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An efficient and safe method for extraction of Mycobacterial Phthiocerol dimycocerosates (PDIM's) and Sulfolipids.

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ABSTRACT

Mycobacterium tuberculosis, the causative agent of tuberculosis, is known for its three distinct characters i.e. resistance, persistence and a rigid cell wall. All these three characteristics, whether directly or indirectly are influenced by the lipid rich cell wall of Mtb. Thus cell wall lipidomic profile of Mtb plays a very dynamic role in its functioning. Out of the total lipid armamentarium of Mtb, PDIM's and sulfolipids plays an important role in the virulence of the bacteria. Present methods of detection and visualization of these two lipids involves harmful radioactive substance. Hence the method of detection by 20% H2SO4 in ethanol followed by charring at 100° C is quite effective as well as safe.

KEYWORDS : Mycobacterium tuberculosis, PDIM's, Sulfolipids, TLC, Lipid Extraction

Introduction

Tuberculosis is scourge for mankind. It is caused by the bacteria *Mycobacterium tuberculosis* and is responsible for the most number of deaths because of a single bacterial infection. As per WHO Report 2014, tuberculosis leads to 1.5 million deaths worldwide[1] and the sudden rise of MDR & XDR tuberculosis have entangled the mankind into a self-created web. It is also estimated that 40% of the Indian population is suffering from tuberculosis although the infection is latent and not active. Environmental stress, low immunity are among various other factors that leads to sudden onset of infection. The devastating situation that we are in because of the MDR & XDR tuberculosis is remnant of the non-compliance of the strictly recommended first line drugs i.e. Isoniazid, Rifampicin, Pyrazinamide and Ethambutol. The resistance mechanism of *Mycobacterium tuberculosis* is the result of thickening of the cell wall of the bacteria and thereby creating a permeability barrier[2]. Cell wall of the bacterium is also responsible for the bacterial's persistence mechanism inside the host. PDIM and Sulfolipids form an important component of the cell wall of Mycobacteria and are also responsible for its virulence[3]. Studies have been performed to know the mycobacterial lipid profile because the mycobacterial lipids (~30% of dry weight) confer to the bacilli resistance to drying, acid or alkaline conditions, and to chemical disinfectants and therapeutic agents[4]. Importance of PDIMs can be attributed by the fact that they are exclusively present in *Mycobacterium tuberculosis* and hence can be said to form the basis of pathogenesis of Mtb. Usually the detection of PDIM and Sulfolipids involves the use of radioactive propionate[5] which makes this method unviable in many of the laboratories that don't have proper infrastructure for the same. Hence an easy efficient and safe method is required to study these important virulence lipids of *Mycobacterium tuberculosis*. Here we have presented a modified method for the detection and visualization of PDIMs and Sulfolipids that doesn't involve the use of radioactive compounds and hence can be used even in the basic laboratories.

Material and Methods

Bacterial Culture and Growth conditions: *Mycobacterium tuberculosis* H37Rv was grown in Middlebrook 7H9 broth (Difco) which was enriched with 10% OADC, 0.05% tween 80, 0.2% Glycerol till OD_{600} reaches approximately ~1. Culture was splitted back to OD 0.3 in 50 mL Middlebrook 7H9 broth. It was further grown for two days. Cells were pelleted down in 50 ml falcon and supernatant was discarded

Lipid extraction: Pellet obtained above was resuspended in 2ml of Methanol: 0.3% aqueous NaCl (10:1). 1 ml of petroleum ether was further added and resulting solution was stirred vigorously until emulsification of two layers takes place. Resulting solution was subjected to centrifugation at 4200 rpm for 10 minutes to separate the two layers. Upper layer of petroleum ether so obtained was pippeted out and the same procedure was repeated once again. Two layers of petroleum ether thus obtained were pooled together and dried under nitrogen and stored at -20° C till further use.

Thin layer chromatography for PDIMs and Sulfolipids: For the detection of PDIM and Sulfolipids TLC was run using petroleum ether/ethyl acetate (98:2, v/v) three times and chloroform–methanol–water (60:12:1, v/v) respectively as the mobile phase.

Visualization of PDIM and Sulfolipids: For the visualization of the resolved lipids a solution of 20% H_2SO_4 in ethanol was prepared. TLC was dipped in the solution for approximately 2 minutes and then kept undisturbed for drying. When the TLC had dried completely it was charred with the help of hot air gun by heating it approximately at 100° C for 5 minutes.

Results and Discussion

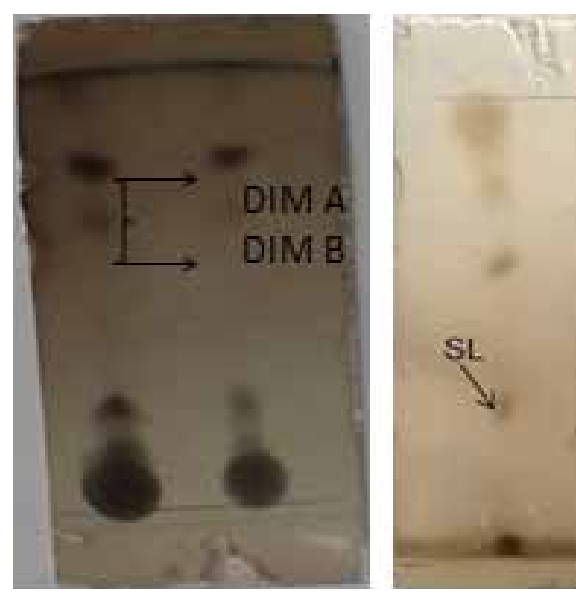

Figure 1 (a)1 Dimensional TLC of PDIM i.e. DIM A & DIM B (b) 1 Dimensional TLC of Sulfolipids of *Mycobacterium tuberculosis*

The spots developed for the PDIM and sulfolipids are clearly visible when developed with the modified protocol i.e. by using 20% H2SO4 in ethanol and charring by hot air gun at approximately 100° C for 5 minutes. This modified protocol helps in the visualization of two important Mycobacterial lipids i.e. PDIM and Sulfolipid by using non-radioactive methods. Lipid spots from our protocol were found to be sharper and had an increased resolution which clearly predicts better extraction and hence can be used as an alternative for the methods that involves the use of radioactive substance. Simplicity of the protocol of the PDIM and sulfolipuds extraction and its visualization makes

it possible to study the various aspects of lipid analysis for not only those laboratories that are lacking proper infrastructure for radioactive studies but also for those which want to shift their work from radioactive to non-radioactive methods. More studies are required to develop similar kind of non-radioactive methods for the visualization of other important mycobacterial lipids such as TDM.

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