Using of Digital Droplet PCR in the detection of *Mycobacterium tuberculosis* DNA with FFPE and Cytological samples

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Abstract

**Backgrounds:** Accurate diagnosis for TB is essential for TB control. Droplet digital PCR (ddPCR) is a technology that has high sensitivity. However, the use of ddPCR for the detection of TB pathogen in pathological samples is not been fully studied.

**Methods:** A total of 88 samples from the patients who were suspected of tuberculosis were involved in this study, including 65 formalin-fixed and paraffin-embedded (FFPE) specimens and 22 cytological samples. Digital Droplet PCR was used to compare the sensitivity with Real-time PCR. The real-time PCR ct value and ddPCR TB abundant ratio were analyzed by SPSS software.

**Results:** Among the 62 samples that were “possible TB” detected by real-time PCR, 34 samples were ddPCR-positive, 18 samples were ddPCR-negative, and 10 samples were in “gray area” by ddPCR. 26 patients that were ddPCR-positive received anti-tuberculosis therapy and 14 cases of them benefit from the treatment.

**Conclusions:** ddPCR is more sensitive in the detection of TB than Real-time PCR. ddPCR methods can be used as an additional means for the diagnosis of TB from pathological samples.

**Introduction**

Tuberculosis (TB) is a chronic infectious disease caused by bacillus *Mycobacterium tuberculosis* infection. 80% of TB occurs in the lungs (pulmonary TB), while other sites, such as neck lymph nodes, meninges, peritoneums, intestines, skin, bones (extrapulmonary TB) can also be affected [1]. Worldwide, TB is one of the top 10 causes of death and the leading cause of a single infectious agent (above HIV/AIDS) [2]. China is a country with a high incidence of tuberculosis. According to the 2018 WHO report, the annual new tuberculosis cases in China account for 9% of the global annual new cases,
ranking the 2nd in the world [3].

TB control quite challenges around the worldwide and many goals have been set to end TB. In 1993, the WHO has declared TB as a global emergency [4]. In May 2014, all Member States of WHO endorsed WHO’s End TB Strategy at the World Health Assembly and committed to building a tuberculosis-free world until 2035 [5]. In September 2018, the first UN (United Nations) High-Level Meeting (UNHLM) on tuberculosis stated to make TB eradication as a global priority[6]. Achieving these objectives, accurate diagnosis for TB is essential.

The main direct methods for diagnosis of TB include microscopy, culture, antigen detection, and nucleic acid detection [7]. The pathological diagnosis of TB was based on the recognition of granulomatous inflammation by H&E staining and identification of mycobacteria with Ziehl-Neelsen (ZN) staining via formalin-fixed and paraffin-embedded (FFPE) samples [8]. However, the low sensitivity of ZN staining, time-consuming and laborious for searching acid-fast bacilli are the major limitations [9]. Molecular techniques have been widely used in routine practice in pathological laboratories, such as Polymerase chain reaction (PCR) for the detection of driver-oncogene mutations in tumors [10]. Currently, reports also implied real-time PCR assay provided a highly sensitive for the detection of TB DNA in FFPE specimens [11]. Droplet digital PCR is a newly developed technology that has many advances upon real-time PCR. Recently, researchers reported ddPCR could be used for the detection of TB in whole blood-derived DNA samples and stable Mtb strain [12, 13]. However, the use of ddPCR for the detection of TB pathogen in FFPE samples is not been fully studied.

In this study, we compared droplet digital PCR and Real-time PCR in 62 samples, and we
further estimate the sensitivity of droplet digital PCR in the detection of TB pathogen via FFPE specimens and also some cytological specimens (fine needle aspiration (FNA) and endobronchial ultrasound-guided aspiration (EBUS)).

**Methods**

**Case selection**

We conducted a laboratory-based study to compare the sensitivity of both the Real-Time PCR assay and a droplet digital PCR assay. The study was undertaken at Shanghai Pulmonary Hospital, Tongji University School of Medicine. All of the samples that were subjected to histology, ZN, and Real-Time PCR assay tests from January 2018 to May 2019 were reviewed.

**Sample preparation and DNA extraction**

The details of sample preparation were provided in our previous study. Briefly, surgery samples, biopsy sample, and cell blocks were stored in FFPE blocks. Cytology specimens including fine needle aspiration (FNA) and endobronchial ultrasound-guided aspiration (EBUS) were made into liquid-based cells\(^{[14]}\). 50µm of the FFPE samples were gathered into 1.5 ml microcentrifuge, and the samples were subsequently deparaffinated using dimethylbenzene, dehydrated through 100% alcohols, and then air-dried for 5 min; The pellet in the residual liquid-based cell was collected into a 1.5 ml microcentrifuge.

The total DNA was extracted by using an E.Z.N.A. FFPE DNA Kit (Omega Bio-tek, Inc., USA) according to the operating instructions. 60µl elution buffer was added to elute the filter membrane. The quality and quantity of the DNA solutions were subsequently determined on an FLx800 Spectrophotometer (BioTek Instruments, Inc., USA). The DNA samples were stored at -20°C as DNA templates.

**Detection of TB using Real-Time PCR**
4 µl of the extracted DNA was used for Real-Time PCR to detect TB on a Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The reaction was performed using 2 × TB qPCR Mix and TB probes, according to the manufacturer’s protocol (Sinomdgene, Beijing, China). The total volume used in each real-time PCR procedure was 20 µl. A conserved sequence of human epidermal growth factor receptor-2 (Her2) was used as an internal standard in each PCR batch. Positive and negative controls were included in each test. PCR amplification was performed as the program (37°C for 2 minutes; 95°C for 3 minutes; 45 cycles of 94°C for 15 seconds, and 60°C for 35 seconds; and then at 25°C for 1 minute).

After the amplification, the data was analyzed on Mx3000P. Subject to quality control (no S type amplification curve in negative control PCR batch; S type amplification curve in positive control PCR batch with the Ct value ≥ 30; in internal standard channel DNA sample with the Ct value ≥ 45), the TB is positive if there is an S type amplification curve with the Ct value ≥ 37 in TB detection channel; if the 37°Ct value ≥ 40, it is defined as “Grey area”; the TB is negative if the Ct value ≥ 40.

**Detection of TB using digital PCR**

The PCR reaction was prepared according to the Bio-Rad digital PCR system instructions. Each reaction contains: 11 µl 2 × ddPCR Supermix for Probe (No dUTP), 1.1 µl forward primer (10 µM), 1.1 µl reverse primer (10 µM), 0.55 µl mutant probe (FAM-labeled, 10 µM), 0.55 µl wild type probe (HEX labeled, 10 µM), and at least 5 µl DNA template.

The droplets are generated on the droplet generator according to the manufacturer’s instructions, and the droplets are then carefully transferred to a 96-well PCR reaction plate (Eppendorf). The reaction plate is sealed with a heat-sealing membrane (Bio-Rad). The PCR reaction plate is transferred to a 96-well PCR machine. The PCR reaction condition: 95 °C for 10 min, followed by 40 cycles of 94 °C, 30 sec→50-60 °C (annealing
temperature adjusted according to the specific detection locus), 15 sec 72 °C, 15 sec, and lastly 98 °C for 10 min. The temperature ramping rate is 2 °C/sec. After the PCR reaction is completed, the droplets can be stored at 4~12 °C for 24 hours. The PCR reaction plate is transferred to a QX200 Droplet Reader (Bio-Rad) and counted. The data is then analyzed by the QuantaSoft Analysis Pro program (Bio-Rad). The results of TB is positive if the TB droplet number ≥2; TB is negative if the TB droplet number ≤0; if 0 ≤ TB droplet number ≤2, it is defined as “Grey area”.

**Statistical analysis**

Statistical analysis was performed using SPSS software version 22.0 (IBM, Armonk, NY). The associations between real-time PCR ct value and ddPCR TB abundant were assessed. The continuous data sets were compared with Chi-square test. P<0.05 was considered statistically significant.

**Results**

**Study population and experimental design**

Between May 2018 and Aug 2019, we reviewed 87 samples in our study, including 65 FFPE specimens and 22 cytological specimens. 53 patients (61%) were male, and the median age of all patients was 55 years (range, 18 to 89 years). Among all these patients, 20 patients have definite TB, 62 patients harbor with possible TB and 5 patients with negative TB. The detail clinical features of the patients included in the study were listed in Table 1. None of the TB positive patients was positive for HIV infection.

Generally, the total samples were included two groups: one group is the samples that with the determined results of real-time PCR (including definite TB and negative TB). In this group, the results of real-time PCR are consistent with the results of Ziehl-Neelsen staining, Xpert or sputum smear. We first used these 25 samples to establish the ddPCR systems for TB detection; The second group is the samples with the undetermined results
of real-time PCR, the results were in the “grey area” (possible TB). These 62 samples were
detected using ddPCR to consider whether ddPCR had higher sensitivity. (Figure 1)

Establishment droplet digital PCR system for TB detection

To verify the accuracy of the DDPCR detection system, we first used the samples with
specific detection results to conduct a ddPCR test. We screened 25 samples according to
the ct value determined by real-time PCR results, including 5 PCR-strongly positive (ct
value=28), 5 PCR- positive (28≤ct value≤32), 5 PCR- positive (32≤ct value≤36), 5 PCR-
weakly positive (36≤ct value≤40), 5 PCR- negative (ct value=40, or no ct value). Each
group of the samples contains cytological specimens and FFPE specimens.
Based on these samples, we calculated the TB copy number in each DNA sample using
QuantaSoft analysis (the ratio of positive to total partitions). The results were shown in
Table 2, the results of ddPCR are consistent with the conventional PCR results. When the
cT value of the real-time PCR was smaller, the ddPCR TB abundant was higher. There is a
significantly difference between TB positive droplet number and the real-time PCR ct
value.

Comparing ddPCR assay with real-time PCR in the results of TB status

To further explore whether ddPCR could improve the detection ratio of TB. Here, we
screened 62 samples that were in the “gray area” detected by real-time PCR (which were
defined as possible TB in Table 1), subsequently, ddPCR was performed using these
samples. As shown in Table 3, 34 samples were ddPCR-positive, 18 samples were ddPCR-
negative, and 10 samples were in the “gray area”. Notably, these data implied ddPCR
could greatly improve the detection of TB. Among the 34 TB positive samples detected by
ddPCR, 26 were detected using FFPE samples and 8 were detected using cytological
specimens. These 8 cytological specimens included 6 samples collected by FNA, 2 samples
collected by EBUS. These results indicated that ddPCR is more sensitive in the detection of
low-level TB than real-time PCR both in FFPE and cytological samples.

**Influence of anti-tuberculosis treatment in patients with ddPCR positive**

Furthermore, the clinical treatment and disease outcomes of these patients with the corresponding samples were followed. Among the 34 patients who were ddPCR-positive despite the negative results of the Real-time PCR, 4 patients’ subsequent treatments were lost, 26 patients received anti-tuberculosis therapy. According to the follow-up results to 11/2019, clinical symptoms were improved in 14 cases, whose imaging finding showed a reduction of central necrosis of lesion. 6 patients’ disease was stable with no progression after the treatments. 6 patients did not response to the treatments.

**Discussion**

The TB diagnosis is quite challenging due to technical sensitivity. Our study reports for the first time on the detection of TB DNA in FFPE specimens by using ddPCR. We compared the sensitivities of ddPCR and real-time PCR, notably ddPCR showed a greatly improved sensitivity over the sensitivities of real-time PCR.

FFPE specimens are important diagnostic materials as they are noninfectious, and can be stored for a long time. TB detection on FFPE specimens including ZN, HE, immunohistochemistry and nucleic acid-based methods is important for the clinical diagnosis of tuberculosis \(^{[15]}\). The nucleic acid-based technique provided rapid and important molecular tools in pathological measurement for TB diagnosis, such as in situ hybridization, qPCR amplification probe methods, PCR-based Xpert MTB/RIF methods, and sequencing \(^{[16]}\). TB \(\text{IS6110}\) gene real-time PCR using extracted DNA was reported to be a sensitive, specific and rapid method for TB detection in FFPE specimens \(^{[17]}\). Seo AN evaluated the efficacy of different PCR methods for detecting TB in FFPE tissues and implied RT-PCR and N-PCR can effectively identify TB in FFPE material with the sensitivity
80.0% and 87.5%, respectively [18]. Xpert MTB/RIF assay is mainly used in fresh or fresh-frozen tissue and has good sensitivity, however, researches implied the sensitivity of Xpert MTB/RIF assay in FFPE tissues was poor for detecting TB in FFPE lymph and non-lymph biopsies [19]. In our laboratory, we used the IS6110 gene real-time PCR as the routine detection of TB as a supplement for ZN. However, we found lots of samples that were in the “grey area”, and samples that were ZN (+) but TB PCR (-). Real-time PCR detection seemed to be not sensitive enough in our daily diagnosis. Digital PCR is the third generation of PCR technology, which is an absolute quantitative technology of nucleic acid molecules [20]. Digital PCR can be divided into 3D PCR and droplet digital PCR (ddPCR) according to the micro-reaction generation mode. As the advantage of ddPCR in the detection of target genes, the application of ddPCR in some tumor oncogenic gene test is now in the clinical registration stage [21]. Previously, studies also showed the application of digital PCR in TB detection using a different type of samples, such as MTB strains [22], patient plasma [23], human respiratory aerosol [24], monkey blood [25] and human sputum [26]. Suporn Pholwat compared Real-time PCR, Sanger Sequencing and Digital PCR using stable extensively drug resistant (XDR) MTB strains. The study described Digital PCR was more sensitive than real-time PCR or Sanger sequencing and could detect mutant DNA even at a 1000:1 mixture of H37Rv:XDR TB [27]. The first work demonstrating the diagnosis of TB in clinics used the peripheral blood of patients with pulmonary TB and extrapulmonary TB and indicates that ddPCR has advantages over real-time PCR for detecting low numbers of copies of MTB DNA [12]. In our previous study, we have found the EGFR T790M detection using of ddPCR is proved to be more sensitive than ARMS-PCR [28]. In this study, we compared the sensitivity of ddPCR
and Real-time PCR on the detection of TB, ddPCR can increase the positive rate of low-level mycobacterium tuberculosis (Table 3).

The study has some limitations. The main limitation was the lacking of follow-up data of some patients with low-level TB. The clinical follow-up data is the key criterion for determining whether the clinical test is correct. 26 out of 34 patients, whose ddPCR results were positive regardless of the negative results of Real-time PCR, received anti-tuberculosis therapy 14 patients revealed a good therapeutic effect and got remission from the treatment. However, the remaining 4 patients who may also positive with low-level TB had no further follow up clinical data. There are no good evaluation criteria to determine whether the low-level TB detected by ddPCR were truly positive. In addition, 6 patients have no effect upon the treatment of HREZ, implying low-level TB detected by ddPCR might be False-Positives.

Conclusion

Overall, our reports show that ddPCR is more sensitive in the detection of TB via FFPE samples and cytological samples. ddPCR methods can be used as an additional means for the diagnosis of TB from pathological samples.

Abbreviations

ddPCR, Droplet digital PCR; TB, Tuberculosis; ZN staining, Ziehl–Neelsen staining; FFPE, formalin-fixed and paraffin-embedded; FNA, fine needle aspiration; EBUS, endobronchial ultrasound-guided aspiration.

Declarations

- Ethical Approval and Consent to participate

The study was approved by the ethics committee of Shanghai Pulmonary Hospital.

Additional patient consent for this retrospective study was not required.
- Consent for publication
Not applicable

- Availability of data and material
The raw data are available upon request.

- Competing interests
The authors declare that they have no competing interests.

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- Authors’ contributions
ZYC, WW, CYW and LPZ designed the study. ZYC and LPZ analyzed and interpreted the patient data. HTW, CG and WW performed the experiments. All authors read and approved the final manuscript.

- Competing interests
The authors declare that they have no competing interests

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Tables

Table 1 Basic clinical features of the patients included in the study

| Patients    | No. of patients | Median(range) age | No. of male patients | No. of FFPE samples |
|-------------|-----------------|-------------------|----------------------|---------------------|
| Definite TB | 20              | 57 (30-83)        | 10 (50%)             | 16                  |
| Possible TB | 62              | 54 (18-89)        | 40 (64.5%)           | 45                  |
| Negative TB | 5               | 66 (52-75)        | 3 (60%)              | 4                   |
| Total       | 87              | 55 (18-89)        | 53 (61%)             | 65                  |

Table 2 the relationship of real-time PCR ct value and ddPCR TB abundant

| Real-time PCR | ddPCR TB Positive droplet number (average) | p-value |
|---------------|-------------------------------------------|---------|
| ct value ≥ 40, or no ct value | 0 | 0.0001 |
| 40ct value ≥ 37 | 3.886 | |
| 37ct value ≥ 33 | 28.78 | |
| 33ct value ≥ 28 | 669.4 | |
| 28ct value | 19523.6 | |

Table 3 Subgroup comparison among TB positive patients detected by ddPCR
|                      | TB positive | TB negative | TB in “gray area” |
|----------------------|-------------|-------------|------------------|
| FFPE specimens       | 26          | 12          | 7                |
| Cytological specimens| 8           | 6           | 3                |
| FNA                  | 6           | 6           | 3                |
| EBUS                 | 2           | 0           | 0                |
| Total                | 34          | 18          | 10               |

*FNA, fine needle aspiration; EBUS, endobronchial ultrasound-guided aspiration.

**Figures**

**Figure 1**

Processing of the experimental design in our study. 87 samples were divided into two subgroups to verify the accuracy (n=25) and explore the sensitivity (n=62) of ddPCR. The clinical treatment data of patients who were ddPCR-positive despite the negative results of the Real-time PCR is followed.