Rapid Tuberculosis Detection Technique for On-site Patient Screening

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Abstract

This paper presents a highly sensitive optical biosensor and novel sample collection strategy to allow for rapid on-site screening of tuberculosis patients. The technique presented herein was field tested and envisaged to be potentially useful in third world countries with high incidences of tuberculosis or at disparate locations void of proper tuberculosis diagnostic laboratories. The rapid screening technique incorporates a fiber-optic based sensor unit, a unique sample collection device and collection strategy to provide for a portable, easy to use and safe diagnostics. The biosensor technique targets trace elements of niacin, a metabolite specific to \textit{M. tuberculosis}, present in sputum samples. Field trial results show that the sensor was able to detect the presence of \textit{M. tuberculosis} in 5 days, significantly reducing the detection time of 6 to 8 weeks when applying the current laboratory-based culture method.

Keywords: biosensors, \textit{Mycobacterium tuberculosis}, rapid screening system, tuberculosis detection.

Received 12 April 2007; accepted 17 August 2007

1 INTRODUCTION

Tuberculosis has often been considered a disease of the past. However, there has been an alarming resurgence in recent years that has warranted strong research and development interests. Based on the World Health Organization (WHO) Report 2004, tuberculosis was ranked the eighth leading cause of death for the year 2002 [1]. In 2005, there were 8.81 million tuberculosis cases, where the largest percentage (34\%) occurred in the South-East Asia region. Total mortality caused by tuberculosis in South-East Asia in that year was 512,000 out of a total of 1.6 million deaths around the world [2]. The estimated tuberculosis incidence rate in the world is mapped out in Figure 1.

Tuberculosis is a contagious pulmonary disease caused by \textit{Mycobacterium tuberculosis} (\textit{M. tuberculosis}). It spreads through the air, either by coughing, sneezing, talking, or spitting. Only a small number of these bacteria are required to cause a healthy person to be infected. The infected person will not necessarily become immediately sick upon exposure. Only when the immune system is weakens; the potential of becoming sick increases. With the emergence of Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS), the number of tuberculosis cases has increased.

In countries or locations where proper biosafety level III laboratory tuberculosis facilities are not available, diagnostics are based simply on patient symptoms or by a Purified Protein Derivative (PPD) skin test. The PPD test has an approximately one-month detection period and frequently gives false positive results [3]. Another commonly used
screening method is another laboratory-based method known as acid fast staining. Its results can be obtained in a matter of days, but gives poor specificity and sensitivity because it detects all acid-fast bacteria instead of only \( M. \) \textit{tuberculosis} \[4, 5\]. Moreover, the acid-fast technique requires a minimum of 5000 bacteria cells to give any satisfactory results \[6, 7\]. In countries with high HIV/AIDS prevalence, acid-fast smearing method has a poor prognosis because these patients tend to excrete significantly fewer organisms per milliliter of sputum \[8\]. Thus, the laboratory-based culture technique is still seen as the preferred gold standard for \( M. \) \textit{tuberculosis} identification. For this, results are obtained after 6 to 8 weeks. This is because \( M. \) \textit{tuberculosis} has a cell generation time of 14 to 20 hours on solid media, which is considered to be significantly longer when compared to other types of bacteria, for example the \textit{Escherichia coli} species (generation time: 20 minutes). In order to conduct safe and accurate cultures, highly trained personnel are essential.

In developing countries, such facilities and manpower resources are rare \[9-13\]. In order to test for drug susceptibility, culture methods require an additional 2 to 6 weeks of tests \[21\]. Molecular methods, on the other hand, give good specificity and are significantly faster than culture techniques. These methods, however, require trained highly trained personnel, purified products, and are relatively expensive to perform. The technique is still classified as under research.

The delay in diagnostic time of the current tuberculosis screening methods results in the delay of identifying high-risk patients. This increases the risk the chances the disease spreading. This situation is especially common in developing countries. To overcome these difficulties, this work aimed at deriving a cheap, rapid tuberculosis screening technique and protocol suited for on-site demands.

### 2 METHODS

The experimental methodology can be categorized into the following parts: chemical formulations, fiber optic-based detection unit, laboratory-based trials, and field trials.

#### 2.1 Chemical Formulations

The \( M. \) \textit{tuberculosis} detection strategy is based on detecting trace niacin compounds in the patient sputum. Niacin was earlier established to be 95% specific of the etiological agent in sputum and therefore deemed as an important marker for the presence of viable \( M. \) \textit{tuberculosis}. The niacin is a product of metabolic reactions stemming from \( M. \) \textit{tuberculosis} binary fission occurrence. The addition of 10% cyanogen bromide (CNBr) and 4% aniline (R-NH2) to a processed human sputum sample that contains niacin will form a chemical reaction producing RNH \(_2\)BrCH:CHCH:NR, a unique yellowish solution. The chemical reaction is detailed as follows \[11\]:

\[
C_5H_5N + CNBr \rightarrow C_5H_5NCNBr \tag{1}
\]

\[
C_5H_5NCNBr + 2 \text{R-NH}_2 \rightarrow \text{RNH}_2\text{BrCH:CHCH:NR} + \text{CN}_2\text{H}_2 \tag{2}
\]

All chemical reagents used were purchased from Merck Co, Singapore. Potassium cyanide solution was prepared by diluting 4 g of potassium cyanide powder (KCN) into 100 ml of distilled water. Bromine-water was prepared by adding 50 ml of bromine solution into a 1000 ml amber flask that contained 150 ml of distilled water. After bromine was added into the water, two distinct layers were formed. From the bottom layer of the bromine water, 1 ml was transferred into an Erlenmeyer flask. Potassium cyanide solution was then added drop by drop until the liquid became colorless. This solution formed the cyanogen bromide solution.

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**Figure 1:** Estimated tuberculosis incidence rate in 2005 \[2\]

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Laboratory-based trials were conducted to determine the sensitivity of the sensor in detecting different niacin concentrations. Concentrations tested were: 0, 1.25, 2.5, 3.75, 5, 6, 8.33, 12, 16, 20, and 25 μg/ml. These different niacin concentrations were obtained by diluting niacin powder (Sigma Co., Singapore) into respective volumes of distilled water. In each test, niacin solution was transferred into the cuvette whereby equal volumes of CNBr and aniline solution were added thereafter. The spectral data of the samples were recorded and evaluated.

Spectral results were evaluated based on differences in intensity ratios (IR) between the control-sample (0 μg/ml niacin) and samples that contained varying concentrations of niacin. The intensity ratio was calculated using the following equation:

\[ IR = \frac{\text{Sample’s absorbance}}{\text{Control’s absorbance}} \quad (3) \]

2.4 Field Trials

Two equations that help estimate the amounts of niacin (N) levels produced by an initial number of *M. tuberculosis* bacteria cells were introduced, as presented in equation (4) and equation (5). The developed equations are based on the binary fission rate of bacterial growth [18, 19]. According to literature, 8.33 μg of niacin is produced by 50 *M. tuberculosis* cells after 3 weeks of incubation on solid culture media, i.e. Lowenstein-Jensen media [20]. Hence, the equations of the niacin production are presented as follows:

\[ N = 3.1 \times 10^{-10} \times a \times (2^t - 1) \mu g \quad (4) \]
\[ x = \frac{i_i - i_L}{g_i} \quad (5) \]

where \( x \) indicates the number of binary fission cycles and is calculated by dividing the logarithmic growth period by the generation time (\( g_i \)), which is 4.5 (fast growers) to 10.5 hours (slow growers) for *M. tuberculosis* growth in liquid media [7]. The logarithmic growth period is the subtraction of the total incubation period (\( i_i \)) by the lag period (\( i_L \)). The lag period of *M. tuberculosis* cultured in liquid medium is about 2 days [21, 22]. These equations were used to estimate the detection time for the developed sensor. Initial numbers of *M. tuberculosis* cells (\( a \)) can be quantified from acid fast staining results. Since acid fast methods can only detect a minimum of 5000 cells, negative acid fast results were assumed to have less than 5000 *M. tuberculosis* cells.

Clinical field experiments were carried out at the Hospital for Pulmonary Tuberculosis, Cisarua, Indonesia in accordance to institutional ethics approval obtained. The experiment was conducted using the developed sensing system (described in Section 2.1) and a specially designed sputum collection system (Figure 2). A total of 125 sputum samples were collected from suspected positive tuberculosis patients, who were diagnosed by medical doctors specializing in pulmonary diseases. Each sputum sample was acid fast tested before culturing. Of these, 77 samples were tested to be *M. tuberculosis* positive, 40 samples were *M. tuberculosis* negative, and 5 samples were *M. tuberculosis* negative but *M. scrofulaceum* positive. Of the 125 samples collected, 3 samples were determined as not useable due to contamination by fungus.
Sputum was collected in the sputum collection system. This novel system provided an all-in-one safe collection unit that could be used with the portable detection unit where there are no biosafety level III facilities for testing.

The sample collection system comprises of several components: a sputum collector (Figure 2a), a filtration tube (Figure 2b), a pre-packed reagent mixer (Figure 2d), and an incubation tube (Figure 2e). Patients were requested to expel sputum into the sputum collector-filtration tube (Figure 2c). For experimental purposes to allow monitoring of the niacin production in a sample, the collected sputum sample from each patient was divided equally to seven different containers. These tubes were then centrifuged for 10 minutes at 8,000 rpm. The supernatant solution that was filtered and collected at the bottom of the sputum collector was disposed. The filtration unit was then transferred to the incubation tube, which contained 1 ml of Middlebrook medium (Figure 2e), a selective broth for \( M.\) tuberculosis. Samples were thereafter incubated at 37°C. Niacin levels were measured on the following days: 0, 3, 4, 5, 7 and 15. These days were selected based on theoretical niacin production levels from equations (4) and (5). The seventh portion of the divided sample was cultured on Lowenstein-Jensen agar media and incubated for 8 weeks. This was used as another control test. All tests were conducted in triplicates. For each sample, the presence of \( M.\) tuberculosis in sputum was confirmed by a tuberculosis microbiologist.

When the niacin levels were to be measured, pre-packed reagents (Figure 2d) were injected into the incubation tube (Figure 2e) and hand shaken to encourage mixing within the chemical reagent mixer (Figure 2c). The sensing probe was then inserted in and the measurements taken in terms of niacin levels.

### RESULTS

#### 3.1 Laboratory-based Trials

Figure 3 shows the intensity ratios at different niacin concentrations. It can be seen that there is an expected downward intensity shift in the 400 to 500 nm wavelength region. In other words, when niacin concentration increases, more light intensity (in the abovementioned wavelengths region) is absorbed in solution. From Figure 3, it can be seen that the biosensor unit was able to detect 1.25 µg/ml of niacin concentration. Figure 4 shows the duration for 1000 cells to produce the varying levels of niacin.

![Figure 3: Niacin spectrum graph.](image)

![Figure 4: Calculated niacin production by 1000 M. tuberculosis cells based on equations (4) and (5).](image)
to be saturating, where as for low concentrations (< 8.33 μg/ml) has a more linear relationship, where:

\[ \text{IR} = 0.56 \times \text{niacin concentration} \]  \hspace{1cm} (6)

Figure 5: Niacin concentration vs. intensity ratio at 472 nm.

3.2 Field Trials

Results of the field trials are presented in Figures 6 and 7. Based on the culture results, 77 samples were *M. tuberculosis* positive, 40 samples were *M. tuberculosis* negative, and 5 samples were *M. tuberculosis* negative but *M. scrofulaceum* positive, a different species of the mycobacterium genus. The detection of *M. scrofulaceum* was based on morphological observations and specific biochemical testing. There were 77 positive *M. tuberculosis* samples, of which 38 samples contained 10^3-10^4 (S34) and the other 39 samples had 10^4-10^5 *M. tuberculosis* cells (S45) (Figure 6). Intensity ratios of samples containing *M. scrofulaceum* were compared against those of *M. tuberculosis* positive samples (Figure 7). The remaining three samples were contaminated by fungus; hence their results could not be used.

![Image of Figure 6: Comparison of IR levels for negative tuberculosis sample and samples containing 10^3-10^4 and 10^4-10^5 M. tuberculosis cells.]

Figure 6: Comparison of IR levels for negative tuberculosis sample and samples containing 10^3-10^4 and 10^4-10^5 *M. tuberculosis* cells.

5 DISCUSSION

Laboratory-based results (Figure 3) show that niacin solutions spectrum changes occur in the 400 to 500 nm wavelength regions, with a maxima at 472 nm. This is because the specific chemical reaction between niacin and the mention reagents produces a yellow color solution.

From Figure 5, it can be seen that the system is more sensitive at lower niacin concentrations (i.e. ≤ 8.33 g/ml). Based on the IR sensed by the detector, the amount of niacin in the sample can be quantified and correlated to the actual number of *M. tuberculosis* cells present in the collected patient sputum samples. This can thus be used as an indication of how pronounced the infection is.

Results of the initial clinical trials for the biosensor are presented in Figure 6. Changes of intensity ratio for S34 and S45 were compared with the negative samples. On day 0, 3 and 4, the intensity ratio level of S34 and S45 did not differ much from the negative samples. There was a slight decrease of 5% in intensity ratio for S34 on day 5, but this drop was not distinct since its standard deviation intersected with the negative samples. On the same day, intensity ratio level for S45 (a decrease of 33%) was distinctive, and its standard deviation did not lie within the negative range. As the samples were incubated further, intensity ratios for both S34 and S45 decreased further to 0.58 and 0.42 OD, respectively, on day 15. This distinct decline in intensity ratio rate indicated the presence of viable *M. tuberculosis*. The intensity ratio level of S34 was 0.27 times lower than S45. However, the declining rates of both samples were generally the same, which means that niacin was being produced at the same rate. This corresponds with the developed niacin production algorithm in equations (4) and (5).

Results from this initial investigation demonstrated that the new biosensor strategy was able to detect *M. tuberculosis* within 5 days from one milliliter of sputum samples containing 10^4-10^5 *M. tuberculosis* cells. Samples containing 10^3-10^5 *M. tuberculosis* cells/ml
(S34) could also be detected in 5 days, but at a slightly lower confidence level. The standard deviation for S34 was 1.6 times larger than that for S45. Thus, for a higher confidence level for S34 samples, a day 7 test would be preferred.

As shown in Figure 7, there were negligible intensity ratio changes from any of the *M. tuberculosis*-negative samples. This was also the case for *M. scrofulaceum*, as niacin metabolite production is absent during binary fission. Since there were no increases in niacin levels, there were correspondingly no spectrum changes detected. *M. tuberculosis* is the only mycobacterium that produces niacin and at the same time connected to pulmonary diseases. This specificity allows the detection strategy to be able to differentiate positive *M. tuberculosis* samples from samples that contains other types of mycobacteria from sputum samples.

Based on experiments, when compared to the acid fast staining method, advantages of the new biosensor includes the ability to differentiate *M. tuberculosis* from other acid fast bacteria. Significantly fewer numbers of bacteria cells are required for detection, thus providing earlier detection. Thus, results can be obtained in 5 days compared to 1 month by the PPD test or 6 to 8 weeks by the laboratory-based culture method. Another important ability is in being able to differentiate between live and dead *M. tuberculosis* cells – a critical problem faced when using immunoassay techniques.

In conclusion, a rapid, portable, highly sensitive method is preferable when using immunoassay techniques. Disposal of used collection units are by proper medical facility or biosafety level III laboratory. Proper medical facility or biosafety level III laboratory providing the appropriate biosafety requirements in the absence of proper tuberculosis Class III laboratory facilities.

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### ACKNOWLEDGMENTS

The authors would like to acknowledge the medical officers and laboratory staff at the Hospital for Pulmonary Tuberculosis, Bogor, Indonesia for helping to conduct the field trials.

### REFERENCES


