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A fast method for the identification of *Mycobacterium tuberculosis* in sputum and cultures based on thermally assisted hydrolysis and methylation followed by gas chromatography–mass spectrometry

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ABSTRACT

A fast gas chromatography–mass spectrometry (GC–MS) method with minimum sample preparation is described for early diagnosis of tuberculosis (TB). The automated procedure is based on the injection of sputum samples which are then methylated inside the GC injector using thermally assisted hydrolysis and methylation (THM). The THM–GC–MS procedure was optimized for the injection of sputum samples. For the identification of *Mycobacterium tuberculosis* the known marker tuberculostearic acid (TBSA) and other potential markers were evaluated. Hexacosanoic acid in combination with TBSA was found to be specific for the presence of *M. tuberculosis*. For validation of the method several sputum samples with different viscosities spiked with bacterial cultures were analyzed. Finally, 18 stored sputum samples collected in Vietnam from patients suspected to suffer from TB were re-analyzed in Amsterdam by microscopy after decontamination/concentration and using the new THM–GC–MS method. No false positives were found by THM–GC–MS and all patients who were diagnosed with TB were also found positive using our newly developed THM–GC–MS method. These results show that the new fast and sensitive THM–GC–MS method holds great potential for the diagnosis of TB.

1. Introduction

The World Health Organization (WHO) declared tuberculosis (TB) a global emergency. It is estimated that around one-third of the World's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). An estimated 9.3 million new cases occur each year with 1.8 million deaths in 2007 [1]. The majority of cases occur in sub-Saharan Africa, South- and South-east Asia, Latin America and the Caribbean. The recent increase in TB is related to the increase in HIV infections [2].

Only early diagnosis of TB followed by appropriate treatment can confine the regions where TB occurs and this remains a challenge [3]. Currently, TB is diagnosed by chest X-ray, direct staining of the mycobacteria in sputum or culture from sputum samples and nucleic acid amplification techniques [4]. However, all these methods suffer from their own limitations. Chest X-ray is not specific enough, a suspected chest X-ray can be caused by other lung diseases and previous TB. Microscopy can detect mycobacteria in sputum, but species identification is impossible and the test lacks sensitivity. The diagnosis of TB in HIV patients is even more difficult since there is a high rate of smear-negative cases among these patients. For those patients, culturing is needed which takes a long time (2–6 weeks) and needs identification of the cultured mycobacteria. Amplification techniques are cumbersome, labor intensive and too expensive in terms of running costs for developing countries [5].

The challenge is to develop a fast and simple specific test for *M. tuberculosis* which is able to reduce the workload of the laboratory personnel while maintaining at least the same sensitivity and reliability as microscopy. Gas chromatography (GC) can be an attractive method for TB diagnosis [6]. It is fast, simple to use and not expensive. GC was already used in the late 1970s for the identification of mycobacteria [7]. Unfortunately, the use of GC suffers from one important drawback: the requirement of complex sample preparation before the actual GC analysis. To overcome this, Syhre [8] used SPME to detect volatile biomarkers of *M. tuberculosis*. Stopforth et al. [9] developed a novel method for sample preparation of non-treated sputum based on stir-bar sorptive extraction and

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thermal desorption. Another method for the reduction of sample pre-treatment using on solid-phase micro-extraction and derivatization of the fatty acids on the fiber was recently developed by Cha et al. [10]. A possible way to reduce the sample preparation even further would be the use of thermally assisted hydrolysis and methylation (THM) with GC or GC–MS quantification of specific *M. tuberculosis* markers. Conventional THM using tetramethyl ammonium hydroxide (TMAH) has already been applied by several authors for the identification of bacteria based on their fatty acid profiles [10,11]. In a recent publication we described the use of THM for polymer analysis [12]. Since then we have further improved the automation of the method turning it into a fully automated method that no longer requires complex sample exchange robots as are required in previously described automated methods [13,14]. In our work, the entire THM procedure is performed inside a programmed temperature vaporizing (PTV) injector with all sample handling done by a standard robotic GC auto sampler. Using this novel approach, not only polymers but also sputum or bacterial cultures can be injected with minimal or no sample preparation. This could eliminate the major disadvantage of using GC for TB diagnosis.

The aim of the present study was to investigate the potential of automated THM–GC-MS as a tool for rapid and reliable identification of *M. tuberculosis* in cultures and sputum from patients suspected of TB. For the identification of *M. tuberculosis* the known biomarker 10-methyl octadecanoic acid or tuberculostearic acid (TBSA) was used. Additionally, other known or proposed biomarkers were investigated to further improve the specificity of the method for *M. tuberculosis*. The experimental settings were optimized for the direct injection of bacterial cultures and sputum into the GC without any sample preparation. Finally, the method was applied for injection of sputum and cultures which were first liquefied and concentrated using the NaOH/*N*-acetylcysteine citrate decontamination method [15]. Results obtained with the novel automated GC method were compared with those of the standard microscopy and culture tests.

2. Materials and methods

2.1. Culture of mycobacteria

M. tuberculosis cultures were obtained from the TB national reference centre, RIVM Bilthoven, The Netherlands. The strains were cultivated at KIT (Royal Tropical Institute) Biomedical research under P3 facilities, in 15 mL Middlebrook 7H9 medium supplemented with OADC (oleic acid albumin dextrose and catalase) (Becton Dickinson, Detroit, MI, USA) and incubated between 35 and 37 °C in a gyrotary shaker at 100 rpm.

2.2. M. tuberculosis suspension for spiking experiments

The *M. tuberculosis* bacteria were killed by heating for 20 min at 80 °C. The suspension was vortexed for 5 min with 25 glass beads (diameter 4 mm) and filtered through a 5 μm filter to get rid of the clumps. The absorbance of the suspension was measured at 420 nm and adjusted to 0.15, which corresponds to 1×10^8 mycobacteria/mL [16]. Two-milliliter sputum samples from non-TB patients were spiked with the heat-killed *M. tuberculosis* bacteria at different concentrations and shaken for 30 min at 250 rpm to obtain a homogenous distribution of the mycobacteria in the sputum.

2.3. Sputum samples

Sputum samples from Dutch patients with pulmonary diseases other than TB were obtained from subjects who were admitted to the Division of Pulmonary Diseases of the Academic Medical Centre in Amsterdam. Sputum samples from patients suspected of TB were obtained from the Pham Ngoc Thach TB and Lung Disease Centre, Ho Chi Minh City, Vietnam. The Medical Ethics Committee of the Academic Medical Centre and the Pham Ngoc Thach TB and Lung Disease Centre approved the study protocol.

2.4. Sample preparation for thermochemolysis

Sputum samples of 1 mL were decontaminated with 0.25 M NaOH + 0.025 M Na–citrate + 5 mg *N*-acetyl-L-cysteine (NALC)/mL expressed as the final concentrations [15]. The samples were shaken for 5 min and neutralized with phosphate buffered saline PBS pH 6 to an end volume of 15 mL. The decontaminated sputum samples were centrifuged for 30 min at 5000 × *g*, supernatant was removed and PBS was added to the pellet to yield an end volume of 110 μL (10× concentration step). 100 μL of each sample was then transferred into small V shaped vial inserts (ATAS GL, Eindhoven, The Netherlands) that fit in the auto sampler vials. The remaining 10 μL of the decontaminated sputum was used for microscopic examination after staining according to the Ziehl Neelsen method (ZN staining).

2.5. Standards and reagents

The 25% aqueous TMAH solution was obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). The water used for dissolving the reagent was deionized before use using a Satorius Arium 611 UV instrument (Satorius, Nieuwegein, The Netherlands). The fatty acid standards used for peak-identity confirmation and quantification: palmitic acid and 17-methyloctadecanoic acid were supplied by Sigma–Aldrich. Chloroform used to dissolve the standard compounds was purchased from Biosolve (Valkenswaard, The Netherlands). All samples, spiked samples and mycobacterium suspensions were made, collected and pre-treated at KIT Biomedical Research.

2.6. Instrumentation

All THM–GC–MS analyses were performed on a Shimadzu GCMS-QP2010 plus (Den Bosch, The Netherlands). The GC system was equipped with a "Focus" XYZ robotic auto sampler (ATAS GL) and an Optic 3 PTV injector (ATAS GL). The PTV injector was equipped with an extra electronic gas control (EGC) unit. One EGC was used to supply the carrier gas, septum purge and the split-flow. The second EGC was used to create a "back flush" to make sure that no water vapor could enter the GC column during the injection and drying of the sample/reagent mixture. To do so, a Y piece (GL Sciences, Tokyo, Japan) was installed in the analytical column, approximately 30 cm below the injector nut. The extra EGC channel of the PTV was connected to this Y piece using a retention gap of approximately 90 cm × 0.25 mm (GL Sciences). During the injection of the sample and the reagent solution as well as during elimination of the water and incubation the back flush was on. After solvent elimination and incubation the backflush flow was switched off and the normal carrier gas flow was restored.

2.7. Automated THM treatment

The automated THM procedure used for the identification and quantification of the *M. tuberculosis* markers was largely identical to that applied previously for the characterization of polar polymers [12]. The entire automated procedure is performed using the OPTIC 3 PTV injector and consists of five subsequent steps: Injection of the aqueous sample solvent mixture into the PTV injector, drying of the sample, injection of the TMAH reagent solution and