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A rapid method for determining *Mycobacterium tuberculosis* based on a bulk acoustic wave impedance biosensor

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Abstract

The bulk acoustic wave impedance biosensor was set up and used to monitor the growth of *Mycobacterium tuberculosis* (M.TB). This sensor is rapid, simple, sensitive (lower limit is 2×10^3 cells ml^{-1}) and cheap (easy to generalize). The typical response curve was different from other bacteria's, such as *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis*. The frequency detection time was used to quantitatively determine M.TB. It was proportional to logarithm of the initial concentration of M.TB in the range of 2×10^3–3×10^7 cells ml^{-1}. The set up sensor was applied to the direct diagnosis of M.TB samples. The interference of other bacteria was eliminated by pretreatment. Our results confirmed that the use of the set up biosensor was reliable, sensitive. It gives out the potential use for determining M.TB.

Keywords: *Mycobacterium tuberculosis*; Bulk acoustic wave impedance biosensor; Quantitative determination

1. Introduction

It is well known that tuberculosis (TB) is a "dead" disease and becomes one of the biggest global health emergencies [1–3]. So determination of TB becomes quite important. Methods for diagnosing *Mycobacterium. tuberculosis* (M.TB) include latex agglutination [4] and ELISA [5,6], the radiometric detection [7], the bacteriological examination, DNA [8–10] and RNA [11] probes, polymerase chain reaction (PCR) [12–14], TB Rapid Cultivation Detection Technique [15,16], such as Bactec MGIT960 system, MB/Bact system, MB RedoxT produce color system, however, these methods exist their drawbacks. Precipitation in gels, and ELISA, do not always indicate TB in high-incidence developing countries; The radiometric detection has the radioactive damage; The bacteriological examination is time-consuming; DNA and RNA probes and especially PCR have a large amount of false positive results, and also these methods are too expensive to generalize in developing countries; Although TB Rapid Cultivation Detection Techniques is in clinical use, the fluorescent reagent which was used in this method is expensive and easily decomposed. In our re-

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search group, we proposed a piezoimmunological (PI) method which responses the PQC mass changes [17,18]. It is simple, but it is not sensitive (limit 10^5 cells ml^{-1}) and the antibody of M.TB is expensive and difficult to acquire.

In this paper, we proposed a new, rapid and sensitive method for quantitative determination of M.TB. The mechanism of the response was based on a bulk acoustic wave impedance sensor to conductivity and permittivity. The conductivity of culture medium (CM) can be changed by the growth of M.TB, which can be monitored by the bulk acoustic wave impedance biosensor through the frequency response curve. In the frequency response curve, the time at which the frequency begins to change is defined as the frequency detection time (FDT) [19]. FDT has the linear relationship with logarithm of the initial concentration of M.TB in the range of 2×10^3–3×10^7 cells ml^{-1}. So, it can be used for quantitative determination of TB.

2. Experimental

2.1. Materials

M.TB ($H_{37}R_a$, dry power,) was purchased from National Institute for the control of Pharmaceutical and Products. *Escherichia coli* (*E coli*) and *Staphylococcus aureus* (*S. aureus*) were acquired from Department of Life Science of Hunan Normal University. Chemicals were all of analytical grade.

2.1.1. Stock culture

M.TB power was dissolved in 0.6-ml physiological salt and inoculated on the decline of Löwenstein-Jensen (L-J) solid culture. After being incubated for 3 weeks, three loops of M.TB were transferred to a 40 ml sterilized conical vial that contained 20 ml sterilized CM. Incubated for 20 days at 37 °C and then stored in a refrigerator as a stock solution. The concentration of stock solution was about 2×10^9 cells ml^{-1} by compared turbidity method and 3.8×10^9 cells ml^{-1} by pour plate counts method (PPC).

2.1.2. Culture medium 1

The CM for M.TB was Sautions liquid CM. The composition of CM was: 99.99% sodium glutamate, 8.0 g; citric acid, 2.0 g; glycerol, 80 ml; dipotassium hydrogen phosphate, 0.5 g; magnesium sulfate, 0.5 g; ammonium ferric citrate, 0.05 g; distilled water, 1000 ml; two drops of Tween-80; pH 7.2 (using ammonia liquor). After being sterilized in autoclaving at 121 °C for 20 min, the CM 1 was ready.

2.1.3. Culture medium 2: Löwenstein-Jensen (L-J)

The composition of CM 2 was: monosodium glutamate, 7.2 g; potassium dihydrogen phosphate, 2.4 g; magnesium sulfate, 0.24 g; magnesium citrate, 0.6 g; glycerol, 12 ml; distilled water, 600 ml; potato starch, 30 g; egg liquid (filtered by sterilized gauze), 1000 ml; 2% malachite green, 20 ml. Being sterilized at 90 °C for 1.5 h, the CM 2 was ready for use.

2.2. Apparatus

A schematic diagram of the apparatus was shown in Fig. 1. The bulk acoustic wave impedance sensor was constructed by connecting an AT-cut coated silver 9 MHz quartz crystal to a pair of Pt net electrode. The sensor was connected to the self-made TTL crystal oscillator. The oscillator frequency of crystal was monitored by a frequency counter (Model SS7201, Shijiazhuang

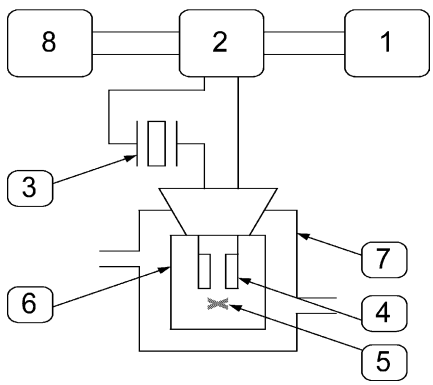

Fig. 1. Schematic diagram of the conductivity sensor: (1) frequency counter; (2) IC-TTL oscillator; (3) AT-cut quartz crystal; (4) Pt net; (5) stir bar; (6) The detection cell; (7) thermostatic water-jacket; (8) d.c. power supply.

fourth wireless factory). A thermostatic water jacket was used to control the temperature at 37±0.2 °C. A 6.2 V direct current was applied to the oscillator to drive the crystal. A LGC number resistance bridge (Th2816, Tonghui Electronics) was employed to measure the resistance and capacitance.

2.3. Procedure

Inoculated different amounts of TB in the sterilized detection cell that contained 3 ml diluting 10 times CM 1. Incubated at 37 °C and recorded oscillating frequency by the set up sensor every 10 min. At the same time connected the sensor to a LGC number resistance bridge, and measured resistance (R) and capacitance (C) in the medium.

3. Results and discussion

3.1. Theory

The proposed method is based on the monitoring change of the CM properties that are caused by the growth of M.TB using the bulk acoustic wave impedance sensor. The theory basis for the bulk acoustic wave impedance sensor responds to the medium properties change was described as follows:

The expression of the oscillating frequency can be represented by Eq. (1)[20].

$$F = F_0\left[1 + \frac{\pi F_0 C_s(2\pi F_0 C_s - YG)}{G^2 + 4\pi F_0^2 C_s(C_0 + C_s) - 2\pi F_0 C_0 YG} - \pi F_0 C_q R_q Y\right] \quad (1)$$

where, F is the oscillating frequency, $F_0 = 1/(2\pi\sqrt{L_q C_q})$ is the resonant frequency of the crystal, L_q, C_q, R_q and C_0 are the dynamic inductance, dynamic capacitance, dynamic resistance, and static capacitance of the crystal respectively, and Y a parameter related to phase shift of the oscillator, $G = k\chi$, where k is the cell constant of the conductivity sensor and χ the specific conductivity, $C_s = k\varepsilon + C_p$, where ε is the solution permittivity and C_p is the parasitic capacitance between the leading wires of the electrode. From Eq. (1) the change of F is mainly dependent on solution conductivity and permittivity if other above-mentioned parameters were kept constant. So the CM causing the conductivity and permittivity change of the detected M.TB can be represented by the sensor frequency change.

3.2. The effect of cell constant on the response

The effect of cell constant on the sensor response was investigated. The experimental results were shown in Table 1. The cell constant caused remarkably effect on sensitivity of sensor response. The sensitivity of the sensor increases consistently with decreasing cell constant from 0.1465 to 1.6749 cm. The cell constant of 0.1465, 0.3183 cm are sensitizer than others, but they are not stable at 37 °C (the noises are about 300, 150 Hz, respectively). When the cell constant is 0.5073 cm, the sensor is sensitive and stable, so the detection cell with cell constant of 0.5073 cm was selected in all experiments.

3.3. The effect of the conductivity of culture medium

The conductivity of CM affected the response sensitivity of the sensor. In order to find the suitable conductivity, CM 1 was diluted 5, 10, 20

Table 1
The effect of the cell constant

Trials	Cell constant (cm)	Frequency shift, mean[a] (Hz)	
		ΔF	noise
1	0.1465	2232	323
2	0.3183	1654	151
3	0.5073	1353	3
4	0.6231	854	3
5	0.8324	622	2
6	1.0566	318	2
7	1.6748	217	2

$K = S/L$. K, S, and L represent the cell constant, the area of the electrode and the distance of two electrodes, respectively. The concentration of M.TB is 3×10^5 cells ml^{-1}.

[a] The mean of three determinations.

times using distilled water, respectively; adjusted pH to 7.2 using ammonia liquor, sterilized in autoclaving at 121 °C for 20 min, cooled at room temperature, then inoculated using the same amount of M.TB ($\sim 3 \times 10^5$ cells ml^{-1}) and the frequency shift curve were determined by the biosensor. The results were shown in Fig. 2. It can be seen that diluting 10 times is better than the two others. If diluted 5 times, maybe the ground conductivity is higher, the frequency shift is not very obvious. If diluted 20 times, perhaps the nutrition is too scanty to supply M.TB normal need, so FDT becomes long and the frequency shift decreases. So CM 1 diluted 10 times was selected for use in our experiment.

3.4. Typical frequency curve for M.TB

Typical frequency curves detected by the bulk acoustic wave impedance sensor were shown in Fig. 2. At first, although the frequency caused a little drift, after several hours, the frequency began to increase obviously. The time that the frequency started to change was defined as the FDT. The frequency continuously increased about 5–6 h, and reached a flat roof. Corresponding to AB stage, the conductivity of CM had little change and M.TB was recovering the physiological activity.

At BC stage, the frequency changed rapidly and named as the active stage, this may be caused by the growth of M.TB. At this stage, M.TB assimilated glutamate, hydrogen phosphate, magnesium ion and other strong electrolyte and synthesized essential macromolecular such as protein enzyme, fatty acid enzyme, etc. This caused the decrease of the conductivity and the frequency rose quickly.

CD is the relative static stage, owing to discharge some poisonous products (such as dissociative fatty acids, peroxides, etc.) and led to the solution properties change (for example, pH of solution varied from 7.2 to 4.8). Also M.TB's physiological activity was restrained and could not absorb nutrition and discharge products. So the frequency reached flat roof.

The frequency curve caused by M.TB was different from those caused by other microorganism, such as *E. coli*'s [19], *P. rettgeri*'s [21], *Proteus mirabilis* (*P. mirabilis*'s) [22], *S. aureus*'s [23], *P. Vulgaris*'s [24], etc. (shown in Fig. 3). For M.TB, it was positive frequency shift and the changed speed was slow. For others', they were quickly negative frequency shift, due to decomposing poor conductive macromolecular into conductive small molecules, such as ammonia, carbon dioxide, organic acid, etc. Furthermore, M.TB has a long generation time (~ 16 h), so the detection time is

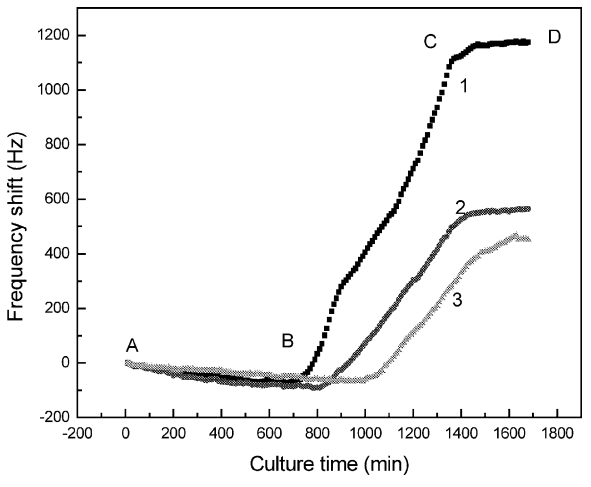

Fig. 2. The effect of the concentration of CM: (1) CM 1 diluting 10 times; (2) CM 1 diluting 20 times; (3) CM 1 diluting 5 times. (The concentration of M.TB is 3×10^5 cells ml^{-1}.)

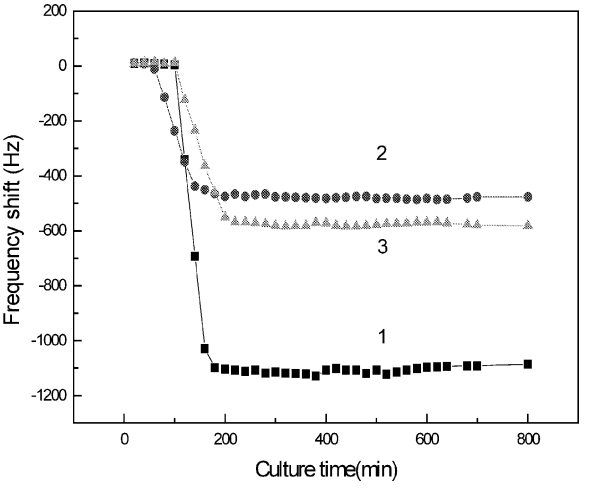

Fig. 3. Typical frequency shift curves for other microbes: (1) *E. coli* (3×10^5 cells ml^{-1}); (2) *P. rettgeri* (1.16×10^5 cells ml^{-1}); (3) *P. mirabilis* (9.53×10^5 cells ml^{-1}).

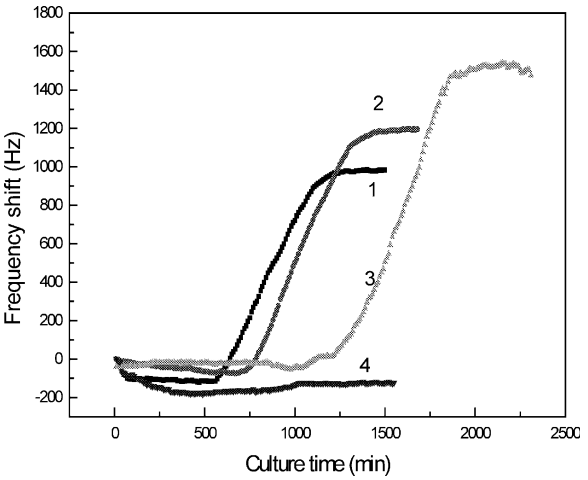

Fig. 4. The response curve for different concentration of M.TB (cells ml^{-1}): (1) 3×10^7; (2) 3×10^5; (3) 2×10^3; (4) blank.

much longer than others'. As shown in Fig. 4, the FDT of high concentration of M.TB was shorter than that of low concentration of M.TB. The concentration of M.TB was determined based on the FDT.

3.5. Determination of M.TB in simulation samples

The linear relationship between the logarithm of initial concentration of M.TB and FDT in the range of 2×10^3–3×10^7 cells ml^{-1} was given by Eq. (2):

$$\log C = 11.23457 - 0.00763\mathrm{FDT}, \quad r = 0.97253 \ (n = 5) \tag{2}$$

where C is the initial concentration of M.TB in the range of 2×10^3–3×10^7 cells ml^{-1}, FDT is the time that the frequency begins to rise rapidly. If the FDT is obtained, the number of the M.TB can be accurately calculated according to Eq. (2). This was the basis for quantitative determination of M.TB.

Table 2 showed the detecting results of M.TB by the FDT, PPC, PI and microscope detection (MD) method. By comparing the results, it is obvious that the FDT method is sensitizer than PI, PPC and MD method.

Table 2
The determination of M.TB concentrations in simulation samples

Simulation samples	M.TB concentration (cells ml^{-1})			
	MD	PI	PPC	FDT
1	−	−	3.2×10^3	3.6×10^3
2	±	+	5.8×10^4	6.3×10^4
3	+	+	4.1×10^5	4.6×10^5
4	+	+	4.7×10^5	5.1×10^5
5	+	+	8.2×10^5	7.4×10^5
6	+	+	3.6×10^6	2.9×10^6

'+' represent positive results and '−' represent negative results; '±' means both the positive and negative results are appeared. The table results are three times mean value.

3.6. R and C curves during inoculation

Resistance and capacitance curve were also recorded during inoculation and were shown in Fig. 5. It can be seen that R and C kept invariable in preparation stage and then increased rapidly in the active stage. M.TB absorbed some strong electrolyte and synthesized some important enzymes and led to the solution's resistance increase and capacitance decrease sharply. Compare to Fig. 2, the oscillating frequency change direction is in the same way of R and the opposite way of C. It coincided with Shen's theory [19]. It can also be seen that C_s changed a little (only 30 nF) and the R

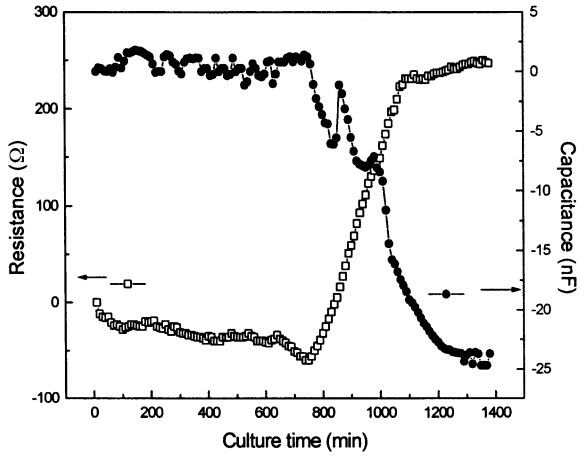

Fig. 5. The change of R and C during the course of M.TB' metabolism: the concentration of M.TB is 3×10^5 cells ml^{-1}. (The cell constant is 0.5073 cm.)

change was about 260 Ω. But frequency shift was about 1400 Hz, so using the bulk acoustic wave impedance biosensor is more sensitive than using impedance sensor.

3.7. Effects of other microbes

The effects of other microbes on determination of M.TB were shown in Fig. 6. Curve 1 and 2 were obtained through following procedure: Put *E. coli*. (3×10^5 cells ml^{-1}), *S. aureus* (3×10^5 cells ml^{-1}), and M.TB (3×10^5 cells ml^{-1}) together and then divided it into two parts. One part was put directly into the sterilized detection cell, connected to oscillator and recorded the frequency curve 1 (Fig. 6). Another part was transferred to the vial, then treated with 2% (w/w) NaOH, stirred homogeneously, waited for 20 min, added 10 ml phosphate buffer solution (pH 6.8), centrifuged, discarded the upper solution. Washed the sediment with sterilized double distilled water 2 times, centrifuged and discarded the upper liquid. Dissolved the sediment with diluting CM in the sterilized detection cell and recorded the frequency curve 2. In curve 1, at first ΔF decreased because of *E. coli* and *S. aureus* and then increased because of M.TB. In curve 2, *E. coli* and *S. aureus* were killed by NaOH, so there was no obvious frequency decrease at first. Curve 3 and 4 were obtained by running the same experiment for 3-ml sputum of normal people and the mixture of 3-ml sputum of normal people with *E. coli* (3×10^5 cells ml^{-1}) and *S. aureus* (3×10^5 cells ml^{-1}). In curve 3, no M.TB, so it was treated as base line; in curve 4, as *E. coli* and *S. aureus* were killed by NaOH, so it was similar to curve 3. Curve 5 was the typical frequency curve of the mixture of *E. coli* (3×10^5 cells ml^{-1}) and *S. aureus* (3×10^5 cells ml^{-1}) and curve 6 was the typical frequency curve of M.TB. From the Fig. 6, it can be seen that bacteria contained in sputum can be killed by pretreatment with NaOH (2%, w/w). Also FDT obtained from the sample processed with NaOH was longer than that of no processed sample. This was because when NaOH killed other bacteria, it affected M.TB physiological activity. FDT was much longer, but it did not influence the detection result. So the interference of other bacteria can be eliminated by pretreatment. We expect to apply this method in the clinic diagnose.

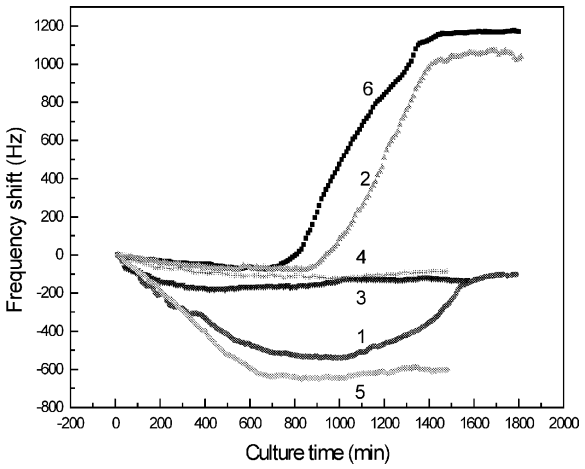

Fig. 6. The response curve for: (1) *E. coli* (3×10^5 cells ml^{-1})+*S. aureus* (3×10^5 cells ml^{-1})+M.TB (3×10^5 cells ml^{-1}); (2) treated with 2% NaOH; (3) sputum of the normal people treated with 2% NaOH; (4) *E. coli*+*S. aureus* (3×10^5 cells ml^{-1}) treated with 2% NaOH; (5) *E. coli*+*S. aureus* (3×10^5 cells ml^{-1}); (6) M.TB (3×10^5 cells ml^{-1}) in diluting 10 times CM.

4. Conclusion

As described above, we proposed a new way to determine M.TB, in comparison with other methods; the bulk acoustic wave impedance biosensor is simple, sensitive, rapid, cheap, and accurate. We expect to apply this method to the clinic diagnose.

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