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Detection of Tuberculosis (TB) using Gold Standard Method, Direct Sputum Smears Microscopy, PCR, qPCR and Electrochemical DNA Sensor: A Mini review

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ABSTRACT

Despite the continued effort globally made to control the growing case of Tuberculosis (TB), it continues to be regarded as the second deadliest disease after the HIV. There are various methods developed to diagnose TB, most of which having the criteria of sensitive, selective, cheap and portable to be used in robust applications. Even with the advancement in medication, the important keys including early stage diagnosis is yet to be considered. In diagnosing TB, the only technique remained as the gold standard method is the culturing method, which is the Acid Fast Bacilli (AFB) staining. On the other hand, molecular technique utilising Polymerase Chain Reaction (PCR) assay is preferred as a non-culturing method. Additionally, as molecular techniques become advanced, real-time PCR or quantitative PCR (qPCR) using multiple probes in one shot has raised interest among researchers, because it can skip the process of gel electrophoresis. Recently, researchers have been working on electrochemical DNA sensors which are sensitive, selective, rapid, cheap and can meet with point of care (POC) testing requirements to diagnose TB.

INTRODUCTION

Mycobacterium tuberculosis (MTB) is an infectious agent, which is the one responsible for causing Tuberculosis (TB). It has been estimated that there are 9.6 million TB cases reported globally in 2014, which equivalent to 133 cases per 100,000 population [1]. This means that about one third of our population was infected by TB worldwide since one patient can transmit the disease to 12-15 people/ year on average through respiratory tract infection [2]. Thus, a rapid diagnosis especially during the early stage is considered crucial for effective TB control, both for treating the infected individual as well as for intervention of the public to decrease further spreading in our population [3].

Traditionally, public healthcare associates microbial culture technique with Acid Fast Bacilli (AFB) staining as the 'gold standard' method [4] to detect the presence of MTB in samples especially from low resource countries [5]. However, this method requires a lot of time with laborious techniques as it involves cell culture, cell enrichment and cell count of a slow growing mycobacterium [6]. Currently, many methods have been improved to rapidly detect the TB disease by developing low time consumption and sensitive diagnosis including the polymerase chain reaction (PCR) based assay [7]. Initially, PCR was conventionally conducted; however, to simplify this process, a quantitative PCR (qPCR) was used to avoid electrophoresis after the thermal cycling [8]. According to Sales and colleagues [8], this method is able to reduce the time of analysis to three days. Nonetheless, despite all advantages, the complex instrumentation of PCR technique and the cost for its

reagent are still of the main obstacles. In recent years, as the sensing technology was improved, the detection in variety of analytes such as enzymes, proteins and DNA was also observed to be more possible. Therefore, interest in electrochemical DNA sensor has been considered as one of the efforts to detect TB in early stage [9]. In this review, specimen collection, gold standard method, conventional PCR, real-time or quantitative PCR (qPCR) technique in detecting TB disease as well as the perspectives of electrochemical DNA biosensor is focused.

Gold Standard Method

The gold standard method for detecting TB involved microbial culture and Acid Fast Bacilli (AFB) staining [10]. Before the culture is grown, the specimen collection must be handled with care and promptly transferred to laboratory, otherwise, the advantages of decontamination technique and sensitive culture media would be a waste. The culture is made from inoculation of centrifuged sediment on Lowenstein Jensen (LJ) media, followed by incubation for growth and finally used for AFB staining [11].

Specimen collection

Most specimens collected from patients are sputum, as it still remains the most critical sample for laboratory testing [3]. Other collected specimens include urine, blood, biopsies and Central Spinal Fluid (CSF). Sputum must be immediately transferred to laboratory after collection or refrigerated if delay is unavoidable to inhibit the growth of other microorganisms [12]. If refrigeration is also not possible within 2 days, suitable preservative treatment with 1 % cetyl pyridinium chloride (CPC) and 2 % sodium chloride solution are recommended [11]. Ryu [3] also stated that sputum can be directly smeared without the need in culture and observed for the present of AFB. This microscopy technique is rapid, simple, and inexpensive; however, this technique has demonstrated a low sensitivity and specificity which leads to poor observation.

Culture of *Mycobacterium tuberculosis* (MTB)

The sputum specimens were transferred into centrifuge tube together with 4 % NaOH for decontamination process. After the specimens were incubated and washed with a blend of distilled water and phenol, the cultures were grown according to a method by Central TB division [13]. During this step, the growth of mycobacteria will be weekly checked within two months.

According to Asmar and colleagues [10], more than 15 different culture media were developed to improve the time for culture to grow. However, due to high price and the limitation of their usage in resource poor countries, Lowenstein Jensen (LJ) solid medium was remained as the gold standard culture medium. LJ media is an enriched egg medium with glycerol and asparagine. LJ contains an inhibitor to prevent the contaminant from outgrowing the MTB [11]. Due to the nature of MTB that requires a long incubation period, culture made in bottles is more preferred rather than in petri dish [13]. The bottles need to be tightly closed to prevent the culture from drying. The growth of MTB is examined by the presence of small, rough, crumbly, waxy, buff pigmented with the appearance of breadcrumb and cauliflower [13]. TB cultures gave the highest sensitivity in detecting active pulmonary TB. Unfortunately, MTB takes 3-6 weeks to give visible colonies, showing a critical disadvantage to this method. From the study by Uddin and colleagues [14], it was observed that out of 915 specimens, 74 (8.1 %) were found to be AFB positive by direct AFB smear. While on culture, out of 915 specimens, 105 (11.5 %) were found to be positive. The study was done by comparing direct sputum smear and concentrated sputum smear with gold standard method. From this study, although concentrated sputum smear has been proven to increase sensitivity of sputum smear technique, if growth period is not considered, culture method can be regarded as more sensitive compared to direct sputum smear microscopy.

Acid Fast Bacilli (AFB) Staining

There are two commonly used procedures for staining, which are the direct microscopy and fluorescent microscopy. Direct microscopy uses carbolfuchsin method and is divided into two; Ziehl Neelsen and Kinyoun methods. According to Tankeshwar [15], both methods are still widely used in developing country. For fluorescent microscopy, fluorochrome procedure using auramine-O or auramine-rhodamine dyes is commonly used [16]. It is recommended by CDC to use fluorochrome staining rather than direct microscopy to detect AFB in primary patient specimens [17].

Carbolfuchsin method

Hot (Ziehl Nielseen, ZN) and cold (Kinyoun) staining conditions are involved in Carbolfuchsin method. ZN staining method has to fix a smear by heating [18] and stained with carbol-fuhscin (a pink dye), making it to bind to the mycolic acid in the MTB cell wall [17]. After staining process has been completed, acid decolourising was applied to remove the pink dye from cells, tissue fibres and untreated mycobacteria, leaving only mycobacteria bond to the dye. The sample was counter stained with methylene blue or other malachite green. The MTB has appeared pink in the contrast background [19]. Even though ZN microscopy was considered as highly specific, the sensitivity displayed was not too promising [20].

In addition, Kinyoun staining method has the same procedure as ZN. Despite that it does not involves heating, the penetration of dye occurred by increasing the concentration of carbolfuchsin primary stain [16]. The comparison result shows that the correlation between ZN and Kinyoun staining analysis of the paired data on the same smears (N = 145) [21] is quite good with correlation coefficients of 0.9 and 0.8, respectively. On the other hand, bacteriological index (BI) and morphological index (MI) indicated that there are no differences observed between both methods. The advantage of Kinyoun staining with good sensitivity (3.6 % false negatives) and very few false positives (0.5 %) was also supported by Sawadogo and colleagues [22] while rate of positive TB has no significant difference both for ZN and Kinyoun. However, study by Van Deun [23] has shown low coloured AFB Kinyoun that gave smear read errors. The heating technique in ZN staining gave better fixation of fuchsin and colour for AFB. However, the disadvantage of using Kinyoun method over ZN method is the use of high concentration carbolfuchsin, which increases the cost of the whole processes.

Fluorochrome staining method

Fluorochrome staining method can be done by flooding Auramine-O on heat fixed smear for 20 minutes to complete smear reaction. After reaction, it is rinsed under running water followed by decolourising using acid alcohol for three minutes before being rinsed again. After quenching with 0.5 % potassium permanganate, it was air dried and examined under microscope with high power magnification. Acid Fast Bacilli (AFB) usually fluoresces as golden with slender rod shaped structure [23]. According to a study by Jagadeesha and Vidya [24], it shows that fluorochrome staining method has better sensitivity than ZN method in detecting AFB, showing 114 (13.24 %) positive samples and 747 (86.75 %) negative samples

compared to ZN staining with 89 (10.33 %) positive samples and 772 (89.66 %) negative samples for AFB.

In addition, it can detect AFB in low densities, more reliable as well as it can easily deal with a large number of samples. Furthermore, Sawadogo and colleagues [22] reported that using same method has led to a positive diagnoses in 15.9 % of their samples; 100 % sensitivity, 95.6 % specificity, and positive and negative predictive values of 75.7 % and 100 %, respectively, in fluorochrome staining result obtained. Kinyoun staining produced a positive diagnosis rate of 12 %, sensitivity of 96.4 %, specificity of 99.5 %, and positive and negative predictive values of 96.4 % and 99.5 %. This proves that fluorochrome staining has better sensitivity than Kinyoun staining.

Conventional PCR

Molecular method for TB diagnosis directly from clinical specimen has been used since the early 1990s. It can be used with DNA from various clinical sample sources with promising result and useful to support the traditional diagnostic method [25]. In addition, this method can shorten the time of diagnosis from 3-6 weeks to 24-48 hours [26]. However, it also demonstrates poor sensitivity especially to microscopy negative samples and extrapulmonary samples [27]. Therefore, this method can be a good alternative for TB diagnosis especially in the area where culture is not available due to lack of laboratory facilities, materials and trained laboratory workers.

In this technique, DNA from clinical sample is extracted and undergoes PCR for amplification assisted by specific primer sequence of mycobacteria with total reaction volume of 25 µl. The reaction involved is denaturation (94˚C/15s), annealing (66˚C/15 s), extension (72˚C/20 s) and final extension (72˚C/3 min) for 35 cycles using thermocycler. The PCR product was observed on 1.6 % agarose gel in 0.5X TBE containing ethidium bromide. Gel can be visualised on UV transilluminator and photographed using gel documentation system. Positive sample is considered by band corresponding to 245 bp (**Fig. 1**).

MTB-1: sequence (5' to 3') CGT GAG GGC ATC GAG GTG GC
MTB-2: Sequence (5' to 3') GCG TAG GCG TCG GTG ACG AA

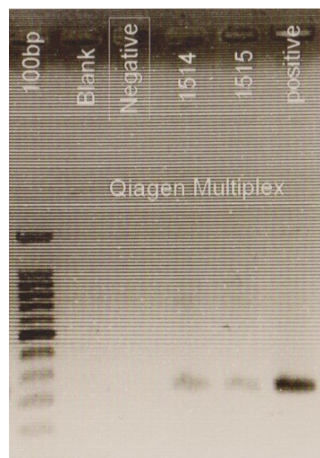

Fig. 1. Representative agarose gel of PCR product with MTB primer (IS6110 gene sequence) with two positive clinical sputum sample (sample no.1514 and 1515) (the author, unpublished results).

Despite its advantage in reducing time, the sensitivity of conventional PCR is comparable to the gold method as tested by Kivihya-Ndugga and colleagues [28]. It can be concluded that PCR can perform better compared to ZN smear microscopy as it can detect similar proportion of culture-positive TB patients with higher specificity. Saxena and colleagues [29] published that after 17 microscopy negative samples were further tested with PCR method, 7 samples were found positive indicating that PCR method is more efficient in detecting MTB.

Gholoobi and friends [30] reported even though PCR is specific, sensitive, rapid, straightforward and is a minimally invasive method for detecting MTB in clinical samples, contamination of specimens with mycobacterial DNA from previous PCRs or contamination of samples during DNA extraction procedures may be the source of false positive results [31] While Saxena and colleagues [29] mentioned that PCR require high cost, it is can be still considered as the best method for TB detection.

Real-time or Quantitative Polymerase Chain Reaction (qPCR)

Recently, real-time PCR or qPCR has been widely used because of its excellence performance to overcome the limitation in conventional PCR method. The qPCR is believed to be more rapid than conventional PCR with excellent reproducibility that only requires less than one hour in diagnosing MTB [32] (after DNA extraction). Despite its short experimental time, qPCR remains as low risk of sample contamination and offers the possibility of quantifying a bacterial load [33].

According to Haron and friends [34] for qPCR optimisation, the sequence of probe can be modified from TB probe of IS6110 gene with quenchers including FAM-GAACGGCTGATGACC AAACT-BHQ-1. A 5` end of the probe was labelled with 6-carboxy-fluorescence-dye (FAM) and a 3` end was labelled with non-fluorescence dye (BHQ-1). The DNA from bacterial isolates and clinical samples were subjected to Real-time PCR using the mixture of TB primers and TB probe. The reaction mixture consists of template DNA, TaqMan MasterMix, primers and probe. Analysis was carried out at 60°C for 1 min. The cycling parameters for Absolute Quantification were done at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. The data were collected during annealing steps.

It was reported in the previous study by Park and colleagues [35] that qPCR has the sensitivity and specificity of 83.7 % and 96.2 %, respectively, while sensitivity and specificity of smear microscopy are only 56.8 % and 99.6 %, respectively. Previous study by Nagdev and colleagues [33] also reported that the overall accuracy of qPCR was found higher than conventional PCR. Thus, it can be concluded that molecular diagnosis of TB can be improved by qPCR.

Electrochemical DNA Sensor

Most previous TB detection methods have been improved to decrease the risk of sample contamination; unfortunately, it is usually centralised in large stationary laboratories due to complex instrumentation and the need for well-trained laboratory staff [36]. To overcome this problem, a biosensor technology seems to be the best way with real-time, sensitive, rapid and accurate detection device. In addition, biosensor is able to deliver point of care diagnostic, which can surpass and

match the conventional standards regarding to time and cost [37].

Biosensor is a short term for biological sensor made up from transducer and biological recognition components such as antibody, enzyme and nucleic acid. The biological element interacts with analyte and the transducer converts biological response into electrical signal [38] proportionate to the concentration of analyte. The signal is later amplified, quantified, displayed and analysed through a processor [39]. This biological recognition component can be combined with different types of transducer. The nanomaterials frequently used as transducing substances are semiconductor nanoparticles, or quantum dots (QDs), carbon nanotubes, silver, silica, graphene, gold nanoparticles, perfluorocarbon and organic polymers [39].

Regarding to MTB, many biosensors were reported and reviewed (**Table 1**) based on electrochemical, piezoelectric, optical, magneoblastic and capacitive [40] transducer. However, electrochemical method has attracted more attention [41] due to its low-cost, rapid response, sensitivity and ease of operation [42]. Electrochemical DNA biosensor involves the hybridisation of nucleic acid attracting many attentions due to its potential application in detecting many diseases and microorganisms. Interactions between nucleic acids and various types of nanomaterial can be easily modified to improve the performance of many biosensors. Unlike enzyme or protein, nucleic acid can be readily prepared and generated in multiple numbers using PCR [39,43].

Table 1. Summary for the Advantage and Disadvantage of each Methods for TB Diagnostic

Method	Sensitivity and accuracy	Time of Diagnosis	Remarks	References
Gold standard method: Culture	sensitive	3-6 weeks	give visible colonies	[10, 3]
Gold standard method: Acid fast bacilli staining (Direct microscopy)	Low concentration dye give smear read errors	3-6 weeks	Use of carbolfuchsin dye increasing the cost	[23]
Gold standard method: Acid fast bacilli staining (Fluorescent microscopy)	Sensitive	3-6 weeks	can easily deal with a large number of samples	[22,24]
Conventional PCR	promising result	24-48 hours	Alternatives diagnosis of TB in rural area	[25,26,27]
qPCR	Overall accuracy higher than conventional PCR	24 hours	Low risk of sample contamination	[33]
qPCR	More rapid than conventional PCR	Less than one hour	Excellent reproducibility	[32,33]
Electrochemical DNA Biosensor	Highest sensitive	Less than one hour	Low cost and able to deliver point of care diagnostic	[37,41,42]

In such a way to improve the sensitivity of biosensor, it is important to utilise materials with a great surface area and good conductivity for better a connection to transducer. According to Hu and colleagues [44], the most excellent candidate in the detection of TB is graphene oxide (GO) with the characteristics of surface area (up to 2,630 m^2/g) and unique *sp2* (*sp2* /*sp3*)-bonded network that make it perfect for biomolecules anchoring and detection. GO was successfully adopted as a platform to discriminate DNA sequences. A plane orientation of DNA nucleobases with respect to graphene surface was the most stable state, which favoured π–π stacking between the bases and aromatic carbons in grapheme [44]. Other than that, Devi and colleagues [45] also reported that gold nanotubes array nanostructures can be a good candidate because of its exceptional biocompatibility and chemical stability of gold materials.

DNA detection using gold nanotubes Array (AuNTsA) was successfully applied for the fabrication of DNA hybridisation biosensor in detecting MTB [46]. The electrochemical DNA biosensor displayed a good linear range of complementary DNA concentration from 0.01 to 100 $ng.ul^{-1}$ with high detection efficiency. Electrochemical DNA biosensor using gold electrode modified of thiol (Au-SAM) can be used to determine hybridisation between ssDNA probe and ssDNA target sequence of MTB with sensitivity value of 0.5152, detection limit of 3.47 $\mu g.mL^{-1}$,and quantification limit of 11.56 $\mu g.mL^{-1}$ [47]. It can be concluded that electrochemical DNA biosensor demonstrates a good stability, high specificity, and provides new alternatives for clinical MTB diagnostics in the future.

CONCLUSIONS

Many literatures stated many advantages of biosensor in clinical medicine and medical diagnostic, especially in TB detection. Nonetheless, researchers are still facing a lot of challenges before DNA biosensor can be truly applied in routine applications. The main issues are sensitivity, selectivity and stability of the device. Even though this study has reported that DNA biosensor can detect the presence of TB in low concentration of DNA, very few evidences were provided differentiating latent TB, active TB, multidrug-resistance TB (MDR-TB) and extremely drug-resistance TB (XDR-TB). Time of detection including sample preparation is very crucial for processes including DNA extraction in gel electrophoresis and polymerase chain reaction, which take longer time compared to Quick real-time PCR (qPCR). To decrease time consumption in DNA biosensor, the number of cycles during PCR step should be reduced. If the time usage can be skipped, extra advantage to DNA biosensor can be materialised as this is a major challenge in TB detection to create a miniature DNA biosensor with point of care (POC) characteristics. Therefore, continuous efforts and collaborations between various fields in TB detection such as material science, molecular biology, physics, chemistry and manufacturing companies are of important as they can give more ideas for improving DNA biosensor in the future.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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