Revisiting a protocol for extraction of mycobacterial lipids

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ABSTRACT
Determination of lipid content of any biological sample is essential for various kinds of studies related to pathogenicity and drug development. Thus, reliable methods for the quantitative extraction of lipids are of critical importance. The mycobacterial cell wall is largely composed of lipids. Commonly used methods to extract lipids, such as the Bligh and Dyer method or the Folch method, yield a low amount of lipids when applied to mycobacterial cells. This study presents an efficient modification of Chandramauli’s method, a less known method developed at this institute earlier that is able to yield a considerably higher concentration of mycobacterial lipids.

INTRODUCTION

With almost 30% of mycobacterial genes devoted to lipid metabolism Mycobacterium tuberculosis is well known for its complex cell wall structure which confers, to the bacilli, resistance to drying, acidic or alkaline conditions, and to chemical disinfectants and therapeutic agents [1]. The most preliminary step for studying the lipid profile is the extraction of cellular lipids. In the search for an efficient method for mycobacterial lipid extraction, three methods were found. These were comprised of two very well known methods given by Folch in 1957 and by Bligh and Dyer in 1959. The Folch’s method, which was developed primarily for brain lipids, involved extraction with chloroform/methanol (2:1, v/v) followed by washing with weak salt solutions of NaCl/KCl/MgCl2 in order to retain acidic lipids. All the steps of washing were performed by centrifugation of the suspensions, but it was also stated that in case there is no time constraint, phases may be allowed to separate by prolonged standing [2]. The method as given by Bligh and Dyer [3] involved lipid extraction from animal tissue (fish muscle) and the solvent system used was slightly different. It recommended the use of a different ratio (1:2, v/v) of the same solvent system (chloroform/methanol, v/v) [2,3]. Though these protocols were developed originally for animal tissues and were validated on samples with low lipid content, they have been employed for extraction of lipids from other sources, including bacteria [4,5]. Chandramouli and Venkitasubramanian in 1974 [6] developed
a method (less known method) inspired by Folch’s method. They added prolonged mixing of cell pellet with the same ratio of chloroform and methanol and allowed phase separation by standing instead of centrifugation. In this study, a comparative analysis of these three methods was carried out for extraction of mycobacterial lipids in order to choose the best suitable method.

**Materials and methods**

**Bacterial cultures**

The different mycobacterial species that were used in the study were comprised of *M. tuberculosis* (H37Rv), *Mycobacterium smegmatis* (ATCC 19420), *Mycobacterium bovis* (ATCC 19210T), *M. avium* (MTCC, IMTECH, Chandigarh, India), *Mycobacterium fortuitum* (ATCC 6841) and *Mycobacterium kansasi* (ATCC 21982). The cultures were maintained on Middlebrook’s 7H9 broth (Difco Laboratories, MI, USA) and were autoclaved prior to lipid extraction to ensure safety during culture handling.

**Extraction of lipids**

Lipid extraction was initially performed with 200 ml of *M. tuberculosis* H37Rv culture for each protocol. The cultures were harvested when the optical density (O.D₆₀₀ = 1) reached 0.4–0.6. Cultures were centrifuged and pellet obtained was dried. Weight of the dried pellet was noted and equal weight of pellets was taken for lipid extraction. Extraction was carried out using all the three aforementioned protocols in parallel, and the methods were followed as were originally specified (Fig. 1). The solvent/sample ratio was also maintained as per the actual protocol. In order to check any protein contamination, the Bradford assay was performed for each lipid extract.

**Thin layer chromatography**

The lipid content obtained in each case was dried under nitrogen, weighed and then analysed by thin layer chromatography (TLC) after dissolving the samples in chloroform. Equal volume (2 μl) of each sample was loaded on TLC plates (Merck). Chloroform/methanol/water (60:12:1, v/v) was used as a mobile phase [7]. The spots were visualized using 0.5% α-naphthol dissolved in 50% methanol followed by charring with 50% concentrated sulphuric acid. For detection of phthiocerol dimycocerosates (PDIM), TLC was run using petroleum ether/ethyl acetate (98:2, v/v) as the mobile phase [8]. The phosphomolybdic acid solution (Sigma) was sprayed for the detection of the spots. After comparison, the selected protocol was used for the extraction of lipids from

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**Fig. 1 – Flowchart of three protocols used in this report.**

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**Fig. 2 – (a) TLC of total lipid extract using different protocols.**

TLC were developed using solvent system (a) and (b) of chloroform/methanol/water 60:12:1(v/v); (b) Solvent system – petroleum ether/ethyl acetate 98:2 (v/v); Lane 1: Folch method, Lane 2: Chandramouli’s method, Lane 3: Bligh and Dyer method; (c) TLC of total lipid extracts obtained from different mycobacterial species using Chandramouli’s protocol: (i) *M. smegmatis*, (ii) *M. fortuitum*, (iii) *M. avium*, (iv) *M. kansasii*, (v) *M. bovis*. Solvent system used – chloroform/methanol/water (60:12:1).
M. smegmatis, M. bovis, M. avium, M. fortuitum, and M. kansasii. The extracts obtained were again analysed by TLC.

**HPLC analysis**

Briefly, extracted total lipids were first saponified by 2 ml of saponification reagent consisting of 25% potassium hydroxide in 1:1 solution of methanol and water and heated to 100 °C for 2 h followed by the addition of 2 ml chloroform and 1.5 ml of acidification reagent (1:1, Conc. HCl/distilled water). The solution was mixed vigorously and the layers were allowed to separate. The lower organic layer was transferred to a new tube and evaporated in a heat block at 85 °C with a stream of nitrogen. Total lipid samples were derivatized to p-bromophenacyl bromide (PABA) esters by adding 20 mg of potassium bicarbonate, 50 µl p-bromophenacetyl-8-reagent (Thermo Scientific Cat. No.4889) and 1 ml chloroform. The reaction mixture was heated at 85 °C for 20 min. Samples were cooled and filtered through a 0.45-µm-pore-size nylon 66 membrane filter. Prepared samples were subjected to HPLC analysis. Separation was made using reverse phase C18 column with 80% acetonitrile – 20% water as mobile phase as published by Singh et al. for mycobacterial lipids at a flow rate of 1 ml/min. UV detection was set at 260 nm [9].

**Results**

A comparative analysis of mycobacterial lipid extracted using these three methods (Fig. 1) was made. Lipid extracts from Chandramouli’s, Folch’s and Bligh and Dye methods were free from any protein contamination. On the basis of dry weight estimation, approximately 42, 30 and 21 mg of total lipid extract was obtained from Chandramouli’s protocol, Folch method and Bligh and Dyer method, respectively. Thus gravimetric analysis states that almost double the amount of lipid could be extracted by Chandramouli’s method in comparison with the others. Further, TLC analysis showed profound bands in case of Chandramouli’s method as compared the other two methods (Fig. 2A). Fig. 2B depicts the spots of PDIM as were visualized on TLC for the extracts obtained using all the said protocols, which is again of higher strength for Chandramouli’s method. The reproducibility of Chandramou-
Chandramouli’s method for extraction of total lipids was checked from different species of mycobacteria and TLC was performed for lipid extracted from *M. smegmatis*, *M. avium*, *M. fortuitum*, *M. kansasii* and *M. bovis* (Fig. 2C). Quantitatively, HPLC revealed higher concentrations of the total lipid extracted by Chandramouli’s method as compared with Bligh and Dyer and Folch’s method (Fig. 3 and Fig. 4).

**Discussion**

While extracting the lipids using the above-mentioned methods, it was found that Chandramouli’s method provides a higher concentration of mycobacterial lipids. The increased concentration of lipid in Chandramouli’s method could be attributed to the use of a higher amount of a non-polar solvent chloroform. This helped in dissolving all the triacylglycerols and non-polar lipids which could be otherwise lost if the solvent system used was highly polar. The Folch’s method employed the same solvent system, but still the concentration of lipids obtained were comparatively less compared with Chandramouli’s, which could be due to longer exposure of the cell mass to the solvent system for a period of 14 h followed by a second extraction for 8 h in the case of Chandramouli’s method. Bligh and Dyer’s method is generally recommended for tissues containing approximately 80% water [3,10]. Also, the majority of evaluations that have been performed for this method have been carried out on samples containing less than 1.5% total lipids. Bligh and Dyer and others have acknowledged that lipid rich samples may require modifications of the original method and that lipid rich samples may present with a reduced yield of lipids [10]. Additionally, it has been reported previously that for samples containing less than 2% lipid, the results of Folch’s and Bligh’s and Dyer’s method did not vary. But for samples containing >2% lipids, Bligh’s and Dyer’s estimates were significantly lower than those of Folch’s (*p* < 0.0001) [11]. In the present study, similar results were also obtained.

**Conclusion**

It is therefore concluded that out of the three methods that are used for extraction of lipids, the method used by Chandramouli and Venkitasubramanian is more suitable and provides a higher concentration of mycobacterial lipids. Although the methods given by Folch and Bligh and Dyer are widely used for eukaryotic as well as prokaryotic cells, when it comes to mycobacteria Chandramouli’s method has an advantage over these prevalent methods in terms of concentration and can be a good alternative for mycobacterial lipidomic studies.

**Conflict of interest**

None declared.

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**References**


