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Full Length Research Paper

Detection limit for differentiating between various *Mycobacterium* species and *Pseudomonas aeruginosa* using gas chromatography-mass spectrometry (GC-MS) metabolomics: A comparison of two extraction methods

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Previously, we investigated the capacity of a metabolomics research approach to characterise and differentiate between various infectious *Mycobacterium* species and *Pseudomonas aeruginosa*, and compared two extraction procedures, prior to gas chromatography mass spectrometry (GC-MS) and statistical data analyses. In the current investigation, we report that the minimum sample material required (detection limit) for this speciation is 250 cells, using the total metabolome extraction method, and 2500 cells, using the fatty acid metabolome extraction procedure. Considering these detection limits, both methods compared significantly well with the currently used tuberculosis diagnostic methods and have the potential to be implemented clinically.

Key words: Detection limit, tuberculosis, metabolomics.

INTRODUCTION

Despite widely available vaccination and treatment strategies, tuberculosis (TB) is still regarded as one of the main health threats worldwide, with Africa accounting for more than 30% of all global cases (WHO, 2010). In a previous study, we investigated the capacity of a metabolomics research approach to characterise and differentiate between various *Mycobacterium* species and *Pseudomonas aeruginosa* using two extraction procedures; 1) extracting the fatty acid metabolome and 2) extracting the total metabolome, prior to gas chromatography mass spectrometry (GC-MS) and statistical data analyses (Olivier and Loots, 2012). By using a mixer mill to enhance the extraction capacity, this study led to the analytical refinement of the well-known Bligh-Dyer fatty acid extraction procedure, allowing a total fatty acid extraction and derivitisation time of only 5 h, and a reduction in the total solvent volume used to only 1.75 mL. Additionally, an alternative extraction approach, using solvent combinations capable of extracting metabolites belonging to all compound classes, was also reported. For the purpose of *Mycobacterium* speciation, the latter "total metabolome" extraction procedure

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Abbreviations: TB, Tuberculosis; **GC-MS,** gas chromatography mass spectrometry; **PCA,** principal component analysis; **AMDIS,** automated mass spectral deconvolution and identification system; **MYCO-LCS,** Sherlock Mycobacteria identification system.

showed advantages over the fatty acid extraction procedure described, as it: 1) was simpler, 2) required comparatively less solvent volumes (only 1.25 mL); 3) was faster (4 h); 4) was more repeatable and 5) isolated metabolites from more compound classes, increasing the chance of identifying metabolite markers. In addition to these parameters, the detection limits, or minimum amount of sample material required for species differentiation, are also of particular importance, should this method later be used for TB research or diagnostics. A low detection limit could potentially allow for the speciation of samples with low bacterial loads, typically occurring in patients with early TB infection, TB/HIV co-infection and TB infected children (Getahun et al., 2007; Marais and Pai, 2007; WHO, 2006). Therefore, in this study, we investigated the detection limits of the previously described extraction methods for differentiating between various *Mycobacterium* species and *P. aeruginosa*.

MATERIALS AND METHODS

Chemicals and reagents

N-methyl-N-(tert.-butyldimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS), pyridine, potassium hydroxide (KOH), methyl-nonadecanoic acid (Me-C19), glacial acetic acid and trimethylpentane were purchased from Merck (Darmstadt, Germany). Methoxyamine hydrochloride and 3-phenyl butyric acid were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Cultures

All the cultured organism samples used in this study were supplied by the Royal Tropical Institute, Amsterdam, The Netherlands. All the bacteria (*Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium bovis, Mycobacterium kansasii* and *P. aeruginosa)* were cultured in Middlebrook 7H9 medium with oleic acid-albumin-dextrose-catalase enrichment, as described previously (Olivier and Loots, 2012). In order to determine the potential detection limits of the previously described extraction methods, 6 x 1 mL repeats of the isolated *M. tuberculosis,* re-suspended in ddH_2O at a concentration gradient ranging from 1×10^1 to 1×10^6 bacteria/mL, including a blank (ddH_2O only), were extracted using the investigated methods and analysed using the GC-MS. In order to verify the differentiation capacity of these extraction methods at the determined detection limits, 6 x 1 mL samples of each of the five isolated bacterial species (*M. tuberculosis, M. avium, M. bovis, M. kansasii and P. aeruginosa*) were re-suspended in ddH_2O at the concentration determined by the detection limit experiments, prior to extraction, GC-MS analysis and statistical data comparisons.

Extraction procedures

The fatty acid metabolome extraction method was performed using 1.25 mL of an extraction solvent mixture consisting of chloroform, methanol and ddH_2O, in 1:3:1 ratio, and added to 250 µL of the earlier mentioned sample suspensions. The extraction was performed by shaking the suspensions in an MM 400 mixer mill (Retsch GmbH and co. KG, Haan, Germany) at a frequency of 30 Hz for 5 min, after the addition of a 3 mm tungsten carbide bead. Following this, ddH_2O and chloroform (250 µL of each) were added to the extract, which was subsequently mixed, and the organic phase collected after centrifugation, and dried under a light stream of nitrogen. Hereafter, 0.5 mL of chloroform, 0.5 mL of methanol and 1 mL of methanolic KOH (0.2 M) was added to the dried extract and the mixture was incubated for 30 min at 60°C. After incubation, 2 mL of hexane, 200 µL of glacial acetic acid (1 M) and 2 mL of ddH_2O was added to the sample mixture and the organic phase was once again collected after centrifugation. The remaining water phase was re-extracted 3 times with hexane and the combined organic phases were dried under nitrogen and re-suspended in 50 µL of trimethylpentane containing 15.6 ng/mL of the external standard (Me-C19).

Similarly, for the total metabolome extraction method, 1.25 mL of the extraction solvent mixture (chloroform, methanol and ddH_2O in a 1:3:1 ratio) was added to 250 µL of the above mentioned sample suspensions after the addition of 50 µL of 3-phenyl butyric acid (26.25 mg/mL) as an internal standard. After mixing, shaking in the vibration mill and centrifugation, the supernatant (total liquid phase) was collected, transferred to a GC-MS sample vial and dried under a light stream of nitrogen. Hereafter, the dried supernatant was derivatised with 50 µL of methoxyamine hydrochloride in pyridine (15 mg/mL) at 50°C for 90 min and then trimethylsilylated using 50 µL of MSTFA with 1% TMCS at 50°C for 60 min.

GC-MS

The prepared extracts (1 µL) were injected on an Agilent 7890A gas chromatograph (Agilent, Atlanta, GA) coupled to an Agilent 5975 mass selective detector equipped with a 7683B injector, 7683 auto sampler and VF1-MS capillary column (30 m × 250 µm i.d., 0.25 µm film thickness) in the splitless mode, using the temperature programs described previously (Olivier and Loots, 2012).

The raw GC-MS data were deconvoluted and analysed using AMDIS software (Automated Mass Spectral Deconvolution and Identification System, V2.65). Alignment of the detected compounds across the samples analysed was achieved by creating a new reference library in AMDIS, which contained the mass spectra of all the compounds detected above a threshold of 0.01% of the total signal, for all the samples analysed. Each analysed sample was subsequently processed using the aforementioned reference library and the resulting output of each sample was combined into a data matrix containing the relative concentrations (normalised with the internal / external standard) for all compounds present or absent in each sample.

RESULTS AND DISCUSSION

Principal component analyses (PCA) were done using the AMDIS generated data matrixes, consisting of the relative concentrations of all the metabolites present in each of the analysed samples. PCA is a mathematical procedure that transforms a number of possibly related variables (compounds) into a smaller number of unrelated variables known as principal components (PCs). The value of these PCs can then be plotted in

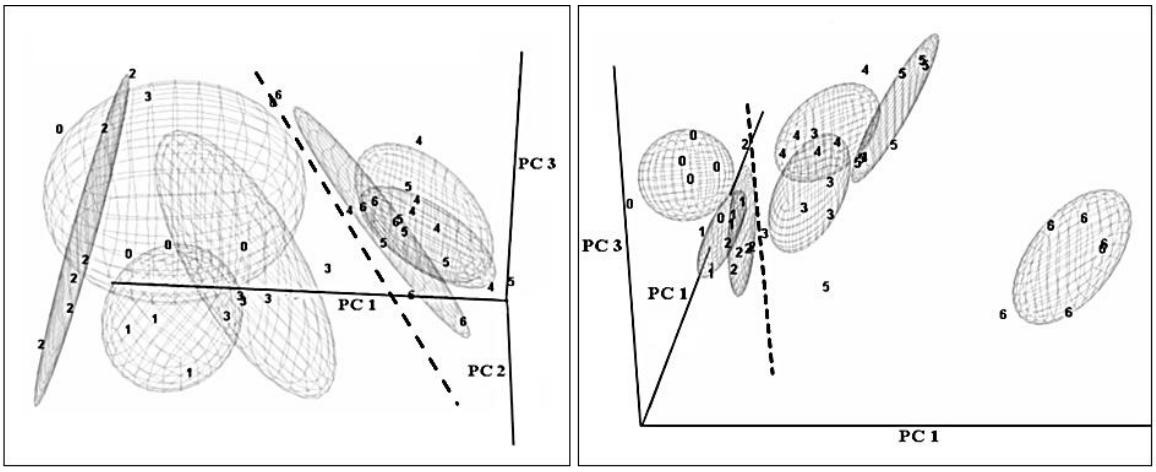

Figure 1. PCA scores plot (PC 1 vs. PC 2 vs. PC 3) of the GC-MS generated data of the concentration gradient samples of *M. tuberculosis* where: 0 = blank; 1 = 1 x 10^1; 2 = 1 x 10^2; 3 = 1 x 10^3; 4 = 1 x 10^4; 5 = 1 x 10^5; 6 = 1 x 10^6 bacteria/mL. The lowest bacterial concentration not overlapping with the blank, and hence the detection limit for the fatty acid extraction method (A), is 1 x 10^4 bacteria/mL, and for the total metabolome extraction method (B), is 1 x 10^3 bacteria/mL.

order to determine whether or not a natural grouping exists between the sample groups, based on their metabolite profiles. The detection limit for each extraction procedure was defined as the group with the lowest concentration not overlapping with the blank samples. Figure 1A represents the PCA output of the *M. tuberculosis* concentration gradient samples analysed after extraction using the fatty acid metabolome extraction procedure. The lowest concentration group not overlapping with the blank, and thus considered the detection limit for this extraction method, was 1 x 10^4 bacteria/mL. Considering that only 250 µL of the sample suspension was used for these extractions, the minimum amount of sample material required for detecting metabolite markers differentiating *M. tuberculosis* from a blank sample is 2500 cells.

Comparatively, considering the results from the total metabolome extraction procedure (Figure 1B), the lowest concentration not overlapping with the blank was 1 x 10^3 bacteria/mL. Since this method also requires only 250 µL of the sample suspension, the amount of sample material required for detecting metabolite markers differentiating *M. tuberculosis* from a sample blank is 250 cells.

In order to confirm that the compounds extracted at these low detection limits are capable of differentiating between the various TB-causing *Mycobacterium* species and *P. aeruginosa*, we repeated the procedure using 250 µL of the separately cultured sample repeats of the five bacterial species prepared at a concentration of 1 x 10^4 bacteria/mL, extracted using the fatty acid metabolome extraction method, and 1 x 10^3 bacteria/mL, extracted using the total metabolome extraction method. The PCA scores plots of the processed GC-MS data (Figure 2A and B) indicates that all the infectious species groups differentiated from one another on the basis of the extracted metabolite profiles, confirming the detection limits for this approach, using both the investigated extraction methods. In both instances, three PCs were used and the total amount of variance shown by the first three PCs (R^2X cum) using the fatty acid extraction method was 65.4%, of which PC 1 showed 35.6%, PC 2 showed 19.7%, and PC 3 showed 10.06%. Similarly, the total amount of variance explained by the first three PCs (R^2X cum) of the total metabolome extraction method data was 78.5%, of which PC 1 showed 50.7%, PC 2 showed 20.9% and PC 3 showed 6.9%.

When considering the detection limits of the two extraction methods investigated in this study, the total metabolome extraction procedure performed comparatively better than the fatty acid extraction method. However, both methods proved to have a better detection limit than the most widely used TB diagnostic method, smear microscopy, which requires 5000 to 10 000 bacteria/mL sputum (Colebunders and Bastian, 2000). Furthermore, smear microscopy cannot differentiate between the various TB-causing *Mycobacterium* species (Ruiz-Manzano et al., 2008), as was done using our approach.

Bacteriological culture is currently considered the diagnostic gold standard for TB diagnostics, with a

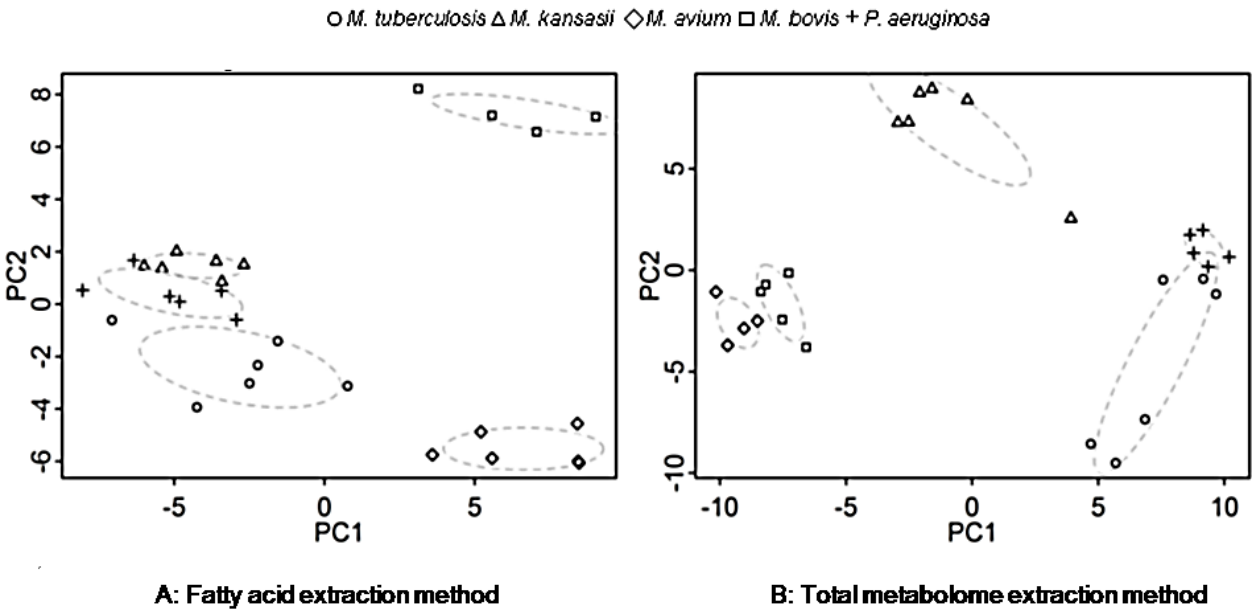

Figure 2. Three-dimensional PCA scores plot of the GC-MS generated data after extraction of the five bacterial sample repeats, showing a clear differentiation between all the sample groups at a concentration of 1 x 10^4 bacteria/mL using the fatty acid extraction method (A) and 1 x 10^3 bacteria/mL using the total metabolome extraction method (B).

reported detection limit of about 10 to 100 bacteria/mL (Getahun et al., 2007; WHO, 2006). This method does, however, require up to 6 weeks for obtaining a diagnostic result (WHO, 2006). Additionally, false negative results are reported for 15 to 20% of all adult TB cases (Frieden et al., 2003; Getahun et al., 2007), whereas 1 to 4% of all cases diagnosed using solid or liquid cultures, are false positives (Getahun et al., 2007). The invention of automated culture systems, such as BACTEC 460, has decreased the mycobacterial speciation time from a few weeks to about 15 days (Cruciani et al., 2004). The high cost involved in the maintenance and infrastructure of these methods does, however, limit their implementation in low-income countries such as South Africa (WHO, 2006). A more recently developed TB diagnostic strategy, based on nucleic acid amplification (NAA), can now detect various TB causing mycobacteria from sputum, at a reported concentration of 10 bacteria/mL sputum, with a sensitivity of 60 to 70% in smear-negative, culture-positive samples (Moore and Curry, 1995; WHO, 2006; Pounder et al*.,* 2010). Pure cultures are, however, still the favoured source of genomic DNA for these NAA assays, due to the presence of PCR inhibitors in sputum, hence, this method does not exclude the timely culturing step (Ahmad et al., 2004). Both extraction methods described in our investigation also require pure bacterial cultures for optimum performance. However, when taking into account that smear-negative, culture-positive sputum samples contain a minimum of 10 cells per sample (Getahun et al., 2007; WHO, 2006), these samples would only require a culturing step of approximately 5 days, prior to using the total metabolome extraction, and 7 days, prior to using the fatty acid metabolome extraction, when considering that the population of these organisms doubles every 15 to 20 h. This timeline is, however, speculative, and should be confirmed.

Furthermore, because the investigated approach does not require the use of expensive DNA primers, the commercial use of either of these extraction methods as part of a metabolomics TB diagnostic approach, would be more economical than NAA and automated culture systems when taking cost per analysis and the required analytical infrastructure into account.

Lastly, this comparison would be incomplete without considering the Sherlock Mycobacteria Identification System (MYCO-LCS), which is the only commercially available TB diagnostic method based on pattern recognition software. MYCO-LCS was developed by the MIDI Research and Development Laboratory for the identification of a variety of mycobacterial species, based on their diverse mycolic acid profiles, using HPLC (MIDI, inc., 2009). For a successful diagnostic outcome, this method does, however, require at least 1 x 10^5 cultured cells per sample (ATC and CDC, 1999).

Considering this, although the total metabolome extraction method could differentiate between the various

Mycobacterium species and *P. aeruginosa* at a lower detection limit than the compared fatty acid metabolome extraction procedure, both methods performed exceptionally well when considering the currently available TB diagnostic methods. With further refinement and validations, this metabolomics approach (using either of the two extraction methods) has the potential to serve as a low-cost TB speciation method, with a low detection limit and a diagnostic time comparable to that of smear-microscopy.

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