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Cell-free DNA next-generation sequencing for *Mycobacterium tuberculosis* obtained from plasma of children with active tuberculosis

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Abstract

Background Difficulties in microbiologically confirming childhood tuberculosis (TB) can result in delayed treatment and increased disease severity.

Methods In this study, we for the first time used whole genome next-generation sequencing (NGS) to detect cell-free DNA (cfDNA) from *Mycobacterium tuberculosis* (MTB) in plasma from children.

Results We enrolled 94 children with active TB and 32 children with other respiratory infections. Combining NGS with probe capture enrichment (targeted cfNGS) showed higher coverage and detecting capability than did NGS alone. The targeted cfNGS showed slightly lower sensitivity (31.9% vs. 44.7%, $P=0.072$) and specificity (96.9% vs. 100.0%, $P=0.236$) to those of sputum tested using Xpert. Agreement between cfNGS-plasma and Xpert-sputum was weak ($\kappa=0.217$). Concordant results were obtained for only 85 children (67.5%; 16 cases positive by both tests and 69 cases negative by both tests). A total of 40 children with MTB culture negative results were tested to have positive cfNGS-plasma or Xpert-sputum outcomes, yielding a significantly increased percentage of children with bacteriological evidence (20.2% [19/94] for MTB culture-positive only vs. 62.8% [59/94] for cfNGS-plasma, Xpert-sputum or culture positive).

Conclusions These data suggest that cfNGS performed well for diagnosing TB using plasma from children. cfNGS may be a new method for diagnosing patients with paucibacillary TB.

Keywords Next-generation sequencing, Cell-free DNA, Tuberculosis, Child, Diagnosis

†Shuting Quan and Xue Tian should be considered co-first authors. Author order was determined by contributions to the study.

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Background

Tuberculosis (TB) remains a major global health threat to children, with an estimated 1.3 million new cases for those aged under 15 years in 2022 [1]. Children are at higher risk for developing severe TB and increased risk of bacterial dissemination owing to their immature immune systems and bacterial dissemination. If diagnosed early, approximately 85% of people who develop TB can be successfully treated [2]. Therefore, accurate diagnostic testing contributes greatly to controlling TB.

To date, *Mycobacterium tuberculosis* (MTB) culturing of respiratory tract specimens remains the gold standard for diagnosing childhood TB. Because of the low bacterial loads and inaccessible respiratory tract samples, microbiological confirmation of childhood TB is low [3]. Since 2013, the World Health Organization has endorsed using the Xpert MTB/RIF assay (Xpert) on sputum samples for early and accurate diagnosis of TB [4]. However, difficulty in acquiring adequate respiratory tract specimens from children inhibits managing TB in these patients. Therefore, a rapid, non-sputum-based diagnostic alternative is urgently needed as a high-priority target product for TB diagnostics. Many diagnostic tests have been developed for use on blood samples; these include interferon-gamma release assays, which measure interferon-γ released by antigen-specific T cells from MTB-infected patients [5] and host-based phenotypic markers, including MTB-specific proteins or immune cell subsets [6, 7]. More recently, screening plasma biomarkers has shown potential application value in diagnosing TB [8, 9].

Cell-free DNA (cfDNA) consists of short double-stranded DNA fragments released into blood plasma from both dying human cells and colonizing or invasive microbes. Compared with complicated and costly biopsies, analyses of cfDNA from bodily fluids, also known as liquid biopsies, have recently emerged as a novel, minimally invasive method of detecting and diagnosing cancers [10, 11]. Some circulating cfDNA from degraded or metabolically active bacteria can also be detected in the blood and other bodily fluids [12]. However, diagnosing diseases on the basis of cfDNA is challenging when using conventional PCR-based testing because of the short lengths and low concentrations of cfDNA molecules released by microbes in the plasma and the high sequence similarity between pathogens [13].

Compared with conventional testing for nucleic acid detection, next-generation sequencing (NGS) is more sensitive and comprehensive for detecting viable but non-culturable or difficult-to-culture microorganisms and investigating previously unknown pathogens [14, 15]. While previously cost prohibitive, this technique has become cheaper in recent years, making it more accessible for clinical use. It shows great potential in cases where there is a strong clinical suspicion of infection, where cultures or other diagnostic tests are negative, or where antimicrobial resistance exists [16]. The class of targeted next generation sequencing (tNGS) is recommended for the detection of resistance mutations to a number of first- and second-line anti-TB drugs by WHO at march 2024. The recommendations will open for faster detection of resistance to a range of anti-TB drugs directly from sputum samples. However, low bacterial loads and difficulty in obtaining sputum specimens in children limit the use of existing tNGS methods in pediatric TB patients [17]. cfDNA-based NGS (cfNGS) is a recent innovation with the major advantage and unique characteristic of a fast run-time. The technique allows isolating DNA directly from samples without lysing microbial cells and can typically be completed within several hours [18]. However, conventional methods for DNA extraction and NGS likely underestimate the proportion of short cfDNA molecules present because they exhibit poor retention of degraded DNA fragments [19]. Therefore, in the present study, we used a targeted enrichment-based method based on TB-capture RNA probe, which targets the whole MTB genome, to achieve higher sensitivity. We compared the accuracy of this targeted cfNGS technique with that of sputum molecular tests using Xpert. To our knowledge, this is the first evaluation of the clinical utility of targeted cfNGS in diagnosing childhood TB.

Methods

Study population and design

This study was performed using samples obtained between January 1, 2019 and December 31, 2021 at the No. 1 People's Hospital of Liangshan Yizu Autonomous Prefecture and Beijing Children's Hospital. We enrolled children who were highly suspected of having active TB as per the following symptoms: cough lasting for >2 weeks, weight loss, malnutrition and abnormal changes on chest radiographs. Finally, we enrolled 94 children with active TB and 32 children with respiratory infections (disease controls). All patients with TB were diagnosed based on the composite reference standard (CRS), which includes: (1) positive bacteriological confirmation results, (2) at least one TB symptom or sign, (3) radiographic evidence consistent with TB, (4) tuberculosis exposure, (5) positive tuberculin skin test or interferon-gamma release assay, and (6) clinical and radiological improvement following anti-TB chemotherapy [20]. Patients with bacteriologically confirmed TB were defined as those with positive MTB culture results (excluding Xpert to avoid inclusion bias). Patients with unconfirmed TB were defined as those clinically diagnosed based on at least 2 of the CRS and without positive MTB culture results. Children with respiratory infections were defined as those who were symptomatic and had confirmed etiological evidence of viral, mycoplasma, or bacterial infections.

TB meningitis, miliary TB, and disseminated TB were defined as severe forms of TB. Plasma and sputum samples were collected from each patient before regular antituberculosis treatment within 1 week of hospitalization, frozen immediately and stored until further use. Further details of the study flow are showed in Fig. 1. The Ethics Committees of Beijing Children's Hospital approved this study, and the guardians of all patients signed written informed consent.

Clinical and laboratory procedures

Demographic information and clinical data on the enrolled patients, including age, sex, and treatment status, were collected from their medical records. Whole blood was collected in EDTA tubes and centrifuged at 3,000 × g for 10 min at 4° C within 4 h of collection. Plasma samples were stored at -80 °C for further analysis. Sputum specimens were usually collected in the early morning and simultaneously subjected to smear microscopy, mycobacterial growth indicator tube cultures and Xpert (Cepheid; Sunnyvale, USA). Xpert was performed per the manufacturer's instructions. Briefly, sputum samples (1–2 mL) were mixed with double volumes of Xpert sample processing reagent and vortexed at 5-min intervals for 15 min, then 2 mL of the mixture was transferred to the cartridge for Xpert testing.

Plasma CfDNA extraction and library Preparation

DNA was extracted from 500 μL of plasma using a cfDNA extraction kit (Matridx, MD005, HangZhou, China) following the manufacturer's operational manual. The extracted 50 μL of cfDNA eluant was stored at − 20℃ to construct the cfDNA libraries. cfDNA libraries were prepared using a cfDNA Library Preparation Kit (Matridx, MD007P) following a method that included end-repair, dA-tailing and sequencing adaptor ligation. Libraries were amplified using the KAPA Library Amplification Kit (Matridx, KK2621) to enable downstream testing.

Hybridization and capture using RNA probes targeting MTB whole genome

Amplified libraries were quantified using the Magic dsDNA HS Assay Kit (Magic-Bio, M331). Every eight libraries were combined into a single pool (≤ 4 μg) comprising 500 ng of each of the amplified libraries, then hybridized overnight using the RNA probe. The RNA probe libraries were designed and synthesized by Agilent Technologies to cover the complete genome of the H37Rv reference strain (Ref No: AL123456.3). Hybridization and capture reagent kits (TargetSeq One Kit-Box1 [for ILM], TargetSeq One Kit-Box2 and Eco Universal Blocking Oligo Kit [for ILM]; iGeneTech) were used per the manufacturer's instructions.

After hybridization, the DNA libraries were captured by the Dynabeads MyOne Streptavidin T1 kit (Thermo Fisher, 65601) and amplified using the Post PCR Primer (for ILM). PCR fragments were purified using the Nucleic Acid Purification Kit (Matridx, MD012), then quantified via qPCR using the KAPA Library Quant Kit (Roche, KK4824) and pooled for sequencing.

NGS and data analysis

All libraries were sequenced on the Illumina NextSeq 550Dx platform using the NextSeq 500/550 High Output Kit (75 Cycles) V2.5. Raw data was first preprocessed by sequence removal followed by computational extraction of human reads aligned to the human genome (hg19). The remaining data were aligned to the pathogens using Kraken2 referring to the NCBI database [21]. Classified sequences were then aligned against the microbial RefSeq database with bowtie2 for verification. BLAST (version 2.9.0+) was used to validate candidate reads when Kraken2 and Bowtie2 were inconsistent [22]. The reads with partial homology, which could potentially align to both MTB and non-tuberculous mycobacteria (NTM), were excluded and only MTB-specific reads was analyzed during this process.

Evaluation of the RNA probe capture capability

We analyzed the detecting capability of RNA probes which were designed to target the MTB whole genome after hybridization and capture (target cfNGS). First, the capability of detecting the MTB whole genome was tested via untargeted cfNGS (sequencing after library preparation without RNA probe capture) and targeted methods (sequencing after RNA probe capture enrichment) in samples containing both 10 ng of human genomic DNA and 1 pg of genomic DNA of H37Rv. The read number and reads per million mapped reads (RPM) of each sample were compared between the two methods. Second, two plasma samples from children with active TB were tested via untargeted cfNGS and targeted methods. Third, samples containing both 10 ng human genomic DNA and 1 pg genomic DNA of any of the following NTM which including *Mycobacterium avium* complex, *Mycobacterium abscessus*, *Mycobacterium kansasii* and *Mycobacterium fortuitum* were used to evaluate the specificity of the RNA probe. The cross-activity of the MTB-specific RNA probe with NTM genome was analyzed.

All mycobacterial genomic DNA were extracted from two isolates from different labs. Samples containing only 10 ng of human genomic DNA or only containing water were also tested as negative controls to verify that nonspecific binding or mapping did not occur in samples without MTB. A positive cfNGS was given to tuberculosis cases when at least one read mapping to MTB.

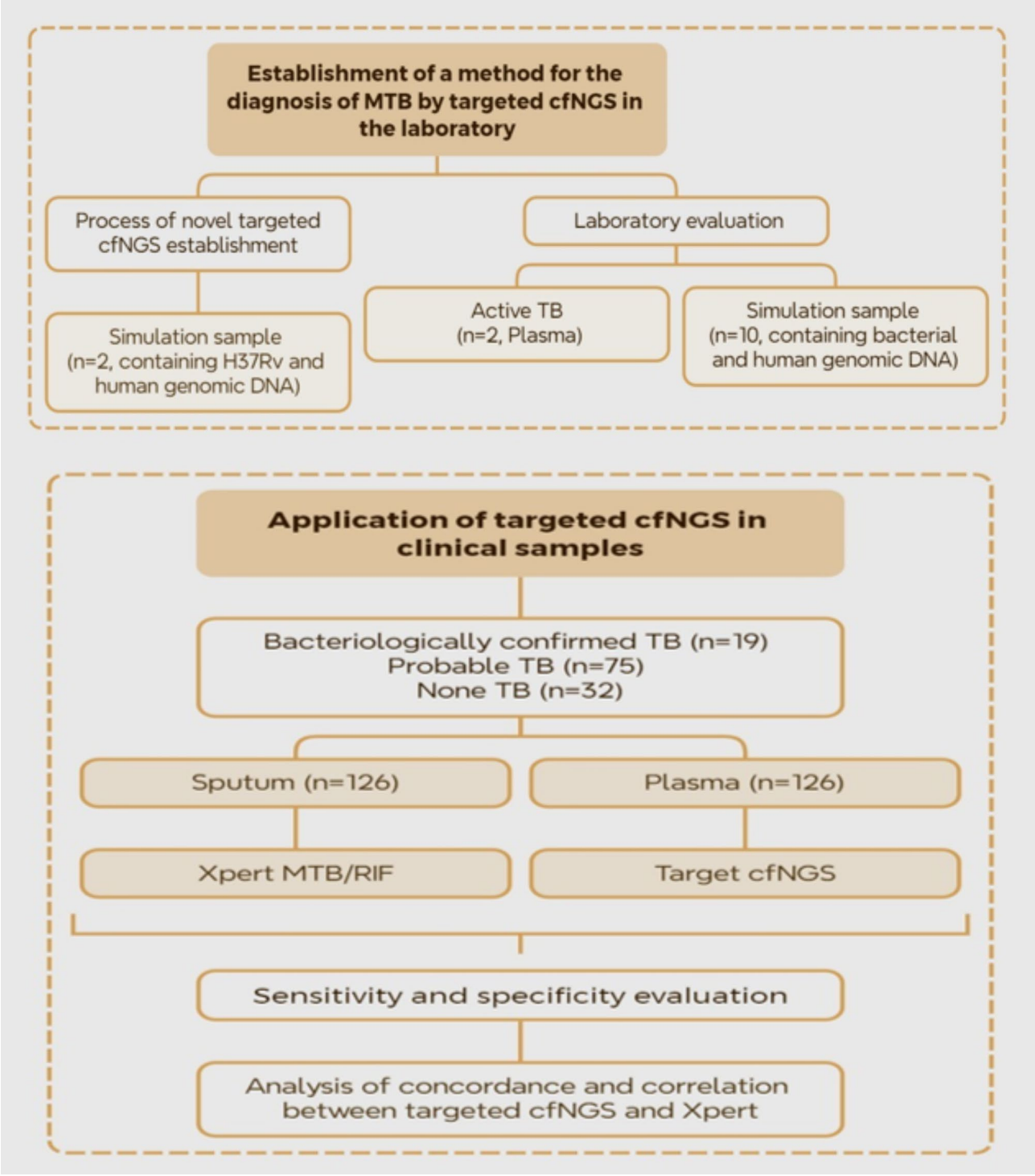

Fig. 1 Flow chat of the study design

Evaluation of CfNGS performance in clinical samples from the study population

The cfNGS performance was evaluated using bacteriological results and clinical evidence as per the CRS. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with 95% confidence intervals (CIs) of cfNGS were calculated in 126 children.

Statistical analysis

To characterize the study populations, we reported the numbers and percentages for categorical variables and

the medians and interquartile ranges for continuous variables. McNemar's test was used to evaluate differences in sensitivity and specificity. Concordance was assessed using the percentage agreement, kappa coefficients, and Spearman's rank correlation coefficients. $P<0.05$ was considered statistically significant. SPSS 23.0 software was used for statistical analysis.

Results

Targeted CfNGS mapping statistics to MTB whole genome

To evaluate the coverage and capture efficiency of the probe, samples containing bacterial and human genomic DNA were tested using targeted and untargeted cfNGS. The average of 59.51% of sequenced reads identified by targeted cfNGS mapped to the MTB whole genome, whereas only 1.24% of reads identified by untargeted cfNGS mapped to the MTB whole genome. After capture (top plot of Fig. 2), the sample exhibits significantly higher coverage across the genome, with some genomic regions reaching depths of several thousand-fold. In contrast, before capture (bottom plot of Fig. 2), the reads are evenly distributed across the genome, but with much lower depth, averaging only 1× at each position. The targeted cfNGS coverage depth was stratified by targeted genomic region (Table 1), which showed higher coverage in the IS*6110* and IS*1081* regions.

Detecting capability of CfNGS for detecting MTB

Two plasma samples from children with active TB were simultaneously tested using both untargeted and targeted cfNGS. Table 2 shows the read numbers and RPMs per sample. The RPMs of MTB from the two samples using untargeted cfNGS were 2.49 and 16.28. After RNA probe capture, the RPMs of MTB in both samples increased to 7,874.63 and 55,562.25. For the samples containing both 10 ng of human genomic DNA and 1 pg of genomic DNA of H37Rv, the RPM of MTB increased from 62.41 using untargeted cfNGS to 148,809.50 using targeted cfNGS.

In the two plasma samples from children with active TB, all reads were finally mapped to MTBC with no reads mapped to NTM. For the samples containing NTM genomic DNA, no reads were mapped to MTB. No negative samples containing only human genomic DNA were mapped to MTB or NTM sequences using the targeted cfNGS.

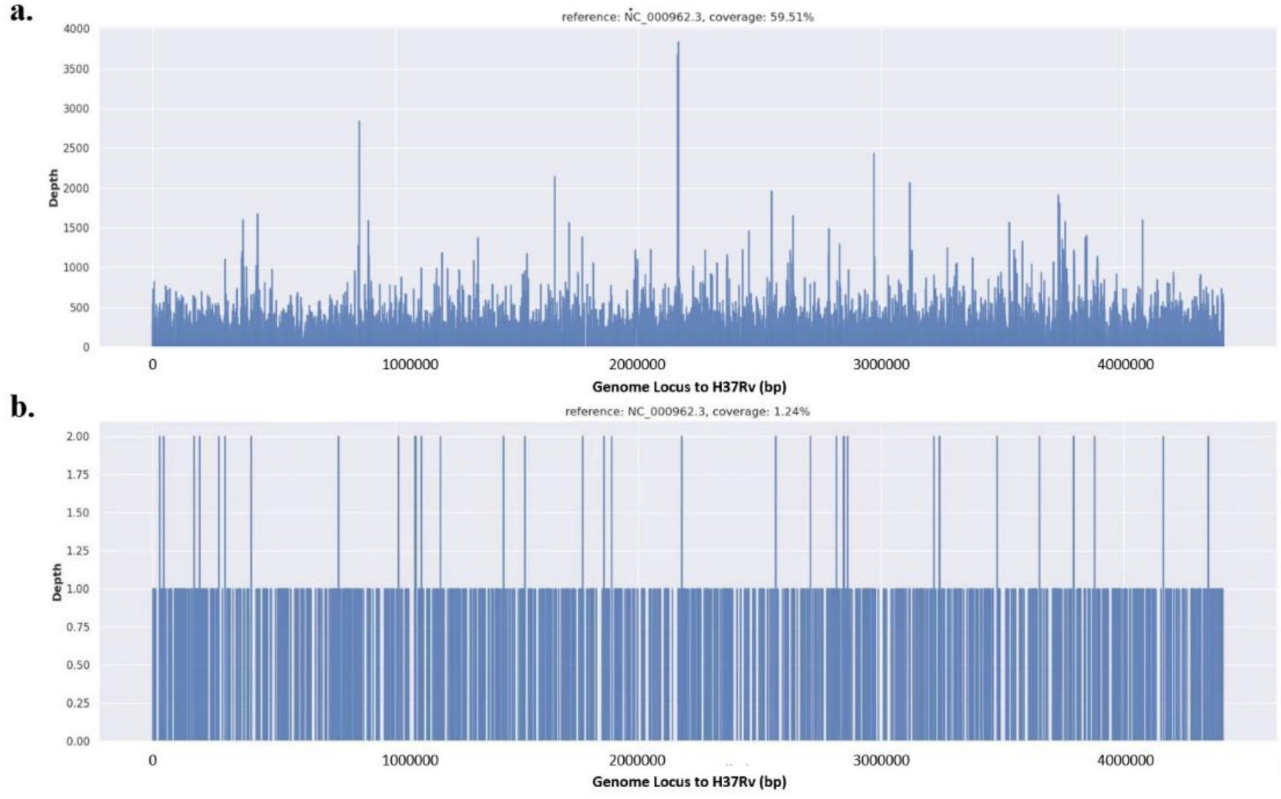

Fig. 2 The evaluation of coverage and capture efficiency of the probe tested using targeted and untargeted cfNGS. (**a**) targeted cfNGS. (**b**) untargeted cfNGS. The MTB coverage plots represent the sequencing read coverage across the entire Mycobacterium tuberculosis genome before and after capture. The y-axis indicates the depth of coverage

Table 1 Coverage as function of genes for targeted and untargeted CfNGS

methods	samples	average coverage depth						coverage percentage					
		IS6110	*IS1081*	*rpoB*	*ESAT-6*	*CFP-10*	*RecA*	*IS6110*	*IS1081*	*rpoB*	*ESAT-6*	*CFP-10*	*RecA*
targeted cfNGS	isolate1	10088.7	1303.3	67.8	26.3	21	43.9	100%	95.33%	57.86%	37.85%	27.39%	48%
	isolate2	5639.7	1056.88	40.5	126	35.1	47	100%	92.26%	51.63%	70.49%	79.87%	37.17%
untargeted cfNGS	isolate1	0.57	0.03	0	0	0	0.02	43.39%	3.34%	0	0	0	2.11%
	isolate2	0.07	0.1	0.04	0	0	0	7.38%	10.01%	4.26%	0	0	0.00%

Application of targeted CfNGS in clinical samples

Study participant characteristics

We enrolled 126 children, including 94 (74.6%) with active TB (19 bacteriologically confirmed cases and 75 unconfirmed cases) and 32 (25.4%) without TB. Table 3 presents the demographic and clinical characteristics of the 126 children.

Pediatric TB diagnosis by targeted CfNGS

Of the 94 children with active TB, MTB was detected in the plasma from 30 children, with the MTB read numbers ranging from 1 to 112,905, resulting in 31.9% (95% CI, 22.9-42.4%) sensitivity. The specificity of the cfNGS-plasma was 96.9% (31/32; 95% CI, 82.0-99.8%). Among the subset of 19 children with bacteriologically confirmed TB, cfNGS-plasma testing yielded a sensitivity of 42.1% (8/19; 95% CI, 21.1-66.0%). Among the 75 children with unconfirmed TB, the sensitivity of the test was lower (29.3%, 95% CI, 19.7-41.1%; 22/75) (Table 4). The sensitivities of cfNGS-plasma in children with severe and mild TB were 38.1% (16/42; 95% CI, 24.0-54.3%) and 26.9% (14/52; 95% CI, 16.0-41.3%), respectively.

Comparison of the 94 pediatric plasma samples for detecting TB using targeted cfNGS showed slightly lower sensitivity (31.9%, 95% CI, 22.9-42.4% vs. 44.7%, 95% CI, 34.5-55.3%, $P = 0.072$) to those of sputum using Xpert. Sensitivity of the Xpert-sputum was significantly higher than that of cfNGS-plasma for children with bacteriologically confirmed TB (84.2%, 95% CI, 59.5-95.8% vs.42.1%, 95% CI, 21.1-66.0%, $P = 0.019$). Sensitivities of both tests were similar in children with unconfirmed TB (34.7%, 95% CI, 24.3-46.6% vs.29.3%, 95% CI, 19.7-41.1%, $P = 0.484$; Table 4).

Concordance and correlation between targeted CfNGS and Xpert

Agreement was weak between cfNGS-plasma and Xpert-sputum ($\kappa = 0.217$ among 126 children). Concordant results were obtained for only 85 children (67.5%; 16 positive on both tests and 69 negative on both tests). 15 children had discordant results cfNGS + Xpert- results (14 with unconfirmed TB and 1 with respiratory infections), and 26 had discordant results cfNGS- Xpert+ (8 with bacteriologically confirmed TB and 18 with unconfirmed TB). Among 14 children with cfNGS + Xpert- results, miliary micronodular lesions were seen in 10 children based on the radiologic findings. The other four children were diagnosed as TB because they all had radiographic evidence consistent with TB and presented with clinical and radiological improvement following anti-TB chemotherapy. Integrating cfNGS-plasma and Xpert-sputum outcomes confirmed that an additional 40 children had TB, resulting in a significant increase in the percentage

Table 2 The reads number and the RPM of the samples tested

Sample type	Sample number	untargeted cfNGS			targeted cfNGS			RPM-fold change of MTBC
		total reads number	reads number of MTBC	RPM of MTBC	total reads number	reads number of MTBC	RPM of MTBC	
Plasma from TB patients	No.1	14,969,630	213	2.49	22,176,044	335,177	7874.63	3162.50
	No.2	14,979,472	45	16.28	17,446,960	152,228	55562.25	3412.91
Simulation sample	MTBC	18,700,833	988	66.00	9,116,651	3,463,768	156194.10	2366.58
	MTBC	18,488,301	881	58.81	12,824,199	2,467,434	141425.00	2404.78
	MAC	17,155,929	0	0	11,832,044	0	0	/
	MAC	15,944,882	0	0	9,282,210	0	0	/
	M.abscessus	15,213,076	0	0	10,084,058	0	0	/
	M.abscessus	7,849,629	0	0	11,327,565	0	0	/
	M.kansasii	17,212,256	0	0	10,869,323	0	0	/
	M.kansasii	18,659,809	0	0	12,283,455	0	0	/
	M.fortuitum	13,080,259	0	0	6,032,459	0	0	/
	M.fortuitum	18,049,492	0	0	19,331,439	0	0	/

TB, tuberculosis; RPM, reads per million mapped reads; MTBC, *Mycobacterium tuberculosis complex*; MAC, *Mycobacterium avium complex*; M.abscessus, *Mycobacterium abscessus*; M.kansasii, *Mycobacterium kansasii*; M.fortuitum, *Mycobacterium fortuitum*

Table 3 Main clinical characteristics of the study population

Characteristic	Total(*N* = 126), n (%)	Bacteriologically confirmed TB(*N* = 19), n (%)	Probable TB(*N* = 75), n (%)	Non-TB(*N* = 32), n (%)
Age mean (interquartile range)	6.6(2.6–11.4)	6.8 (1.5–12.0)	6.9(3.0-11.5)	5.9(2.0–9.0)
Gender				
Male	68(54.0)	8 (42.1)	39 (52.0)	21 (65.6)
Female	58(46.0)	11(57.9)	36 (48.0)	11 (34.4)
BCG vaccination				
Yes	27 (21.4)	7 (36.8)	17(22.7c)	3 (9.4)
No	50 (39.7)	9 (47.4)	25(33.3)	16 (50.0)
Unclear	49(38.9)	3(15.8)	33(44.0)	13(40.6)
Tuberculin skin test				
Positive	68 (54.0)	12 (63.2)	52 (69.3)	4 (12.5)
Negative	54 (42.9)	7 (36.8)	22(29.3)	25 (78.1)
No data	4(3.1)	0(0.0)	1(1.3)	3(9.4)
Interferon-γ release assay				
Positive	76 (60.3)	16 (84.2)	54 (72.0)	6 (18.8)
Negative	34 (27.0)	2 (10.5)	11(14.7)	21 (65.6)
No data	16(12.7)	1(5.3)	10(13.3)	5(15.6)

TB, tuberculosis; BCG, Bacillus Calmette-Guérin

of children with bacteriological evidence of TB (62.8% [59/94] vs. 20.2% [19/94]; Fig. 3a).

Comparison of detected MTB CfDNA concentrations across groups

To further analyze the detected TB-specific cfDNA concentrations in patients with different clinical phenotypes, the absolute numbers of the detected MTB reads were compared between subgroups and did not significantly differ between children with bacteriologically confirmed TB and unconfirmed TB ($P = 0.0263$) or between children with severe TB and mild TB ($P = 0.2125$). Analysis of MTB read numbers in children using a different semiquantitative scale for Xpert, patients with higher semiquantitative scores were more likely to have more MTB reads (Fig. 3b, $P = 0.0035$).

Discussion

We developed a novel diagnostic procedure based on NGS and cfDNA analysis using plasma from children with active TB to improve TB diagnostic methods. Because newborns, infants, and younger children have difficulty producing sputum and are particularly susceptible to severe TB types owing to delayed diagnosis, these children may benefit the most from this diagnostic tool, which requires only 0.5 mL of plasma. Additionally, it may be a good alternative to MTB cultivation, which

Table 4 Performance of different assays for pediatric tuberculosis

Patient Group	cfNGS-plasma	Xpert-sputum	*P* value
Total TB cases			
Sensitivity % (95% CI), n/N	31.9(22.9–42.4), 30/94	44.7(34.5–55.3), 42/94	0.072
PPV %, (95% CI), n/N	96.8(81.5–99.8), 30/31	100.0(89.6–100.0), 42/42	
NPV %, (95% CI), n/N	32.6(23.6–43.1), 31/95	38.1(27.9–49.4), 32/84	
Bacteriologically confirmed TB			
Sensitivity %, (95% CI), n/N	42.1(21.1–66.0), 8/19	84.2(59.5–95.8), 16/19	0.019
PPV %, (95% CI), n/N	88.9(50.7–99.4), 8/9	100.0(75.9–100.0), 16/16	
NPV %, (95% CI), n/N	73.8(57.7–85.6), 31/42	91.4(75.8–97.8), 32/35	
Unconfirmed TB			
Sensitivity %, (95% CI), n/N	29.3(19.7–41.1), 22/75	34.7(24.3–46.6), 26/75	0.484
PPV %, (95% CI), n/N	95.7(76.0-99.8), 22/23	100.0(84.0-100.0), 26/26	
NPV %, (95% CI), n/N	36.9(26.8–48.2), 31/84	39.5(29.0–51.0), 32/81	
Specificity			
%, (95% CI), n/N	96.9(82.0-99.8), 31/32	100.0(86.7–100.0), 32/32	0.236

TB, tuberculosis; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value

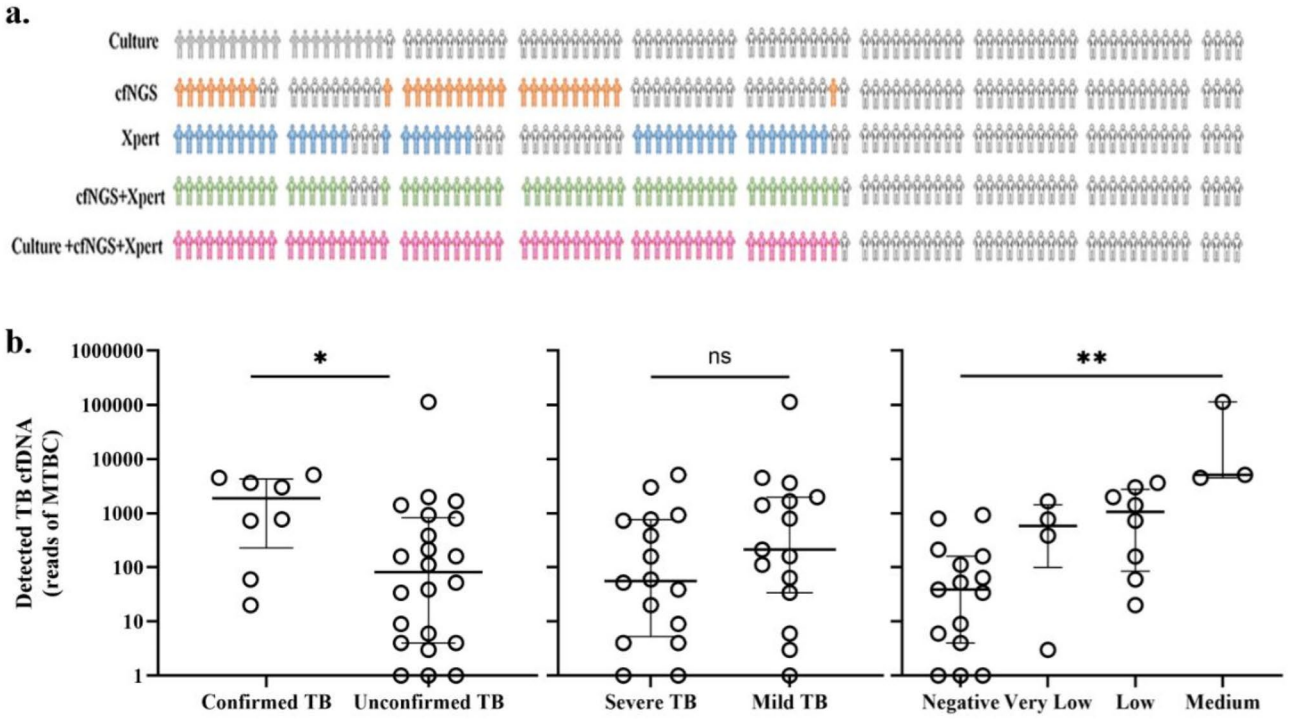

Fig. 3 Diagnostic value of targeted cfNGS in clinical samples. (**a**) assay data for children with active TB. Patient with positive results were filled with color. (**b**) comparison of detected TB cfDNA absolute numbers across groups (bars indicate median and interquartile range [IQR] of sample means of cfDNA-positive samples)

requires at least 6–8 weeks to report negative culture results.

Microbiological confirmation of childhood TB is usually blocked because of the difficulties in acquiring adequate sputum samples. New methods for detecting microbial cfDNA from patients' body fluids (e.g., pleural effusion, urine, cerebrospinal fluid, and blood) can improve the positive detection rates of pathogens [23–25]. NGS has recently been used to detect short cfDNA molecules of MTB [26]. Limited samples were used to identify MTB-derived cfDNA by NGS and the results showed that urine cfDNA is a potentially powerful biomarker for diagnosing TB [19]. However, data on diagnosing TB in children using cfDNA remain limited. Only a few studies with a small sample size of children have reported results [27]. Using 250 μL of plasma in EDTA, MTB cfDNA was detected in two of four children (50%) with smear-positive culture-confirmed TB at the time of initial TB diagnosis [27]. Another study reported that the sensitivity of cfDNA detection on plasma was higher

than Xpert assay on gastric aspirate (28.57% VS. 15.38%, $P > 0.05$) [27]. Therefore, the diagnostic value of cfDNA in patients with a lower bacillary burden requires further study.

Performing NGS alone may make it difficult to detect genomes from pathogens present in low copy numbers, and optimization is needed to improve sensitivity. The method of enriching MTB reads after NGS library preparation has shown great promise [28]. In the present study, we developed a probe capture enrichment method using oligonucleotide capture probes targeting the whole MTB genome. This target enrichment sequencing is a recent methodology for detecting samples containing low genomic copy numbers of a particular pathogen versus the host [29]. Here, we modified the common process of target enrichment sequencing which included cfDNA library preparation and amplification, probe hybridization and capture, amplification of captured library to consequent enrichment and sequencing. Library amplification was performed twice before and after probes capture. Therefore, the modified protocol has higher sensitivity and less contamination. The targeted NGS method yielded a 3,000-fold increase in MTB reads from plasma of pediatric patients with paucibacillary characteristics. Target enrichment sequencing is gradually used to the deep-sequencing of interest with great depth [30, 31]. Although this method has been used successfully on pathogens such as bacteria, fungi, and viruses, our study was the first to explore probe capture enrichment on the sequencing of MTB cfDNA in children [32].

Microbial cfDNA analysis is an alternative approach that provides rapid information on infections at various locations in the body when biopsies are not possible or etiological results are unavailable. Recent studies have reported the diagnostic value of cfDNA from MTB in patients with active TB. One study enrolled adults with TB pleurisy and found that MTB cfDNA showed higher sensitivity (75.0%) than did MTB cultures (26.7%) and Xpert (20.0%) [33]. Another study which enrolled 10 HIV-positive patients with pulmonary TB reported a combination of DNA extraction and single-stranded sequence library preparation methods, and verified that urine MTB-derived cfDNA was a potentially powerful biomarker [19]. In this study, the sensitivity of the cfNGS-plasma was 31.9% (95% CI, 22.9-42.4%) in children with active TB, which is lower than the previously reported data in adult patients. Considering the low bacterial load in the respiratory samples of children, the lower sensitivity of the cfNGS method may be due to the decrease of cfDNA which was released into plasma. Indeed, we observed that read numbers of MTB cfDNA in TB children were correlated with bacterial loads in sputum detected by Xpert. The positive correlation between extracellular DNA fragments from microorganisms and the bacterial numbers in the respiratory tract also confirmed the potential role of cfNGS as a new method for diagnosing pulmonary TB. However, unlike Xpert, which enables detecting dead bacteria after treatment, cfDNA can be cleared rapidly from plasma after treatment [34]. Therefore, detection of MTB cfDNA circulating in the bloodstream can reflect present infection conditions and cfDNA is also a potential biomarker for monitoring TB treatment.

We found similar sensitivities in unconfirmed TB children and low concordance for both cfNGS using plasma and Xpert using sputum. The ability to combine the specificity of target enrichment and with the high throughput nature of NGS has been shown to result in comparable detection sensitivity with Xpert. As a rapid testing using noninvasive samples, Xpert-sputum was preferred for diagnosis in children with negative MTB culture results. Considering the case detection gap for children and the fact that these technologies are not largely available at lower-level healthcare centers where the majority of TB cases present, targeted cfNGS was able to detect MTB in cases missed by Xpert, because sputum Xpert was able to detect the largest number of cases.

Targeted cfNGS emerged as an important method for pathogen identification, especially in conditions where the pathogen load is expected to be low. Firstly, the cost of probe synthesis and NGS sequencing has significantly decreased. Taking into account the possibility of pooling multiple libraries for hybridization, along with personnel costs, we estimate the total cost to be below 500 RMB (approximately 80 USD) per sample. Moreover, with the increasing automation of NGS workflows and further reductions in sequencing costs, the overall expenses are expected to decrease even further. Regarding the experimental timeline, it is true that the capture method involves relatively complex procedures. However, there are now automated systems available that can assist in completing parts of these processes. In terms of turnaround time (TAT), it is already possible to deliver results within 24 h, which meets the diagnostic needs of tuberculosis patients. In summary although targeted cfNGS is more complex and expensive, it requires a lower level of laboratory safety and the time to results will be greatly reduced (traditional MTB culture methods require at least 6–8 weeks to report negative culture results and a biosafety containment level BSL-3 laboratory [35], making this approach feasible for clinical diagnostics.

The likelihood that positive results in children with negative culture or Xpert tests represent contamination is minimized in our study due to the stringent quality control measures in place. The use of multiple diagnostic methods, such as combining cfNGS with traditional tests like culture and Xpert, provides a robust cross-verification framework, which reduces the chance that

contamination influenced the results. Additionally, the clinical context and radiographic evidence supporting the diagnosis in these cases further reduce the likelihood of contamination being a primary factor. To mitigate the risk of contamination, several precautions were taken throughout the laboratory workflow: (1) We used dedicated areas for different stages of the experiment (e.g., DNA extraction, library preparation, and PCR amplification) to avoid cross-contamination. (2) We included multiple negative control samples (e.g., blank water and human DNA samples without microbial input) in each batch of experiments to monitor for any potential contamination. (3) In the data analysis phase, reads that could not be confidently mapped to MTB were excluded, and strict thresholds were applied to further minimize the possibility of contamination-derived reads being considered in the final analysis.

We also observed that one child without TB was tested positive by cfDNA with 64 reads mapped to MTB. The patient was excluded the diagnosis of active tuberculosis because she presented with atypical clinical presentations, inconsistent radiographic evidence with TB, and negative laboratory results including IGRA, TST, molecular test and MTB culture. The child was not treated with anti-tuberculosis drugs but clinically and radiologically improved during hospitalization. Therefore, the possibility may be related to experimental factors. Although we implemented stringent contamination prevention measures in our laboratory (as previously described), the possibility of cross-contamination during sample handling or processing cannot be entirely ruled out. Additionally, given the nature of NGS technology, multiple samples are sequenced on the same chip simultaneously, raising the potential for issues such as index hopping or carry-over contamination, which could lead to false-positive results.

Limitations

The use of cfDNA as a diagnostic biomarker of TB remains in its infancy. NGS is more expensive than culturing tests and has a longer run-time, although it can be completed with shorter turnaround time of < 2 days. Data are limited regarding the underlying value of cfDNA in pediatric patients with TB. However, cfNGS is gaining attention and shows great potential. Standard laboratories with specialized equipment, trained lab technicians and bioinformatics expertise are also needed, which are not available for resource-limited settings.

Conclusion

In conclusion, cfNGS performed well for diagnosing TB using plasma from children. cfNGS is a potential new diagnostic tool for patients with paucibacillary TB.

Abbreviations

TB	Tuberculosis
NGS	Next-generation sequencing
cfDNA	Cell-free DNA
MTB	*Mycobacterium tuberculosis*
Xpert	Xpert MTB/RIF assay
CRS	Composite reference standard
RPM	Million mapped reads
NTM	Non-tuberculous mycobacteria
PPV	Positive predictive value
NPV	Negative predictive value
CIs	Confidence intervals

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Author contributions

Lin Sun and Adong Shen conceptualized and designed the study, drafted the initial manuscript, reviewed and revised the manuscript. Shuting Quan, Xue Tian, Xuemei Yang, Baixu Sun and Xi Zeng collected and analyzed the data, drafted the initial manuscript. Min Fang and Li Duan, enrolled the subjects and collected samples. Kun Duan, Jichao Wang, Xue Fu and Fang Xu performed the tests and analyzed the data. Weiwei Jiao, Yuting Sun and Hui Qi drafted the initial manuscript, reviewed and revised the manuscript.All of the authors have approved the submitted version.

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Data availability

The datasets generated and analysed during the current study are available in the SRA repository with accession number PRJNA1094919.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Beijing Children's Hospital affiliated with Capital Medical University (ID:2018-96). The patients' guardians provided written informed consent. In cases of illiteracy, a witness attested to informed oral consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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