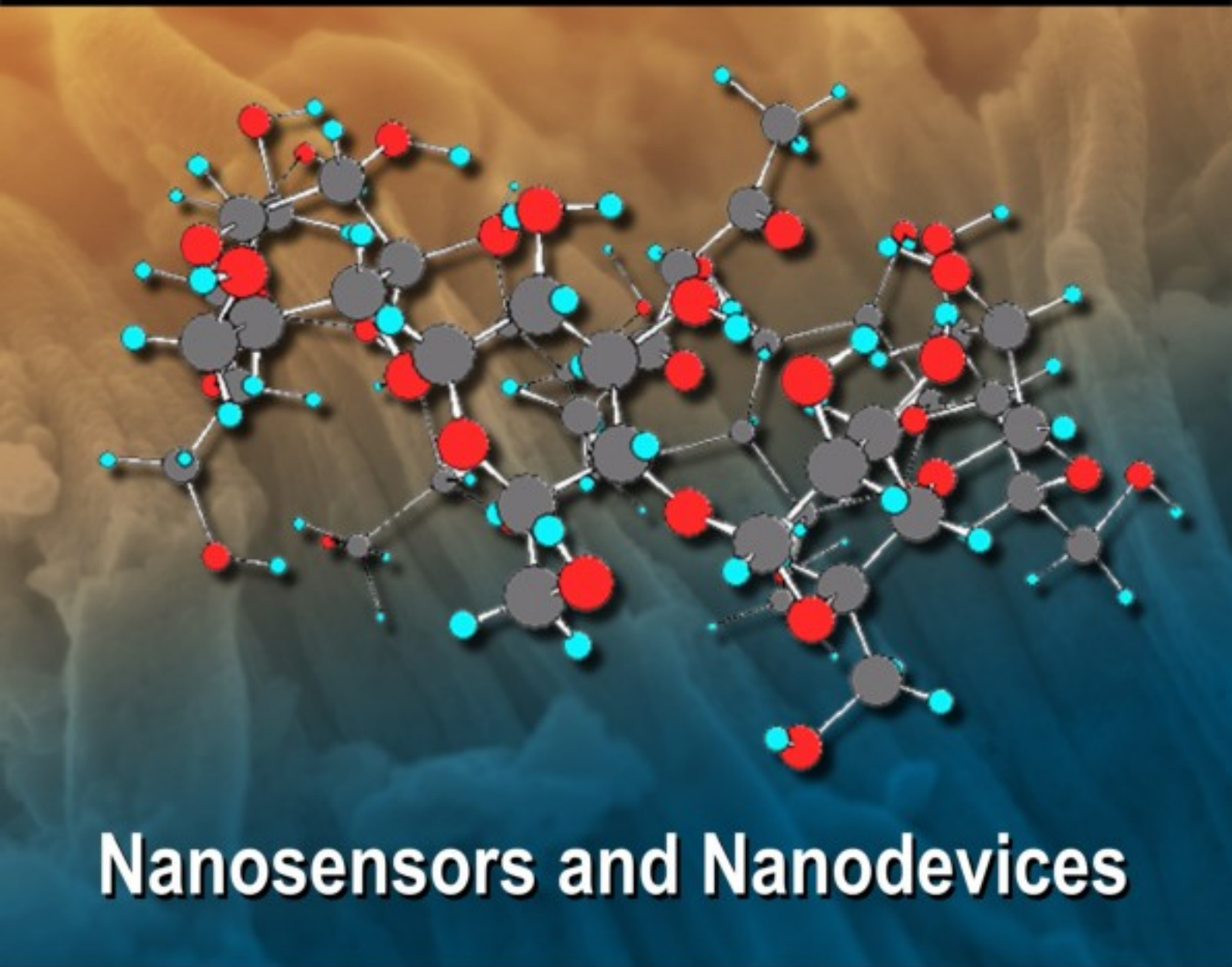
ISSN 1726-5479

SENSORS & TRANSDUCERS

vol. 146
11/12



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Sensors & Transducers

Volume 146, Issue 11
November 2012

www.sensorsportal.com

ISSN 1726-5479

Sensors & Transducers Journal (ISSN 1726-5479) is a peer review international journal published monthly online by International Frequency Sensor Association (IFSA).
Available in electronic and on CD.

Sensors & Transducers Journal

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Volume 146
Issue 11
November 2012

www.sensorsportal.com

ISSN 1726-5479

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Sensors & Transducers Journal, Vol. 146, Issue 11, November 2012, pp. 156-163

www.sensorsportal.com

Sensors & Transducers

ISSN 1726-5479

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Gold Nanoparticle Amplification Combined with Quartz Crystal Microbalance DNA Based Biosensor for Detection of *Mycobacterium Tuberculosis*

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Received: 17 October 2012 /Accepted: 23 November 2012 /Published: 30 November 2012

Abstract: Tuberculosis is among the top ten causes of global mortality and morbidity, constitutes an important public health problem in Thailand. This study demonstrates a rapid and sensitive quartz crystal microbalance (QCM) biosensor combined with gold nanoparticle amplification for diagnosis *Mycobacterium tuberculosis*. The functionalized gold electrode of the quartz crystal was immobilized using the specific thiol modified oligonucleotide probe. The sensitivity was also improved by using gold nanoparticle as mass enhancement at 3' end of DNA target sequence. The target IS*6110* amplified DNA was hybridized to a probe for 5 min after application. DNA hybridization was indicated by resulted in frequency changes of the QCM. The QCM could clearly identify the data indicated that QCM could detect serial dilution of *M. tuberculosis* DNA limited as 5 pg of genomic DNA. The technique showed a significant specificity since no cross-hybridization with other mycobacteria was found. In testing in direct clinical sputum samples, the sensitivity of QCM was 100 %, and the specificity was 100% by comparison with culture. The label free QCM was successfully developed with high sensitivity, specificity, low cost and convenience, this technique may prove to be a powerful tool for an early diagnosis of *M. tuberculosis*. *Copyright © 2012 IFSA.*

Keywords: DNA biosensor, Quartz crystal microbalance, Tuberculosis, *Mycobacterium tuberculosis.*

1. Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis*. It is among the top ten causes of global mortality and morbidity, constitutes an important public health problem in Thailand. Mycobacterium needs 1–2 months in culture to grow. Besides, The Ziehl-Neelsen (ZN) stain for direct specimen examination, a conventional diagnostic tool, lacks sensitivity. A rapid and timely diagnosis of tuberculosis is thus essential to combat this disease. The need for rapid and sensitive detection of *M. tuberculosis* has resulted in the introduction of various molecular PCR methods in the routine workflow of laboratories showing promise for the detection of mycobacteria in clinical samples [1-4] but involves in the use of ethidium bromide staining which is carcinogenic agent in gel electrophoresis [5, 6].

Recently, there has been an increasing interest real time quartz crystal microbalance QCM based biosensor technology by this biosensor is one of the candidate devices of biosensor technology for detection of DNA hybridization that is rapid and sensitive detection among them, especially QCM based biosensor by using oligonucleotide hybridization detection method. The system using a QCM based biosensor in a flow cell might be developed for automated or continuous operation. The relationship between the oscillation frequency change of a quartz resonator in contact with liquid and accumulated mass had first realized by Kanazawa and Gordon in 1985 [10] that derived a relationship by expressing the change in oscillation frequency of a quartz crystal in contact with a fluid,

$$\Delta F = f_0^{3/2}\left(\frac{\rho_L \eta_L}{\pi \mu_q \rho_q}\right)^{1/2}, \quad (1)$$

where ΔF = measured frequency shift (Hz);
$f_0^{3/2}$ = resonant frequency of the unloaded quartz crystal (Hz);
ρ_L = density of liquid in contact with the quartz crystal;
η_L = viscosity of liquid in contact with the quartz crystal;
μ_q = shear modulus of quartz crystal = 2.947×10^{11} g/cm^2×s^2
ρ_q = density of quartz crystal = 2.648 g/cm^3

The penetration depth of this sheer wave depends on $\left(\pi f_0 \mu_q \rho_q\right)^{-1/2}$. Kanasawa and Gordon (eq. 2) demonstrated stress that ΔF in solution is linear function of $(\rho_L \eta_L)^{1/2}$ except for salts and high polymer solutions. They, in fact, tested the linearity of depence of the frequency decrease on $(\rho_L \eta_L)^{1/2}$ using many solvents selected on the basis of their different viscosity, density and electrical conductivity [7].

This biosensor has its own advantages that the detection method is label-free from radioactive or fluorescent tags [8]. There are many reports about the development of QCM specific DNA-based biosensor for detection many pathogenic bacteria in flow injection analysis such as *Staphylococcus epidermidis* [9], *Escherichia coli* [10], and *Pseudomonas aeruginosa* [11] by using of PCR for the preparation of bacterial target DNA.

In this study, a rapid and sensitive QCM combined with gold nanoparticle (AuNPs) amplification was developed as a new method for detection of *Mycobacterium tuberculosis*. The advantage of this study is that it uses a gold nanoparticle amplified with specific nucleic acid target which can be extended to develop new methods that are highly sensitive, specific, cheap, easy to use, and rapid for detection of other diseases.

2. Materials and Methods

2.1. Chemicals and Reagents

The chemicals and reagents used in the study were included 98% sulfuric acid (Sigma-Aldrich, USA), 30 % hydrogen peroxide (Merck, Germany), sodium chloride (Merck, Germany), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (Sigma, USA), Potassium dihydrogen phosphate (Merck, Germany), gold colloidal (sigma, USA).

2.2. Fabrication of QCM Sensor System Assay

The gold electrode surface of quartz crystals were cleaned by using Piranha solution (30 % H_2O_2: H_2SO_4 = 1:3) for 30 seconds. The crystals were then thoroughly washed with distilled water and used immediately afterward. Initially, immobilization of thiol-modified oligonucleotide probe on gold electrode surface by using HEPES buffer (0.05 M HEPES, 0.2 M NaCl, pH 7.5) was passed through the flow system. The inlet/outlet pumps were controlled to deliver 50 µL of solution to the detection cell. After the baseline was stabilized, 1.5 µM of thiol-modified oligonucleotide probe (SH-$(CH_2)_6$-5'-TTTTTTGTGGCCATCGTGGAAGCGA-3') in buffer (1M KH_2PO_4, pH 3.8) was added into the cell through the reagent reservoir and left to react on the surface of the gold electrode surface to form self assembly monolayer (SAM) for 5 min followed by immobilization buffer and distilled water to remove unbound for 30 seconds. After washing step, the resonant frequency (F1) was measured. The quartz crystal at this stage was ready for hybridization. Then the PCR product was injected to hybridize on the surface of gold/thiol modified probe for 5 minutes, after remove unbound the new resonant frequency (F2) was measured. The frequency change ΔF = F2 - F1. To improve the signal, the gold nanoparticle conjugated with primer enhancement sequences (Block: 5'-ATCGTGGTCCTGCGGGC TTTTTTT TT-3'-$(CH_2)_3$-SH) was added on the flow QCM sensor for 5 minutes after that, the bacterial DNA was hybridized with the optimum DNA probe and then the new resonant frequency (F3) was measured.

2.3. Samples

All clinical samples (10 samples as positive culture, 8 samples as negative culture, and H37RVKK11-20 as standard strain) were provided from National Tuberculosis Reference Laboratory (NTRL), Bureau of Tuberculosis, Department of Disease Control, Ministry of Public Health, Thailand. All samples were extracted genomic DNA following by Rienthong et al., 2009 [12]. Briefs, Sputum samples were decontaminated with N-acetyl-L-cysteine-sodium hydroxide. After centrifugation, the crude cell lysates were suspended in 300 µl of distilled water, heat killed at 95 ^oC for 20 min in Thermoblock, and then sonification for 15 min at the highest speed in an ultrasonic bath, followed by spinning the samples in a standard centrifuge with an aerosol-tight rotor at approximately 10,000 g for 5 min. The supernatant was used for the PCR amplification. The total of sputum specimens were compared with gold standard by using cultivation on Lowenstein-Jensen slants and incubated at 37 ^oC for 8 weeks. And the standard strain (H37RVKK11-20) cultivation was extracted genomic DNA following by kaewphinit et al, [13, 14].

2.4. PCR Amplification of DNA Target

The PCR amplification was performed 50 ng of DNA in a final volume 25 µL. The reaction contains genomic DNA in 10x PCR buffer, 50 mM MgCl2, 20 mM dNTP, 20 µM each of primers (RMTB: GTGGCCATCGTGGA AGCGA, FMTB: AAAGCCCGCAGGACCACGAT), 1 unit of *Taq* DNA

polymerase and distilled water. The PCR amplification was performed using DNA thermal cycle apparatus. Each cycle consist of pre-denaturation at 94 °C for 5 minutes, denaturation at 94 °C for 1 minute, annealing at 53 °C for 1 minute, extension at 72 °C for 1 minute and Post-extension at 72 °C for 7 minutes. The PCR product (209 bp in size) were analyzed using electrophoresis in 2 % agarose gel at 100 volt for approximately 30 minutes prior to ethidium bromide staining and record the DNA band under ultraviolet light.

2.5. Preparation of AuNPs Conjugated with Thiol Modified Oligonucleotide

Au colloidal was particle size (monodisperse) as 10 nm conjugated with 18-thiol terminated oligonucleotides were prepared following by Mirkin and college [15]. Place 20 µl of 5 nmol of thiol-modified DNA probe (Block) mixed 4 mL of Au colloidal in a tube, then the solution stand well by incubated shaking (100 rpm) at 45 °C for 24 h. The solution was then transferred to buffer I (0.1 M NaCl, 10mM phosphate buffer, and 0.01 % SDS, pH 7), and allowed to stand for an additional 48 h. The solution was centrifuged for 20 min at 12,000 rpm twice to attain red precipitates. The resulting precipitates were then washed with 500 µL of a buffer I solution and then resuspended in 750 µL of a buffer II (0.3M NaCl, 10mM phosphate buffer, and 0.01 % SDS, pH 7) and immediately used.

2.6. Sensitivity and Specificity of Detection

To determine detection sensitivity limits, 10-fold serial dilutions (10^{-1} to 10^{-5}) of 50 ng of total DNA of *M. tuberculosis* standard strain culture were used as DNA template PCR tests performed under optimized conditions. The specificity of PCR primers was examined using 50 ng of total DNA extracted from other mycobacterium. These included infectious *M. intracellulare*, *M. fortuitum*, *M. avium*, *M. kansasii*, and *M. gordonae*.

All PCR products were analyzed by 2 % agarose gel electrophoresis, and by QCM assay.

2.7. Bacterial DNA Samples Analysis and Comparison

The AuNPs combined with QCM assay in this study for identification of *M. tuberculosis*, 10 culture-positive and 8 culture-negative from sputum samples were detected individually using the QCM assay.

3. Results and Discussion

3.1. Comparison of Sensitivity with Gel Electrophoresis

Using equivalent quantities of DNA extracted from *M. tuberculosis* (H37RVKK11-20) infected samples as DNA templates at various dilutions, detection limits for PCR product (209 bp) was at 10^{-4} (5 pg of genomic DNA) (Fig. 1a. and 1b.). This assay was corresponded to the detection limit for PCR methods followed by electrophoresis, as described above.

This study used 10 nm diameter of AuNPs optimal as mass enhancement was signal amplification for the surface immobilization of QCM biosensor and the consequent sensitivity improvement because the hybridization was highest maximum value when the average diameter of nanoparticles was 20 nm and then decreased with the increasing of particle size [16]. Therefore, the frequency shift of the sensor was decreased accompanied by an increase of nanoparticle size applied in the static solution [16, 17]. The particle with an average diameter of 20 nm afforded the best hybridization rate, probably as

previous report [16]. In addition, gold nanoparticle modified blocking oligonuclotide deposited onto the piezoelectric sensor were depended on the DNA hybridization efficiency between the specific probe and target sequences [18, 19]. The gold nanoparticles could be attributed to the steric hindrance effect which the larger nanoparticles could not move as independently as the smaller nanoparticles and the larger nanoparticles connected to the DNA target hinder the approach of further nanoparticles [17].

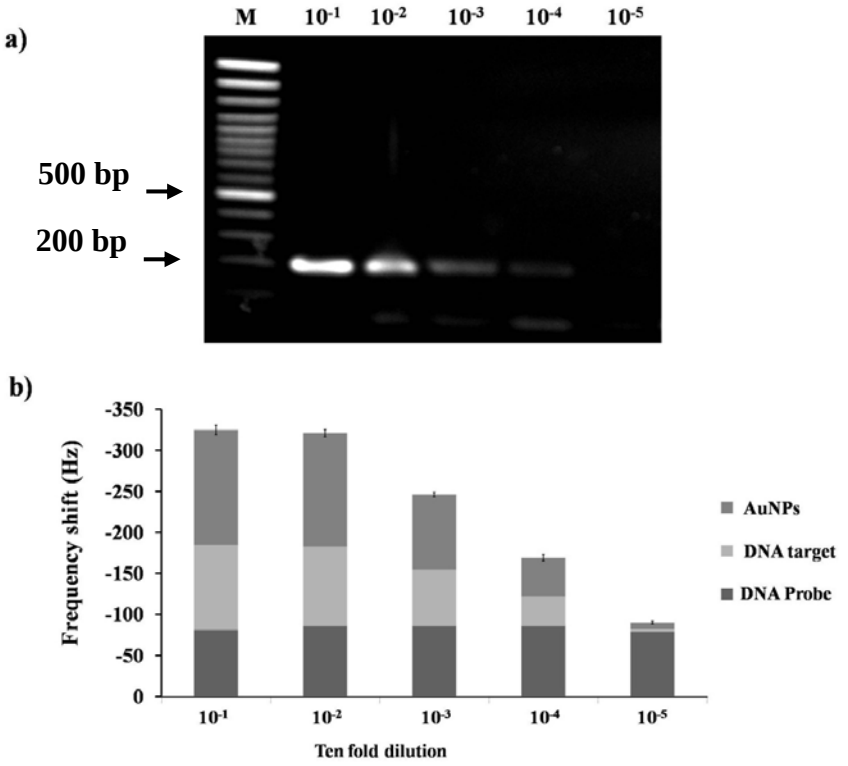

Fig. 1. Detection sensitivity data of *M. tuberculosis* DNAs at concentration range of 10^{-1} to 10^{-5} dilutions (initial concentration was 50 ng) obtained from (a) PCR-gel electrophoresis, (b) QCM assay; Lane M represent DNA ladder marker.

3.2. Specificity of QCM Assay

Specificity test was manipulated by using 50 ng each of *M. tuberculosis* DNAs and of other mycobacteria (i.e. *M. intracellulare*, *M. fortuitum*, *M. avium*, *M. kansasii*, and *M. gordonae*). The data revealed that no cross-reactions were obtained from gel electrophoresis (Fig. 2a.) AuNPs combined with QCM assay (Fig. 2b).

The dilutions of genomic DNA could be performed to reach the appropriate concentration within the limit of this sensor. The binding of target DNA sequences was inhibited when an excess amount of target DNA sequence complementary to the DNA probe. This is due to the formation of double-stranded in the solution by the two single strands of the DNA target at high concentration. Hence, blocking oligonucleotides capture with AuNPs were used for protection of the DNA fragments form self-assembly. Also, AuNPs as mass enhancement was signal amplification. Therefore, the thermal denaturation plus block oligonucleotides for preparation of target DNA could differentiate *M. tuberculosis* from other microbacteria. This result showed that the frequency shift of *M. tuberculosis* was lower than the frequency shift of other microbacteria. This result was similar to the report of previous study [13, 14, 20-22] which used IS*6110* target for detection of *M. tuberculosis* by PCR technique.

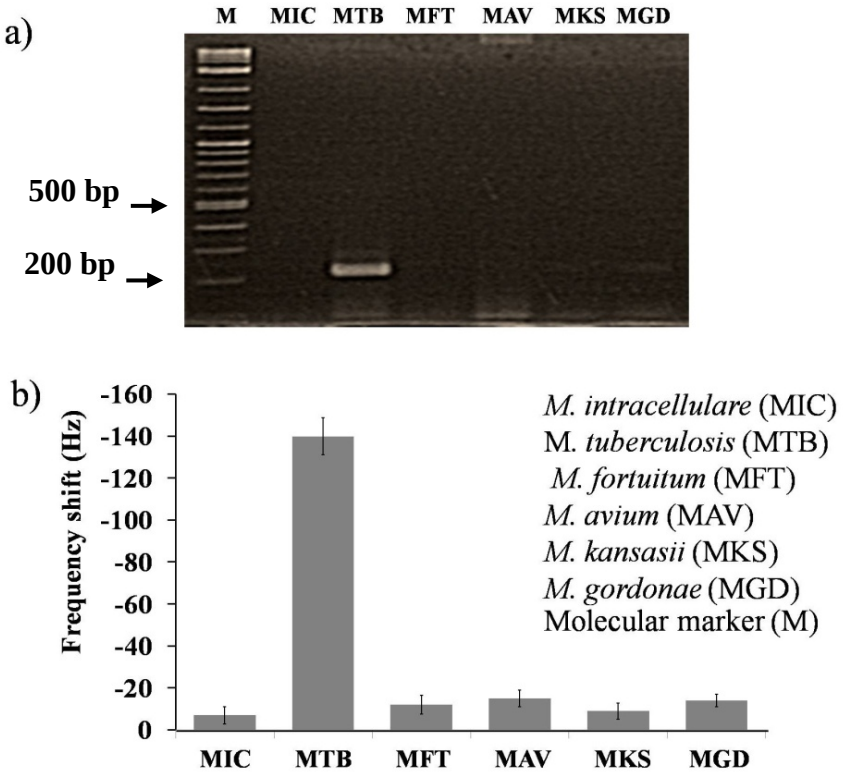

Fig. 2. Specificity data test of the LAMP method by using 100 ng each of DNA templates and for detection of *M. tuberculosis* by **(a)** gel electrophoresis or by **(b)** QCM assay. Lane M represents DNA ladder marker. Lanes 2-7 represent DNAs of M. *tuberculosis* (MTB), *M. intracellulare* (MIC), *M. fortuitum* (MFT), *M. avium* (MAV), *M. kansasii* (MKS), and *M. gordonae* (MGD), respectively.

3.3. Bacterial DNA Samples Analysis and Comparison

A total of 18 clinical sputum samples using culture diagnosis results were confirmed from National Tuberculosis Reference Laboratory (NTRL), Bureau of Tuberculosis, Department of Disease Control, Ministry of Public Health, Thailand. After PCR-amplification all genomic DNAs were hybridized with DNA probe and enchantment by using AuNPs, results were measured by QCM assay and compared with those obtained from traditional culture assays. In brief, the sensitivity and specificity of this assay for clinical samples diagnosis of MTB DNAs were 100 % and 100 %, respectively, as compared with culture assay (Table 1). However, it had been reported that IS*6110* and its homologue could be detected by nested-PCR in some mycobacteriae other than *M. tuberculosis* [23]. It meant that the specificity of these detection methods based on IS*6110* was not only on account of IS*6110* itself but also the possession of IS*6110* among bacteria strains. Therefore, this developed method had possibility to get false-positive results in clinical specimens containing non-*M. tuberculosis*.

The normal clinical sample for diagnosis of tuberculosis was lymphnode aspirates, cerebrospinal fluid, ascitic fluid, pleural fluid, sputum, and others. The sputum specimen has the highest possibility to get heavy contamination with other organisms. The result showed that satisfied performance of this biosensor system still remained in case of sputum samples.

In addition, the AuNPs amplification combined with quartz crystal microbalance DNA based biosensor for detection step confirms the identity of the specific amplicon by hybridization and avoids the use carcinogens such as ethidium bromide. The test platform can be adapted easily for rapid detection of other mycobacteria agents simply by designing appropriate sets of PCR primers which it constitutes a highly sensitive, and rapid alternative for detection of *M. tuberculosis*.

Table 1. Clinical samples identified with AuNPs combined with QCM assay, tested with specific carrying the IS*6110* probes for genotyping, compared with the culture assay.

Culture assay*	**QCM assay**	
	Positive	**Negative**
Positive (10)	10	0
Negative (8)	0	8
Total (18)	10	8
* Culture diagnosis results were confirmed from National Tuberculosis Reference Laboratory (NTRL), Bureau of Tuberculosis, Department of Disease Control, Ministry of Public Health, Thailand.		

4. Conclusions

The AuNPs combined with QCM biosensor appears to be a suitable and convenient tool for monitoring hybridization of complementary stands of oligonucleotides compared to other biosensor methods. The method demonstrated the sensitivity and specificity of the detection. this study was new finding direct detection PCR combined with AuNPs which could be beneficial for further routinely clinical development as portable device for sensitivity, specificity, cheap, easy to use, and rapid for detection of *M. tuberculosis*.

Acknowledgments

This work was supported by Faculty of Medicine, Srinakharinwirot University and I would also like to acknowledge Bureau of Tuberculosis, Ministry of Public Health Thailand.

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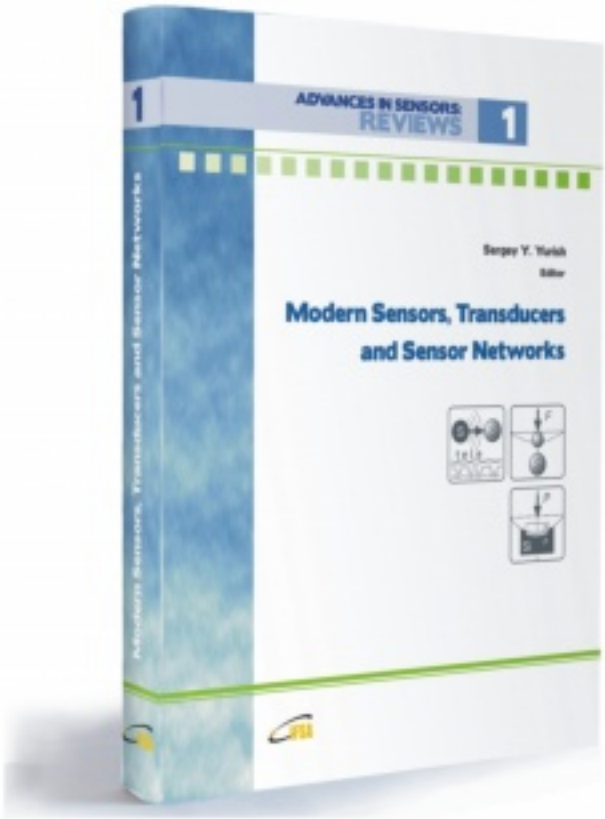

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