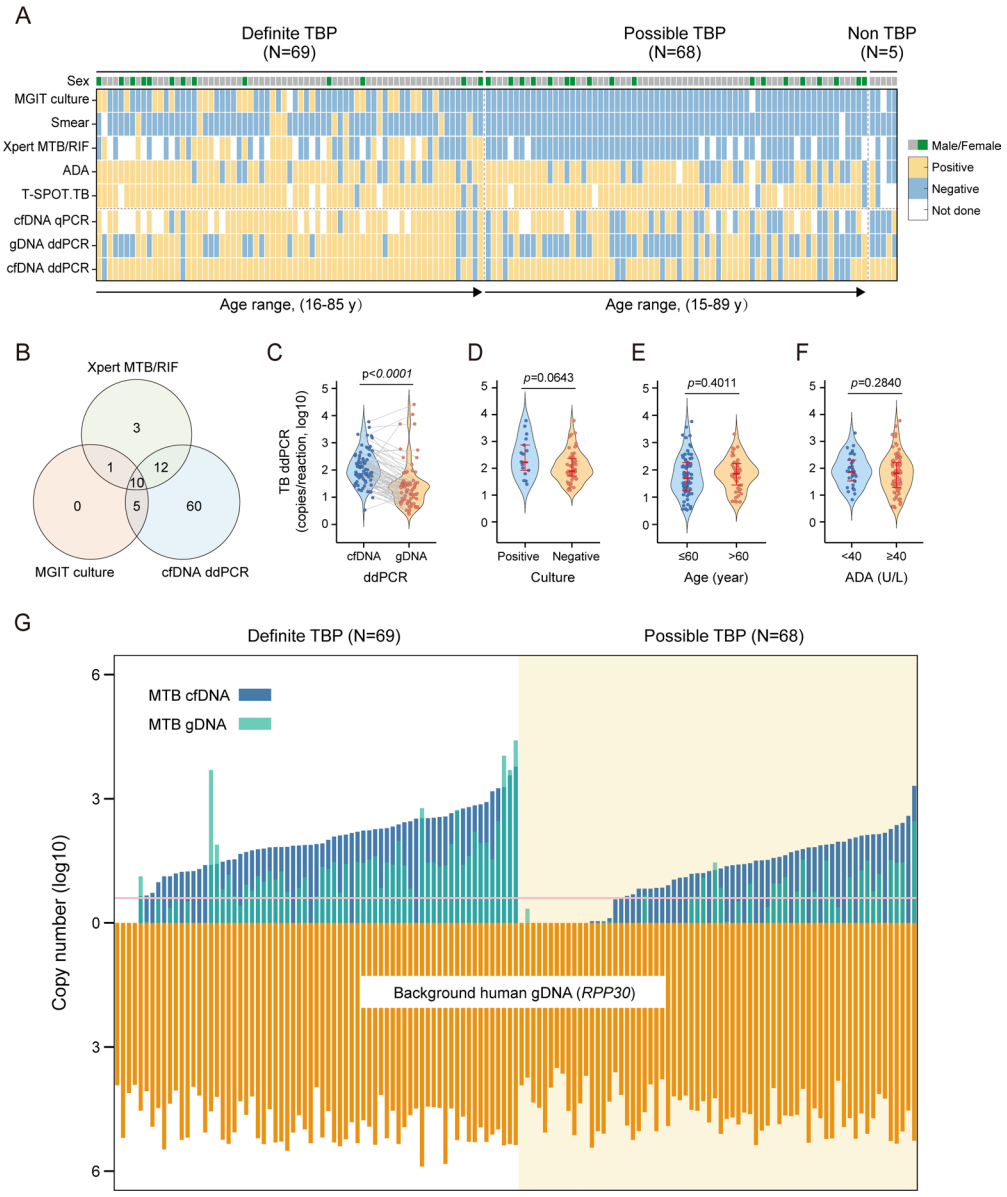

Figure 4 Performance of MTB cfDNA ddPCR assay in patients with tuberculous pleurisy (TBP). (A) Detection results of MGIT culture, smear, Xpert MTB/RIF, adenosine deaminase (ADA), T-SPOT.TB assay, cfDNA qPCR, gDNA ddPCR and cfDNA ddPCR in patients with definite TBP, possible TBP and non-TBP cases. The figure includes all definite and possible TBP cases and five non-TBP patients who had positive cfDNA ddPCR results. (B) Venn diagram of positive diagnostic tests for MGIT culture, Xpert MTB/RIF and cfDNA ddPCR among 87 patients with TBP, including 45 definite and 42 possible TBP cases. (C) Comparison of nucleic acid content in paired pleural effusion samples positive for both cfDNA and gDNA detection. (D) Comparison of cfDNA content in pleural effusion samples with culture-positive versus culture-negative results. (E) Comparison of cfDNA content in pleural effusion between patients aged ≤60 years and >60 years. (F) Comparison of cfDNA content in pleural effusion samples with ADA <40 U/L versus ADA ≥40 U/L. (G) Nucleic acid distribution of MTB cfDNA and gDNA in patients with definite and possible TBP using ddPCR. The bar plot shows the copy numbers of MTB cfDNA (blue) and MTB gDNA (green) across individual patient samples. The pink line represents the limit of detection for the ddPCR assay, defined as four copies per reaction. Patients are categorised into two groups: definite TBP (left) and possible TBP (right). Orange bars represent the copy numbers of background human gDNA (*RPP30*). cfDNA, cell-free DNA; ddPCR, droplet digital PCR; gDNA, genomic DNA; MTB, *Mycobacterium tuberculosis*; qPCR, quantitative PCR; TB, tuberculosis.

0.2 genome copies per reaction, a sensitivity unattainable with unfragmented gDNA. These findings highlight the necessity of tailoring molecular assays to the specific status of MTB cfDNA in pleural effusion, thereby achieving enhanced detection sensitivity for TBP diagnosis.

Excess background DNA can compromise sensitivity and specificity of a molecular assay by competing for primers, promoting non-specific amplification and distorting quantitative measurements.[24 25] ddPCR mitigates these issues by partitioning the reaction mixture into microreactions, thereby reducing background DNA interference and enhancing assay reliability.[26 27] In this study, TBP pleural effusion samples exhibited significantly higher human DNA levels than those from cancer, VAP and other diseases. This is likely due to the high lymphocyte count, which releases abundant human DNA into TBP-associated pleural effusion,[6] therefore inhibiting bulk solution-based qPCR while not affecting compartmentation solution-based ddPCR. Our titration experiment revealed that at varied MTB DNA

Table 2 Diagnostic performance comparisons of TB ddPCR assay with diagnostic tests in diagnosis of pleural TB

		Definite TBP cases				Total TBP cases				
	Number	Sensitivity†	Difference (95% CI)	P value*	Sensitivity vs possible TBP cases	Sensitivity†	Difference (95% CI)	P value*	Negative predictive value vs total TBP cases	Specificity
Mycobacterial culture	321	35.3% (25.0 to 47.2); 24/68	−58.9% (-71.2 to −46.4)	<0.0001	0; 0/67	17.8% (12.2 to 25.1); 24/135	−67.6% (-74.5 to −58.9)	<0.0001	62.6% (57.0 to 67.9); 186/297	100.0% (98.0 to 100.0); 186/186
Smear microscopy	336	14.7% (8.2 to 25.0); 10/68	−79.5% (-88.6 to −68.6)	<0.0001	0; 0/67	7.4% (4.1 to 13.1); 10/135	−78% (-85.2 to −71.0)	<0.0001	98.1% (95.1 to 99.2); 203/207	100.0% (98.1 to 100.0); 201/201
Xpert MTB/RIF	300	52.0% (38.5 to 65.2); 26/50	−42.2% (-63.0 to −29.3)	<0.0001	0; 0/57	24.3% (17.1 to 33.2); 26/107	−61.1% (-67.3 to −46.4)	<0.0001	70.4% (64.8 to 75.5); 193/274	100.0% (98.0 to 100.0); 193/193
Adenosine deaminase assay	334	76.8% (65.6 to 85.2); 53/69	−17.4% (-28.3 to −5.5)	0.0075	65.2% (53.1 to 75.5); 43/66	71.1% (63.0 to 78.1); 96/135	−14.3% (-24.5 to −6.2)	0.0019	83.0% (77.6 to 87.3); 190/229	95.6% (91.6 to 97.6); 190/199
T-SPOT.TB assay	306	95.3% (87.1 to 98.4); 61/64	1.1% (-4.8 to 10.9)	0.6875	93.8% (85.0 to 97.5); 60/64	94.5% (89.1 to 97.3); 121/128	9.1% (2.2 to 16.3)	0.0169	93.6% (87.4 to 96.9); 103/110	59.9% (52.4 to 66.9); 103/172
cfDNA qPCR	309	88.7% (77.4 to 94.7); 47/53	−5.5% (-12.4 to 1.5)	0.25	61.0% (48.3 to 72.4); 36/59	74.1% (65.3 to 81.3); 83/112	−11.3% (-15.3 to −4.0)	0.001	87.0% (82.0 to 90.8); 194/223	98.5% (95.6 to 99.5); 194/197
gDNA ddPCR	345	72.5% (61.0 to 81.6); 50/69	−21.7% (-31.0 to −11.2)	0.0001	36.8% (26.3 to 48.6); 25/68	54.7% (46.4 to 62.8); 75/137	−30.7% (-38.0 to -22.5)	<0.0001	77.0% (71.6 to 81.6); 207/269	99.5% (97.3 to 99.9); 207/208
cfDNA ddPCR	345	94.2% (86.0 to 97.7); 65/69	…	…	76.5% (65.1 to 85.0); 52/68	85.4% (78.5 to 90.4); 117/137	…	…	91.0% (86.6 to 94.1); 203/223	97.6% (94.5 to 99.0); 203/208

The values are percentages (95% CIs) and numerators/denominators unless otherwise stated.
*Sensitivity comparison between cfDNA ddPCR and other diagnostic methods was conducted using McNemar's test. A p value <0.05 was considered indicative of a statistically significant difference.
†The composite microbiological reference standard included a positive result for the detection of *M. tuberculosis* in the sputum, pleural effusion or pleural tissue on any of Zieh–Neelsen stain microscopy, Xpert MTB/RIF or MGIT culture.
cfDNA, cell-free DNA; ddPCR, droplet digital PCR; gDNA, genomic DNA; gDNA, genomic DNA; TB, tuberculosis; TBP, tuberculous pleurisy.

concentrations, ddPCR yielded consistent results regardless of the human DNA levels, whereas qPCR exhibited marked increases in Ct values and even failed to yield positive results as human DNA levels increased (online supplemental figure S7). Additionally, in the clinical study, no samples were found to be ddPCR-negative but qPCR-positive. Among ddPCR-positive but qPCR-negative samples, MTB cfDNA levels were significantly lower than those in qPCR-positive samples (median: 7.1 vs 75.1 copies/reaction), and human DNA content exceeded MTB cfDNA by over 5000-fold, leading to severe qPCR inhibition (online supplemental figure S9). This discrepancy likely explains why qPCR assays for MTB cfDNA rarely achieve 80% sensitivity,[18 19 28 29] while ddPCR demonstrated superior sensitivity exceeding 90% in our study and in a recent report.[13] Notably, our ddPCR assay outperformed the previously reported ddPCR assay, achieving a sensitivity of 94.2% (95% CI 86.0% to 97.7%) compared with 90.5% (95% CI 82.8% to 95.1%), attributed to the inclusion of shorter amplicon lengths. Furthermore, ddPCR analysis confirmed the relative abundance hierarchy of human DNA, MTB cfDNA and MTB gDNA in pleural effusion, reinforcing the importance of targeting MTB cfDNA with ddPCR for optimal sensitivity.

The cfDNA ddPCR assay demonstrated a specificity of 97.6%. Among the 208 patients in the non-TB group, five yielded positive ddPCR results. One patient with a malignant tumour had positive pleural effusion for both cfDNA (11.1 copies/reaction) and gDNA (11.5 copies/reaction). The remaining four patients, diagnosed with either malignancies or pulmonary infections, showed elevated cfDNA levels (>20 copies/reaction) but tested negative for gDNA. These discordant results, along with variable IGRA and ADA levels, raise the possibility of false-positive cfDNA signals, potentially attributable to sample contamination or concurrent subclinical TBP.

Despite its diagnostic potential, MTB cfDNA presents challenges in clinical practice due to its susceptibility to degradation from fragmentation, lack of protective elements and vulnerability to nucleases.[17] Additionally, in TBP, immune cells actively degrade MTB cfDNA to maintain homeostasis.[30] Moreover, its sensitivity to temperature and storage conditions, along with the requirement for dedicated extraction protocols, further complicates its application.[31] In this study, we carefully evaluated sample storage conditions and compared two cfDNA extraction methods, revealing significant effects of these pre-analytical steps on cfDNA yield. These findings emphasise the critical role of pre-analytical optimisation in ensuring the overall performance of the MTB cfDNA ddPCR assay and facilitating the establishment of a standardised diagnostic workflow. Further refinement of this workflow could provide a robust protocol suitable for diverse clinical settings.

There are still several limitations of the study that warrant consideration. First, further validation in larger and more diverse patient cohorts is required before clinical adoption. Second, as participants were recruited from a designated TB hospital with high TB prevalence, generalisability to other settings may be limited; however, this also reflects the diagnostic challenges frequently encountered in referral centres and underscores the clinical utility of highly sensitive molecular diagnostics in such contexts. Third, while ddPCR offers high analytical sensitivity, it requires skilled personnel and a contamination-controlled laboratory environment to ensure accuracy and reproducibility, which may pose barriers to widespread use in resource-limited settings. Nevertheless, with proper workflow standardisation and integration into centralised diagnostic laboratories, ddPCR holds strong potential to complement existing TBP diagnostic methods, particularly in paucibacillary cases or when conventional results are inconclusive.

CONCLUSIONS

Confirmative diagnosis of TBP has been a longstanding difficulty due to the low diagnostic sensitivity, often leading to the misconception that TBP primarily stems from a delayed hypersensitivity reaction rather than direct pleural cavity infection.[4 32] Although improvements in culture media have enhanced MTB detection,[33] culture methods are limited to viable bacilli. Molecular methods offer earlier diagnosis with greater sensitivity but have traditionally relied on bacterial gDNA, which does not adequately reflect the total bacterial DNA species in pleural effusion. Our characterisation of MTB cfDNA and the development of an optimised ddPCR assay offer a new approach to molecular testing, with potential implications for TBP diagnosis and for improving understanding of TBP pathophysiology. By leveraging the unique properties of MTB cfDNA, this assay may help address existing diagnostic gaps and could serve as a promising tool to improve patient care in high-prevalence TB regions.

Acknowledgements We thank the staff at the Department of Pulmonary Medicine and Tuberculosis at the Fuzhou Pulmonary Hospital for their efforts in collecting the samples.

Contributors SZ, YX and QL conceived and designed the study. MH, JS and XC contributed to patient recruitment and management and collection of specimens and clinical data. SZ, YP, RP, XH, ZF and NL executed and performed quality control of laboratory work. SZ and YX analysed the data. SZ, YX and QL wrote the manuscript. All authors interpreted the results, critically revised the article for important intellectual content and approved the final version to be published. SZ, MH, YX and QL had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. QL is the guarantor of the study and accepts full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript. I used ChatGPT solely for the purpose of language refinement in the submission. No other use of AI was involved.

Funding This work was supported by the Major Science and Technology Project of Xiamen (grant no. 3502Z20231025), the Guiding Project of the Fujian Scientific and Technological Program (grant no. 2023D012), the Fuzhou Municipal Science and Technology Program (grant no. 2023-S-042), the key technological innovation and industrialisation projects in Fujian Province in 2023 (grant no.2023G051) and the Natural Science and Technology Major Project of the Xiamen (grant no. 3502Z20231034). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involved human participants and was approved by the Medical Ethics Committee of Xiamen University (approval number XYDD2021016) and the Medical Ethics Committee of Fuzhou Pulmonary Hospital (approval number 2022-001-01). Written informed consent was obtained from all participants, except for those aged ≤18 years, for whom consent was provided by their legal guardians. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer-reviewed.

Data availability statement Data are available upon reasonable request.

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