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Research Article

Dielectrophoresis Concentration Method for Increased Sensitivity of the Loop-Mediated Isothermal Amplification Test for the *Mycobacterium tuberculosis* Complex

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Abstract

Background: An effective concentration method is required to detect *Mycobacterium tuberculosis* (MTB) in paucibacillary specimens. Dielectrophoresis (DEP), a phenomenon in which a force is exerted on a dielectric particle subjected to a non-uniform electric field, is useful for concentrating bacterial specimens.

Objectives: To investigate whether the procedure using the DEP method increases nucleic acid amplification test (NAAT) sensitivity.

Methods: First, the capture rates were examined for multiple electrode settings by calculating the bacterial load before and after DEP using quantitative real-time PCR. These results were used to determine the optimal electrode setting. Second, conventional loop-mediated isothermal amplification (LAMP) was performed using 30 µL DNA purified using a PURE DNA extraction kit from 60 µL Mycobacterium bovis bacille de Calmette et Guérin culture fluid. The minimally-diluted specimen (theoretical concentration, 10^3 cfu/mL), which showed 10 consecutive negative results by LAMP, was subjected to DEP. One milliliter of the specimen was concentrated to 50 µL in the DEP chip, and LAMP was performed in 30 µL of the specimens without DNA purification.

Results: At 73.2-84.9%, the capture rate was the highest in the setting with 100 kHz frequency. LAMP with DEP was performed using conventional LAMP-negative specimens, and eight of ten tests (80%) were positive. The sensitivity was higher than that of the conventional LAMP method ($p = 0.0007$).

Conclusion: The DEP method has the potential to increase NAAT sensitivity for the MTB complex in paucibacillary specimens.

Keywords: *Mycobacterium Tuberculosis* Complex; Dielectrophoresis; Concentration; Nucleic Acid Amplification Test

Abbreviations

TB: Tuberculosis; MTB: *Mycobacterium Tuberculosis*; DEP: Dielectrophoresis; NAAT: Nucleic Acid Amplification Test; BCG: Bacille de Calmette et Guérin; LAMP: Loop-Mediated Isothermal Amplification

Introduction

Tuberculosis (TB) remains a serious global health concern, with 1.5 million reported deaths in 2019 [1]. Accurate and rapid diagnosis is important to control TB. Bacterial culture and Nucleic Acid Amplification Tests (NAAT) are the definitive diagnostic tools for active Mycobacterium Tuberculosis (MTB) [2]. Liquid culture shows the highest sensitivity among MTB laboratory examinations; however, it takes several weeks to detect MTB. Thus, NAAT is essential for rapid diagnosis, but unfortunately, it has a lower sensitivity than that of bacterial liquid culture. In reality, approximately 20-50% of pulmonary tuberculosis cases globally are diagnosed clinically without bacteriological confirmation [1]. Low bacillary burden and insufficient MTB recovery from specimens are among the reasons why NAAT is not as sensitive as liquid culture [3,4].

To increase the diagnostic sensitivity of active MTB, many new molecular methods have been developed. For example, Xpert MTB/RIF Ultra (Cepheid, Sunnyvale, CA, USA), which amplifies multiple target genes, has a higher sensitivity than that of Xpert MTB/RIF [5]. Other molecular methods have also been developed [6]. However, to date, none of the molecular methods has a higher sensitivity than that of liquid culture [5,7].

Another strategy for improving the sensitivity of bacterial confirmation tests is to modify specimen processing. Centrifugation is generally performed to concentrate and recover MTB. However, centrifugation is not an efficient technique to collect bacteria, particularly in low bacillary specimens [4,8], necessitating the development of other concentration methods.

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The Dielectrophoresis (DEP) method is a good strategy for concentrating target cells [9,10], thereby increasing the sensitivity of the bacterial confirmation test. DEP is a phenomenon in which a force is exerted on a dielectric particle subjected to a non-uniform electric field. The DEP method can be efficacious for target cell discrimination and isolation [9-13]. For mycobacteria, *M. bovis* bacille de Calmette et Guérin (BCG) and *M. smegmatis* have been captured using DEP methods [14,15].

In this study, the optimal DEP electrode setting for *M. bovis* BCG was examined by calculating the capture rate using quantitative real-time PCR. It was also verified whether specimen processing using the DEP method increases the sensitivity of NAAT using Loop-Mediated Isothermal Amplification (LAMP) in a paucibacillary *M. bovis* BCG specimen that showed negative results using the conventional LAMP method.

Materials and Methods

Specimen Preparation

M. bovis BCG was cultured at 37°C in MycoBroth (Kyokuto Pharmaceuticals, Tokyo, Japan) until the optical density at 530 nm (OD_{530}) of the culture medium reached 0.10. The MycoBroth culture fluid was centrifuged, and the cell pellets were resuspended twice in DEP buffer. The DEP buffer consisted of 8.5% (w/v) sucrose, 0.3% (w/v) dextrose [11], and 1.0% Tween 20, and the buffer was deionized using SMNUPB ion-exchange resin (Mitsubishi Chemical, Tokyo, Japan). The final conductivity of the DEP buffer was 10 μS/cm.

Dielectrophoresis Device Fabrication

The function generator was the prototype of the ConseeMo α instrument (AFI, Kyoto, Japan). The ConseeMo α is a compact device that can concentrate bacteria in a specimen using a microfluidic chip. The dedicated X-23 chip (AFI, Kyoto, Japan; Figure 1) has a 50 μL capacity, and was used after loading with bovine serum albumin (BSA; 2 mg/mL; Thermo Fisher Scientific, Basingstoke, UK) for 1 h to minimize cell adhesion to the DEP chip.

Evaluation of Optimal Dielectrophoresis Electrode Settings for *M. Bovis* BCG by Quantitative Real-Time PCR

To identify the optimal DEP electrode setting for *M. bovis* BCG, the capture rates in different electrode settings were calculated using quantitative real-time PCR (three replicates per electrode setting). One milliliter of the prepared specimen was introduced in a continuous 0.5 mL/h flow into the DEP chip with multiple electrode conditions; the frequencies were 1, 50, 100, 150, and 200 kHz, with a fixed voltage of 20 V_{pp} (peak to peak). Capture rates were calculated from the bacterial loads before and after DEP using real-time PCR. Bacterial DNA from 50 μL of the initial prepared specimen without DEP and the concentrated specimen were heated in boiling water for 10 min, followed by sonication for 10 min. Next, real-time quantitative PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and MTB-F and MTB-R primers [16] on a QuantStudio 3 system (Thermo Fisher Scientific). Briefly, the PCR reaction mixture (25 μL final volume) was comprised of 12.5 μL 2x Master Mix, 0.5 μL 10 μM forward and reverse primer, and 2 μL extracted DNA sample. Then, PCR was performed under the following conditions: initial denaturation and activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

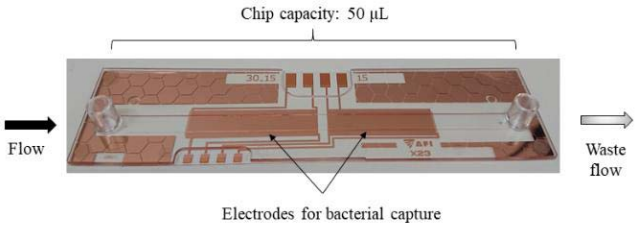

Figure 1: Image of the dedicated X-23 chip for DEP (AFI, Kyoto, Japan). A specimen was introduced *via* continuous flow into the DEP chip, and target bacterial cells were captured in the chip by the DEP phenomenon.

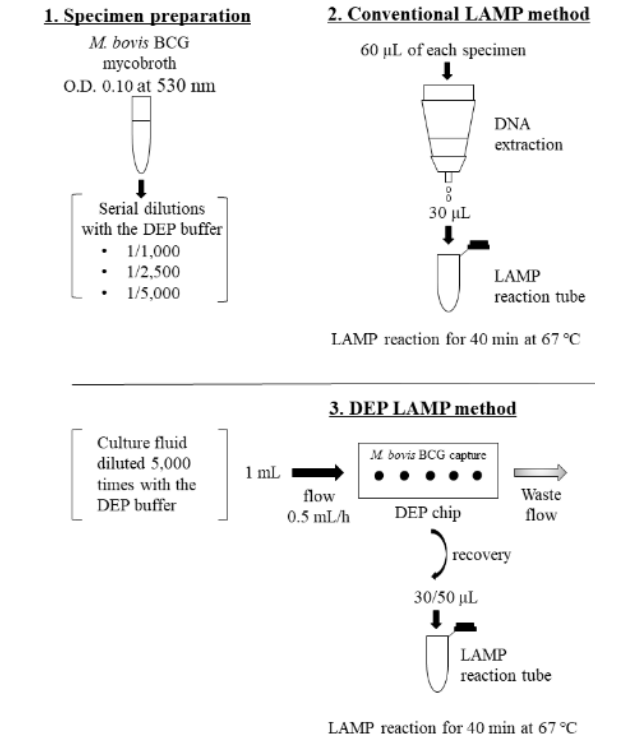

Figure 2: Overview of the conventional and DEP/LAMP methods. Conventional LAMP was performed using 30 μL purified DNA, prepared by subjecting 60 μL *M. bovis* BCG fluid to DNA extraction using a PURE DNA extraction kit. The culture fluid diluted 5,000 times with DEP buffer showed 10 consecutive negative results using the conventional LAMP method. This sample was subjected to the DEP method. One milliliter of the specimen was concentrated to 50 μL in the DEP chip and LAMP was performed in 30 specimens without DNA purification.

Paucibacillary Specimen Preparation

M. bovis BCG culture fluid prepared as described above was serially diluted in DEP buffer (1/1,000, 1/2,500, and 1/5,000). The single minimally-diluted specimen that showed 10 consequtive negative results by conventional LAMP was subjected to the DEP method (Figure 2). The theoretical concentration of BCG in the 1/5,000 diluted specimen was 10^3 cfu/mL.

Conventional Loop-Mediated Isothermal Amplification Method

Conventional LAMP was performed using a commercial LAMP kit for MTB detection (Eiken Chemical, Tokyo, Japan) as described previously (Figure 2) [17]. Briefly, the DNA from 60 μL specimen was

purified using a PURE DNA extraction kit (Eiken Chemical). Then, 30 μL extracted DNA was added to the LAMP reaction tube (Eiken Chemical). The LAMP reaction for each specimen was performed with incubation at 67°C for 40 min, and the result was determined by real-time turbidity measurements and visual fluorescence observation under ultraviolet light using Loopamp EXIA (Eiken Chemical). Positive results were considered as those with differential calculation values, and the maximum turbidity value calculated by the moving average on differential values was ≥0.10.

Loop-Mediated Isothermal Amplification Using the Dielectrophoresis Method

The electrode condition providing the highest capture capacity was determined from the quantitative real-time PCR results.

One milliliter of the prepared specimen was introduced in a continuous 0.5 mL/h flow into the DEP chip with the above electrode condition. After the DEP procedure, 30 μL of the concentrated specimen was inserted into the LAMP tube directly from the DEP chip to avoid the loss of *M. bovis* BCG in procedures such as pipetting or DNA purification. Then, the LAMP reactions were performed as described above (Figure 2).

Statistical analysis

The sensitivity of the LAMP test with the DEP method was compared to the sensitivity of the conventional method using Fisher's exact test. A *p*-value of <0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA).

Ethical considerations

Ethical approval was not required for this laboratory-based in vitro study.

Results

Capturing Capacity in Each Electrode Condition

The capture capacity for each electrode condition is shown in Figure 3. The highest capture capacity (73.2-84.9%) was achieved using the setting with 100 kHz frequency and 20 V_{pp} voltage.

Minimally-Diluted Specimen Preparation

The culture fluid was diluted 5,000 times with the DEP buffer before the specimen showed 10 consecutive negative results using the conventional LAMP method (Table 1). This single specimen was subjected to the DEP method.

LAMP Test by The DEP Method

Eight of ten (80%) tests yielded positive LAMP results in the specimens prepared using the DEP method **(Table 1)**. The sensitivity was significantly higher than that of the conventional method (p = 0.0007).

Discussion

The LAMP sensitivity was 80% after bacterial concentration using the DEP method and the optimal electrode setting in the minimally-diluted specimen. This sample showed negative results by conventional processing. Our result revealed that modifying the specimen concentration can improve the NAAT sensitivity in a paucibacillary specimen. During specimen processing, it was important that the concentration efficacy was increased and minimal target cells were lost.

The DEP method was effective for the capture of *M. bovis* BCG, as described previously [14], leading to specimen concentration. The efficacy of target cell capture in DEP depends on cell polarization, the conductivity medium, and the electrode condition [12,14,15]. Cell polarization is associated with parameters such as size, geometric shapes, and electrical properties of the cell wall and cytoplasm [14,18]. Cell capacitance might be influenced by factors such as drug exposure or live/dead cell status [14,18]. In this study, the condition of *M. bovis* BCG was post-liquid culture without anti-tuberculosis drug exposure.

Table 1: LAMP test results: Conventional *versus* DEP methods.

Method	Test	Df	Tt	Result
Conventional	1	0.003	-	-
	2	0.003	-	-
	3	0.047	-	-
	4	0.023	-	-
	5	0.088	-	-
	6	0.089	-	-
	7	0.003	-	-
	8	0.003	-	-
	9	0.095	-	-
	10	0.003	-	-
DEP	1	0.002	-	-
	2	0.209	22:30	+
	3	0.179	34:06	+
	4	0.166	34:06	+
	5	0.182	25:12	+
	6	0.192	28:00	+
	7	0.098	-	-
	8	0.127	33:48	+
	9	0.203	26:54	+
	10	0.131	31:42	+

LAMP: Loop-Mediated Isothermal Amplification; DEP: Dielectrophoresis; Df: Differential Calculation Value; Tt: Threshold Times (min); -: negative, +: positive

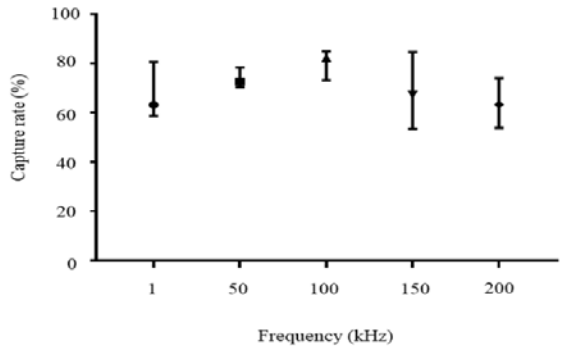

Figure 3: The capture rate for *M. bovis* BCG at multiple DEP electrode settings. The median capture rate with the range is shown. The highest capture rate (73.2-84.9%) was obtained using 100 kHz frequency and 20 V_{pp} voltage.

In terms of medium conductivity, a lower-conductivity medium is required to efficiently capture the target cells [12,15]. The DEP buffer was deionized by ion exchange resin to reach a conductivity of 10 μS/cm (as low as possible). As for electrode conditions, the optimal electrode conditions for capturing the MTB complex were determined based on the results of the capture capacity of multiple electrode settings using quantitative real-time PCR.

It is also important to maximally recover target cells during processing. In the DEP method, the chip was loaded with BSA and 1% Tween 20 was added to the buffer to minimize tight cell adhesion to the DEP chip itself. In addition, the specimens were transferred to the LAMP reaction tube directly from the DEP chip without DNA purification. Although we were concerned that these procedures might interfere with the LAMP reaction, it worked normally.

In the conventional method, 30 copies of *M. bovis* BCG are inserted to the LAMP tube from the specimen with a theoretical concentration of 10^3 cfu/mL. This copy number is higher than that of the theoretical limit of detection, i.e., 0.38 genomes/tube [19]. The reasons might be as follows: 1) the actual limit of detection with the LAMP test has been reported to be higher (10^2-10^3 cfu/mL) because the concentration is not increased through the PURE DNA extraction method [20,21] or 2) in this study, a certain number of bacteria might have been lost during procedures such as resuspension or dilution.

Although the concentration and recovery procedures mentioned above were performed in the minimally-diluted specimen, which showed negative LAMP results by the conventional method, the DEP method yielded negative results two out of ten times (Table 1). This result was obtained because not all *M. bovis* BCG cells were captured in the DEP chip, and/or the *M. bovis* BCG recovery from the chip was insufficient, as shown in the results of capture capacity by quantitative real-time PCR.

This experiment was conducted using *in vitro* specimens containing only *M. bovis* BCG cells and DEP buffer. When the DEP efficacy is verified using clinical specimens, particularly sputum, the efficacy might decrease because of the influence of mucus and different cell types and substances. Appropriate processing of clinical specimens for the DEP method will also be needed.

Conclusion

The DEP concentration method was effective for increasing the NAAT sensitivity in paucibacillary MTB complex specimens. The utilization of the technology will be expected to further increase the sensitivity of NAAT.

Acknowledgment

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Conflicts of Interest

None to declare.

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