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Research Article

Extraction, Purification and Identification of Phenolic Glycolipids in Various Mycobacterial Species

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Abstract

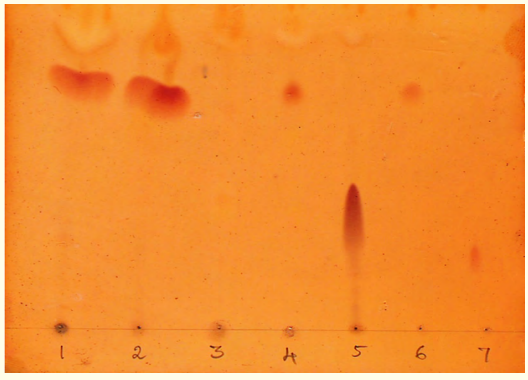
Background: Phenolic glycolipids (PGLs) were first discovered as cell-wall constituents of *M. bovis*, *M. bovis* BCG, *M. marinum* and *M. kansasii*. PGLs were also isolated from *M. leprae*; have been used as a species-specific serological marker and play a role in the mycobacterial pathogenicity. They are also found in *M. gastri*, *M. microti*, *M. africanum* and *M. tuberculosis* strain Canetti. *M. tuberculosis, M. bovis* and *M. marinum* did not accumulate the PGLs; possibly due to a loss of its ability to synthesize those lipids over an extended period of laboratory culture. However, a previously published study from our group has been reported which shows the presence of mycoside B in *M. bovis* AN5 type strain and other seventeen field strains obtained from IVRI, India. Besides looking for the presence of these lipids qualitatively, it is thought to be more useful to quantify these lipids by simplified analytical and preparative procedures to get a clear idea for the presence as well as the extent of distribution of these characteristic lipids.

Methods: We have identified PGLs in clinically important mycobacteria- *M. bovis*, *M. bovis* BCG, *M. microti* and *M. kansasii* by employing 2D-thin layer chromatography (TLC) and column chromatography.

Results: We have quantified the yield of PGLs which ranged from 0.9 to 1.7 mg/100 mg cells (dry weight) of *M. bovis*, *M. bovis* BCG, *M. microti* and *M. kansasii*. Purification of PGLs was also done by column chromatography.

Conclusion: We have first reported the PGLs extraction and purification by TLC and column chromatography methodology in *M. bovis*, *M. bovis* BCG, *M. microti*, and *M. kansasii* and suggested that it is conventional, fast and very simple method which can be particularly useful in PGLs analysis.

Graphical Abstract



Unidimensional TLC of purified PGLs from different mycobacteria.

Keywords: *Mycobacteria; Phenolic Glycolipid Lipid; Thin Layer Chromatography; Column Chromatography; Purification; Identification*

Abbreviations

PGL: Phenolic Glycolipids; TLC: Thin Layer Chromatography; ETZ: Electron Transparent Zone

Introduction

Mycobacterium generally contains novel lipid components which are not found in any other living organisms [1]. Mycobacterium cell envelopes are extremely rich in lipid moieties, approximately 30 - 60 percent have been recorded [2]. A discontinuous distribution of these unusual lipids has great potential for both classification and identification of mycobacterial species [3-5]. Most of these lipids are phthiocerol dimycocerosates (Wax esters; PDIMS), glycopeptidolipids or Mycoside-C [6], dimycocerosates of glycosylphenolphthiocerols (mycoside A,B,G,L etc) [6-8] trehalose dimycolate (Cord Factor), sulpholipids, phospholipids, covalently bound mycolic acids [9], fatty acid profiles [10,11], triacyl glycerols and isoprenoid quinones [6]. Phenolic glycolipids (PGLs) are essential for survival of the mycobacterial species in the harsh intracellular environment and their pathogenesis. The 'waxiness' of *mycobacteria* due to their high lipid content was recognized long ago [12] and probably plays a role in their resistance to chemical or enzymatic degradation when the *mycobacteria* are in the host tissue. The majority of isolates of *M. bovis* from tissues of infected animal yield a highly characteristic PGLs, the presence of which allows one to distinguish *M. bovis* from other *mycobacteria* [13]. The *Mycobacterium tuberculosis* complex contains the additional species-*Mycobacterium africanum, Mycobacterium bovis* and *Mycobacterium microti*, all of which share a high degree of genetic relatedness [14]. *M. bovis* can also be distinguished from *M. tuberculosis* by presence of PGL. The finding that single representatives of *M. africanum* and *M. microti* also produce the same PGL indicates a closer affinity of these species to *M. bovis* than to *M. tuberculosis*. There are few reports which showed that PGLs are absent in type of *M. bovis* strains [7] and *M. marinum* [15]. It is possible that these strains have lost the ability to synthesize PGLs over an extended period of laboratory culture. Contrary to the report that PGL is absent in M. bovis MNC433 strain (AN5) [16]; have shown the presence of phenolic glycolipids in *M. bovis* AN5 strain and 17 other field strains from IVRI, India. In the case of lepromatous leprosy, PGLs are involved in the stimulation of the suppressor T-cells [17] Our knowledge on the role of cell wall components in pathogenesis is still inadequate in spite of great efforts over many years, although twin questions-whether the mycosides (both C-mycosides of *M. avium* group and PGLs) might play a primary role in pathogenesis. In depth study of the PGLs metabolism may help in evaluating the precise function of these lipids in their interaction with the living environment. Moreover, further knowledge of the PGLs may lead to potential diagnostics and drug targets.

Materials and Methods

Culture

M. bovis BCG (Danish) and *M. microti* strains were obtained from the Mycobacterial Repository Centre, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra. *M. kansasii* (T3) was obtained from National Institute of Research in Tuberculosis (NIRT), Chennai. *M. bovis* (AN5) was obtained from IVRI, Izatnagar. Mycobacterial strains were cultured in Sauton's medium at 37°C till log phase growth. *M. microti* was grown on modified Sauton's medium [18]. After four weeks culture was harvested, autoclaved and dried at 45°C for 18 - 20 hrs used for lipid analysis.

Extraction of PGLs

The extraction method employed for PGL was according to published protocol [19]. Usually organisms were treated with biphasic mixtures of methanolic saline and petroleum ether (b.p.60 - 80°C) followed by extraction of cell debris and residual aqueous phase with monophasic chloroform-methanol-saline according to well-proven method of Bligh and Dyer [5] with slight modifications [20,21]. The petroleum ether extract from first stage is composed of readily extractable non-polar lipids and addition of chloroform and saline (0.3%

w/v aqueous sodium chloride) to second monophasic extract gives the remaining more polar lipids from small amounts of mycobacterial cells.

In brief, 100 mg each of *M. bovis* BCG, *M. bovis* (AN5), *M. kansasii* and *M. microti* in 8.5 ml test tube with PTFE (Poly Tetra Fluoro Ethylene)-lined cap was added 2 ml of aqueous methanol (100 ml of methanol added to 10 ml of 0.3% saline). Free non - polar lipids were extracted by partitioning between aqueous phase and 2 ml of petroleum ether (bp 60˚ - 80˚C) for 15 minutes. The mixture was centrifuged for 5 min at 2000 rpm at room temperature. The upper petroleum ether layer were separated and stored in small screw capped vial. The lower layer was re-extracted with a further aliquot of 2 ml petroleum ether for 15 min. The combined petroleum ether extracts was evaporated to dryness under a stream of nitrogen at 37˚C and examined for PGLs.

TLC analysis of PGLs

The non-polar lipids extracted with petroleum ether was dissolved in a small volume of chloroform and spotted on the corner of a 5 x 5 cm silica gel G, F254 - precoated aluminium plates and developed with chloroform-methanol (96 : 4,v/v) once in the first direction followed by toluene-acetone (80 : 20, v/v) thrice in the second direction [5]. The plates were sprayed with α-napthol-sulphuric acid reagent and heated at 120˚C for 10 min. The presence of PGL was confirmed by comparing the mobility of authentic mycoside B obtained from Dr. Delphi Chatterjee, USA.

Purification of PGL by Column Chromatography:

The non-polar lipid of *M. bovis* (AN5), *M. bovis* BCG, *M. microti* and *M. kansasii* extracted with petroleum ether was dissolved in 0.5 ml of chloroform and processed by florisil column chromatography [22]. A chromatographic column of 1 cm x 10 cm was packed with florisil upto 8 cm and washed with about 15 ml of chloroform. The reconstituted non-polar lipid fraction was applied on to the top of the column and sequentially with 10 ml aliquotes of hexane:chloroform (1 : 1,v/v), pure chloroform, and 1%, 2% and 5% methanol in chloroform in that order. The eluates were collected in 15 ml glass vials and dried under nitrogen at 37˚C. The weights of the fractions were determined by using preweighed vials.

The dried eluates (five in number) of respective mycobacterium were applied as spots on a 8 x 4 cm,Silica gel F254 percoated aluminium strips and developed respectively once with chloroform : methanol (96 : 4 v/v) and the presence of PGL was detected using α-naphthol-sulphuric acid reagent as described above.

The lipid fractions of each mycobacterium showing positivity of PGL were pooled, reconstituted in a small volume of chloroform, streaked on plastic backed silica gel precoated TLC strips (10 x 8 cms) and developed unidimensionally once with chloroform : methanol (96 : 4 v/v). The PGL was located by staining the small cut portions of the plate in chloroform and dried under nitrogen at 37˚C. The final purified product was rechecked for the purity of PGL by analytical TLC.

Quantitation of PGLs

The PGL content of the lipid material obtained after column chromatography and purification by TLC was determined by chemical assay of rhamnose content of the PGLs sample. In brief, an aliquot (0.2 ml) of the PGL sample reconstituted in chloroform-methanol (2 : 1,v/v) was hydrolysed at 105˚C for 5h with 0.5 ml of 1M sulphuric acid. The hydrolysates were extracted with hexane to remove the liberated fatty acids. The released deoxy sugars in acid phase were estimated by the method of Dische [23].

Results

Analysis and Identification of Phenolic Glycolipids (PGL)

The presence of PGL was noticed in the petroleum ether extract from *M. bovis* (AN5), *M. bovis* BCG, *M. kansasii* and *M. microti*. PGL was identified by the colored spots in the chromatograms sprayed with α-naphthol reagent and charred at 120˚C for 10 minutes. The different

mycobacterial species gave different colored spots with varying Rf values. A brown coloured spot was developed in *M. bovis* (AN5) and *M. bovis* BCG chromatograms. R_f value of PGL in M. bovis (AN5) was 0.69 in chloroform: methanol (96 : 4 v/v x1); and 0.4 in toluene : acetone (80 : 20, v/v x 3) solvents (Figure 1A). R_f value of PGL in *M. bovis* BCG was 0.37 in the first direction and 0.26 in the second direction (Figure 1B). *M. microtii* derived PGL gives a similar color to that of M. bovis and changes to reddish orange at room temperature. The R_f value of mycoside B of *M. microtii* were 0.38 and 0.40 respectively (Figure 1C). *M. kansasii* initially develops a light brown color and finally on leaving at room temperature for a period of 16 h changes to a more distinctive brown colour. The R_f value of PGL (mycoside A) were 0.63 and 0.30 respectively (Fig.1D). The presence of PGL was confirmed by cochromatographing authentic mycoside B and PGL - I samples.

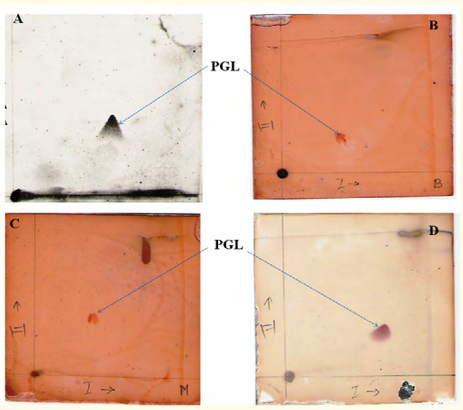


Figure 1: *Two dimensional TLC of most polar class of lipids in non-polar lipid fractions of various Mycobacteria.*
(A) M. bovis AN5 (B) M. bovis BCG (C) M. microti (D) M. kansasii

Unidimensional TLC of Different Eluates of Florisil Column Chromatography

The different eluates of florosil column chromatography of petroleum ether extracted non polar lipid fraction from *M. bovis* (AN5), *M. bovis* BCG, *M. kansasii* and *M. microti* were examined for the presence of PGL by unidimensional TLC with single development with chloroform: methanol (96:4, v/v). The eluates with 1% and 2% methanol in chloroform contained most of the major PGL (mycosideB), 5% methanol in chloroform eluate contained less significant amounts of mycoside B ; while they also contained more polar minor variants of mycoside B (Figure 2A). *M. bovis* BCG contained PGL in 1% and 2% methanol in chloroform (Figure 2B). *M. microti* contained PGL in eluate of 1% methanol in chloroform; 2% and 5% methanol in chloroform eluate contained very less amount of PGL (Figure 2C). Similarly the petroleum ether extract from *M. kansasii* contained PGL in eluate of 2% methanol in chloroform; 1% and 5% methanol in chloroform eluate contained very less amount of PGL (Figure 2D).

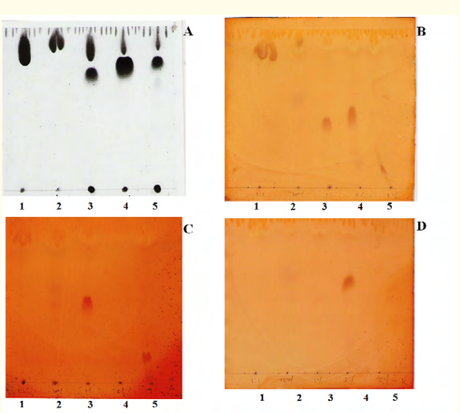


Figure 2: *Single dimensional TLC of lipids of different eluates from florisil column chromatography in different mycobacteria.*
(A) M. bovis AN5 (B) M. bovis BCG (C) M. microti (D) M. kansasii
Lanes: 1. n-Hexane : chloroform (1 : 1 v/v).
2. 100% chloroform.
3. 1%methanol in chloroform.
4. 2% methanol in chloroform.
5 5% methanol in chloroform.

Unidimensional TLC of mycobacterial PGL

The PGL eluates obtained by florisil column chromatography of petroleum ether extract from *M. bovis* (AN5), *M. bovis* BCG, *M. kansasii* and *M. microti* were examined by unidimensional TLC with single development with chloroform: methanol (96 : 4 v/v). R_f value of PGL found in *M. bovis* (AN5) was 0.65, *M. bovis* BCG was 0.67, *M. kansasii* was 0.58 and *M. microti* was 0.60 (Figure 3).

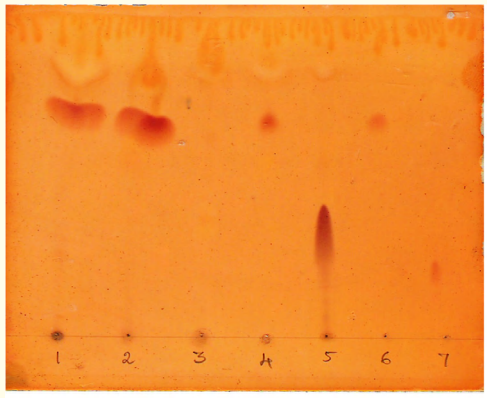


Figure 3: *Single dimensional TLC of purified Phenolicglycolipids from different mycobacteria.*
Lanes: 1. M. bovis AN5 2. M. bovis BCG 3. M. kansasii. 4. M. microti.
5. PGL-1 (Reference). 6. MycosideB (Reference) 7. Deacylated Mycoside B

Quantification of phenolic glycolipid content of mycobacteria

The phenolic glycolipid content of the lipid material obtained after column chromatography and purification by TLC was determined by gravimetry as well as chemical assay of rhamnose content of the glycolipid sample (Table 1). There was not much difference between the values obtained by gravimetry and chemical assay. As much as 1.70 mg of phenolic glycolipid were obtained from 100 mg of *M. kansasii* by dry weight while the content was at a lower side in *M. microti* (0.9 mg/100 mg dry weight). 1.3 to 1.58 mg was recovered from 100 mg dry organisms of *M. bovis*.

Strains	Gravimetric	Chemical Assay
M. kansasii	1.70	1.64
M. bovis AN5	1.58	1.60
M. bovis BCG (Danish)	1.30	1.32
M. microti	0.90	0.92

Table 1: *Quantification of PGLs in Mycobacterial staining (mg/100 mg dry wt).*

Discussion

Lipid profiles of *mycobacteria* have been increasingly used in the chemotaxonomy of *mycobacteria* [12]. PGL is present in a few mycobacterial species like *M. tuberculosis Canetti, M. bovis, M. marinum, M. microti, M. kansasii and M. leprae* [6,16]. PGLs (Dimycocerosates of

glycosyl phenolphthiocerols) were discovered as cell-wall constituents of *M. bovis, M. bovis* BCG, *M. marinum and M. kansasii*. PGLs were also isolated from *M. leprae* which is species-specific serological marker and are involved in the stimulation of the suppressor T-cells, in case of lepromatous leprosy. The functional activities of these PGLs over the immune cells stimulation emphasized the role played by these molecules in the mycobacterial pathogenicity.

The quantification of cell wall lipids at various phases of mycobacterial growth will help in assessing the importance of these lipids in the cell wall of the organisms. In this study we have extracted and purified the significant amounts of PGLs. Yield of PGLs ranged from 0.9 to 1.7 mg/100 mg cells (dry weight) of *M. bovis, M. bovis* BCG, *M. microti and M. kansasii*. Daffe and Cho, (1991) have reported similar yields of triglycosyl PGL in *M. tuberculosis* strain *Canetti* [24]. Watanbe., *et al*. (1997) while working on structures of PGLs from *M. kansasii* have recovered PGLs to the tune of 0.6% by dry weight of the cells [25]. Hunter and Brennan (1981) working on PGLs from *M. leprae* found PGL-I to the content of 0.3 - 0.4% by dry weight [17]. Venkatesan., *et al*. (1988) reported the PGL content is directly proportional to number of bacilli growth [22]. Effective chemotherapy has been observed to lower the concentration of PGL-I in infected tissues and blood [26], suggesting that synthesis of PGL-I might be a quantitative indicator of the overall viability of the leprosy bacillus. Ramsesh., *et al*. (1987) have suggested that PGL-I can be used to quantitate the metabolism of *M. leprae* in macrophages *in vitro* [27]. Inhibition of PGL-I synthesis does not necessarily mean the killing of bacilli as these are only free extractable lipids. Selectively inhibiting synthesis of the PGLs by blocking the biosynthetic assembly at various points should be a convenient tool for testing their importance in pathogenicity as almost all the species that synthesize PGLs are more or less pathogenic [28]. However, Rastogi and David (1988), have reported evidences to prove that mycosides may not play a primary role in pathogenicity and that the lipid substances responsible for electron transparent zone (ETZ) formation around the bacilli in resident macrophages and phagosome-lysosome fusion inhibition are not identical [29]. Here we hypothesized that cumulative effect of PGLs and other lipids might be involved in pathogenicity of these mycobacterial species. This aspect needs further study as the intermediates in mycoside synthesis might also contribute to early events in ETZ formation.

Quantifying these lipids in clinical samples from patients suffering from mycobacterial infections, at various points of antimicrobial therapy will be useful in assessing the chemotherapeutic efficacy of the antimicrobials and the clinical activity. For column chromatography various substances have been used, the most common being silicic acid-celite (2 : 1 w/w) and florisil only [22,30]. The addition of celite ensures rapid elution. Miniature columns have been employed to elute very small amount of PGL using small aliquots of eluting solvents. The mycobacterial cell envelopes are abundant in lipids amounting to nearly 40% [2], of dry weight of the organisms. The non-polar lipids in the petroleum ether extract in the study comprises of about 25 - 30% of total lipids. Of these the neutral lipids like triacyl glycerol usually eluting with hexane-chloroform (1 : 1 v/v) and chloroform are the major constituents in the non-polar lipid fractions. In this study we have reported the PGLs extraction and purification by TLC and column chromatography methodology in *M. bovis, M. bovis* BCG, *M. microti*, and *M. kansasii* and suggested that it is conventional, fast and very simple method which can be particularly useful in PGLs microanalysis and radioactive studies.

Conclusion

Mycobacterial cell wall consists nearly of 40% of lipids by dry weight and has been used in the chemotaxonomy and pathogenesis. PGLs were discovered as cell-wall constituents of *M. bovis*, *M. bovis* BCG, *M. marinum* and *M. kansasii*. PGL isolated from *M. leprae*, is species-specific serological marker and are involved in the stimulation of the suppressor T-cells, in case of lepromatous leprosy. PGL might play a role in the mycobacterial pathogenicity. In this study we have extracted, quantified and purified the PGLs by TLC and column chromatography which could help in assessing the importance of these lipids in the cell wall of *M. bovis*, *M. bovis* BCG, *M. microti* and *M. kansasii* and suggested that it is conventional, fast and very simple method which can be particularly useful in PGLs analysis.

Conflict of Interest

There is no conflict of interest among the authors.

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