

ORIGINAL ARTICLE

GCMS Based Detection of Lipid Biomarkers of *Mycobacterium tuberculosis* in the Serum Specimen

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Received: March 5, 2016

Revised: May 20, 2016

Accepted: May 27, 2016

Published online: June 18, 2016

ABSTRACT

AIM: The present study is focused on the identification of high abundant and low abundant biomarkers of *Mycobacterium tuberculosis* from serum specimen using Gas chromatography and Mass spectroscopy.

METHODS: The TB positive and negative sera were screened on the basis of sputum smear microscopy and the in house developed liposome based antibody detection kit. The lipid fraction was isolated from the collected sera and derivatized. In GCMS analysis, the derivatized lipid samples were analyzed through Gas Chromatograph Mass spectrometer (Shimadzu QP-2010 plus with Thermal Desorption system TD 20). Split or splitless mode of sample injection was performed to identify the high abundant and low abundant biomarkers of tuberculosis.

RESULTS: The study identified lipid biomarkers specific for *M. tuberculosis* in the serum of tuberculosis positive subjects. The study

revealed that lipids like C₁₇H₃₄, C₂₁H₅₂O₆, C₂₉H₆₀, C₃₄H₇₀, C₄₄H₉₀ was identified in the split mode of sample injection, whereas C₂₉H₆₀, C₃₄H₇₀, C₄₄H₉₀, C₁₄H₂₃BrO, C₁₁H₂₄O₂, C₁₈H₄₄O₅, C₁₄H₃₀O₃S and C₂₄H₃₉N were identified in the splitless mode of sample injection. Under split injection mode of analysis, C₁₇H₃₄, C₂₁H₅₂O₆ and C₃₄H₇₀ lipids were identified as low abundant lipids. The molecules like C₁₄H₂₃BrO, C₁₁H₂₄O₂, C₁₈H₄₄O₅ and C₂₂H₃₉N were identified as low abundant lipids even after the splitless mode of analysis.

DISCUSSIONS: The GCMS analysis revealed the presence of lipid biomarkers of *Mycobacterium tuberculosis* in the circulation of selected tb positive sera samples. The study further identified the low abundant and high abundant biomarkers of tuberculosis. The detection of characterized low abundant biomarkers may help in identifying the disease in sputum smear negative cases.

Key words: Lipid biomarker; GCMS detection; VOC (volatile organic compound); MTB (*Mycobacterium tuberculosis*)

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Joseph AZ, Jain A, Pachauri M, Kumar A, Prasad GBKS, Bisen PS. GCMS Based Detection of Lipid Biomarkers of *Mycobacterium tuberculosis* in the Serum Specimen. *Journal of Respiratory Research* 2016; 2(2): 47-55 Available from: URL: <http://www.ghrnet.org/index.php/jrr/article/view/1630>

INTRODUCTION

Mycobacterium tuberculosis is the causative pathogen of tuberculosis (TB) in more than 90% of the infected persons. These bacteria remain in a dormant state in granulomas for life with the ability to persist in host tissue referred to as latency, is central to the disease. The *Mycobacterium tuberculosis* possess cell wall lipid molecules which are responsible for the pathogenesis, virulence and invasiveness. Gas chromatography is being used for the analysis of cellular fatty acids and alcohols of *Mycobacterium* species. It is already known that isolates of *Mycobacterium* complex had a relatively high concentration of hexacosanoic acid (1-13%) and low level of tetracosanoic acid

(0.1-3%)^[1]. Enzymes are being used in the sample to enhance the microbial growth and generate higher volatiles in the head space of analysis tube. The fingerprint analysis of the volatile lipids in the enzyme enriched samples is normally used for the confirmation of diagnosis of tuberculosis. More than 100 volatile biomarkers of tuberculosis were detected in the head space in *Mycobacterium avium* subsp Paratuberculosis with variation in the release in different mycobacterium species^[2]. GC-MS based method was used to identify the VOC (volatile organic compound pattern) linked with the disease conditions. Nano Artificial NOSE (NA-NOSE) was developed to detect *M. bovis* infection in cattle based on the volatile lipid pattern^[3-4]. The other commonly used methods for the detection of *Mycobacterium* sp using VOC are gas chromatography with mass spectrometry (GCMS), proton transfer reaction mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS), laser spectrometry, ion mobility spectrometry (IMS) or differential ion mobility spectrometry (DMS)^[5]. The highly stable hydrophobic waxes of cell wall components of *Mycobacterium* like Phthiocerol dimycocerosates (PDIMs) are also being explored for diagnostic purpose^[6]. Several analogues of major PDIMs such as C34 and C36 viz. phthiocerols, phthiodiolone, phthiocerolone, phthiotriol, mycolic acid and ester of phthiocerol form mycocerosate have been identified^[7-8]. The physical property of mycocerosic acid is analogous to the 2, 4, 6, trimethyl nonacosonate. Thermochemolysis GC-EI/MS have been employed for analyzing mycocerosic acid components of the phthiocerol dimycocerosate (PDIM) family of lipids to study for diagnosis of tuberculosis in sputum^[9]. Although several volatile lipid moieties specific for mycobacterium have been identified in the culture, and sputum of the infected subject. However, the present study is unique by itself in identifying high and low abundant lipid biomarkers of tuberculosis in the circulation of the tuberculosis infected subjects.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Research Ethical Committee at the Gajra Raja Medical College in Gwalior, M.P. India. Written consent was obtained from all the studied patients for sample collection and subsequent analysis.

Sample collection

The sera samples were collected from tuberculosis patients ($n = 10$ samples) at a minimal volume of 0.5 mL from the primary health centre of Madhya Pradesh region of India. The patients were clinically diagnosed by sputum smear and culture with pulmonary tuberculosis. None of the patients had taken antibiotics before sampling. The sera samples of healthy cases were also collected from the same region ($n = 10$). For spiking experiments, sera specimens were obtained from patients with pulmonary infection (but free of TB), who were treated at Gajra Raja Medical College, Gwalior.

Spiking of negative samples

The spiking of negative sample with MTB is performed as per method described earlier^[10] with minor modifications having final concentration of 1×10^6 mycobacteria/mL sera.

Extraction of lipids from serum

Briefly, 1 mL of normal human serum was added to 10 mL of chloroform-methanol 2:1 (v/v). The mixture was agitated in a orbital shaker at 70 rpm for 12 hours. The organic extract was filtered through the 0.2 μ syringe filter and the lipid extract was evaporated to dryness. The lipids were derivatized for GCMS analysis.

Culturing of *M. tuberculosis*

M. tuberculosis strain H37Rv (ATCC27294) was cultured in a Lowenstein-Jensen agar culture medium (2×10 CFU/mL)^[11]. The cells were then harvested by centrifugation ($10,000 \text{ g} \times 20 \text{ min} \times 4^\circ\text{C}$). The cell pellet was isolated and washed with 100 mL of PBS at pH 7.2 followed by resuspending it in TNF buffer. The *M. tuberculosis* cells were heat inactivated and lyophilized.

Extraction and isolation of lipid antigen(s) from *M. tuberculosis*

Heat inactivated mycobacterial cell (5 g) was placed into a glass reagent bottle, and 100 mL of organic solvent of chloroform-methanol mixture (2:1) was added to it. This mixture was stirred at a temperature of 25°C for 60 minutes and filtered through Whatman number 1 filter paper. The organic phase was dried by evaporating the solvent in a rotary solvent evaporator at 45°C . Neutral lipids were removed from the dried mixture by adding 50 mL of chilled acetone while the mixture was vortexed for 10 min and then filtered through Whatman no. 1 filter paper. This step was repeated 3 times. The contents of the flask were filtered through Whatman filter paper no. 1, and the filtrate was discarded. The lipids present on the filter paper were dissolved with chloroform-methanol (2:1) and transferred to a round-bottom flask. The solvent was evaporated on a rotary evaporator under reduced pressure at 45°C . The crude preparation of lipid mixture was reconstituted in 10 mL of chloroform-methanol (2:1) and stored at a temperature of -20°C for further use^[11].

Derivatization

The lipid samples were mixed with 20 μL of -obis (trimethylsilyl) -trifluoroacetamide (BSTFA) (Fluka). The samples are capped, wrapped with Teflon tape, and heated at 45°C for 12 hours to convert the targeted analytes to their trimethylsilyl derivatives. The samples were further reconstituted in hexane and GCMS profiling was performed.

GCMS method

The lipid samples were analyzed through Gas Chromatograph Mass spectrometer (Shimadzu QP-2010 plus with Thermal Desorption system TD 20). The column used was Rtx-5MS, cross bonded with 5% diphenyl and 95% dimethyl polysiloxane (30 m, 0.25 mm ID, 0.25 μm) and had minimal bleed even at a temperature range of 350°C - 33°C . The column was conditioned with a temperature 150°C and injection temperature was optimized at 260°C . The carrier gas Helium was purged at a flow rate of 16.3 mL/min and 1.21 mL/min flow was maintained in the column was conditioned in the column. The column oven was programmed at 150°C (hold time, 5 min) at rate of $15^\circ\text{C}/\text{min}$ to 310°C (hold time 8 min). The sample injection was programmed at either split/splitless mode. The GC run time was optimized to 25 min. The analytes were analyzed through FTD detectors. The mass spectrum of the molecules was analyzed through QP 2010 plus programmed for data acquisition from 5-23.6 min. The ion source temperature was equilibrated to 230°C and the interface temperature was maintained at 280°C . The data acquisition was set a speed of 1,250 with a rise time of 0.5 sec. The mass fragmentation acquisition was set at a range of m/z 40– m/z 650. The mass spectrometric results were recorded.

Data mining and logical analysis

The mass spectrometry data were matched with the NIST Database, Wiley and the relative hits according to the retention time and mass fragmentation pattern was recorded. The data were compared with the lipid molecules of *Mycobacterium tuberculosis* cell lipids and further compared with the sera lipids of the healthy individuals. The molecules which were very specific to tuberculosis were used as biomarker.

RESULTS

The biomarkers of tuberculosis were identified by in the sera samples of tuberculosis confirmed cases by GC-MS. Sera samples were extracted with organic solvents, lipid fraction was subjected to GCMS analysis and molecules were identified through mass spectrometric databases and respective controls like sera of healthy individuals, *M. tuberculosis* cell lipids. The biomarkers specific for the tuberculosis infection were identified.

Identification of potential biomarkers of tuberculosis

The initial study identified and characterized the lipids of mycobacterium origin in the sera of the suspected individuals. The injection volume of the sample was only 1 μ L. The sample was analyzed in the split mode of 1/10 ratio or split less mode. The mass spectrum of the individual lipid molecule separated in the Gas chromatograph and the mass spectrum of the individual molecule was determined. The molecules were identified using mass spectrometric databases.

The split mode of injection in GCMS analysis lead to the detection of $C_{17}H_{34}$ lipids in TB positive sera, however, this lipid was not detected in the *Mycobacterium tuberculosis* cell wall extracts (Figure 1a, 1b) and Table 1 and 2. Another biomarker with a molecular formula $C_{21}H_{32}O_6$ was detected in the sera of the TB positive subjects. This molecule had a similar mass fragmentation pattern of hexopyranose. The *Mycobacterium tuberculosis* cell wall extract also showed the presence of a molecule with hexopyranoside unit in it (Figure 2a-d and Tables 1, 2). The sera of the TB positive cases showed the presence of higher alkane, $C_{34}H_{70}$. This molecule had a similar mass fragmentation pattern of tetratriacontane lipids. These lipids are also found in the cell wall extract of *M. tuberculosis*. The higher alkanes are found to be the building blocks of Phthiocerol lipids (Figure 3a-3d) and Table 1. The other higher alkanes like $C_{29}H_{60}$ and $C_{44}H_{90}$ were also identified in the sera of the TB infected cases. These molecules serve as building blocks of phthiocerol lipids (Figure 4a-4d) and Table 1, 2; (Figure 5a- 5d) and Table 1, 2. To the best of our knowledge this is the first report of above mentioned biomarkers from tuberculosis sera hence considered as novel in nature. Another lipid $C_{22}H_{39}N$ was detected in the sera of TB infected cases (Figure. 6a-d). This molecule had a close similarity with hexadecylaniline.

The splitless mode of injection in GCMS showed the presence of $C_{18}H_{44}O_5$ (Figure 7a, b). This molecule is similar to the mannopyranose, however, the molecule was not detected in the lipid extract of *Mycobacterium tuberculosis* under the identical GCMS conditions. In splitless mode, biomarkers $C_{14}H_{23}BrO$ (figure not shown), $C_{11}H_{24}O_2$ (figure not shown) and $C_{14}H_{30}O_3S$ (figure not shown) were detected. In splitless mode of injection, molecules like $C_{18}H_{44}O_5$, $C_{13}H_{20}$, $C_{22}H_{39}N$ were identified. To the best of our knowledge this is the first report of these biomarkers in the sera of tuberculosis infected cases and the method is novel in isolating and identifying the low abundant and high abundant lipid biomarkers.

Identification of high abundant and low abundant biomarkers of tuberculosis

The high abundant and low abundant biomarkers of *Mycobacterium tuberculosis* were identified from the sample injected and analysis was performed under split and splitless mode. Under split mode of analysis the high abundant lipids like $C_{29}H_{60}$ and $C_{44}H_{90}$ lipids were identified at a level of 50.02% and 29.15%, respectively whereas low abundant molecules like $C_{21}H_{32}O_6$, $C_{17}H_{34}$, $C_{34}H_{70}$ and $C_{21}H_{44}$ were identified in the sera of the infected at a level of 0.76%, 1.46%,

2.98 and 4.74% of total lipid content (Table 3, 4). Under splitless mode injection, the molecules like $C_{13}H_{20}$, $C_{12}H_{16}O$, $C_{18}H_{44}O_5$, $C_{22}H_{46}$, $C_{19}H_{29}ClO$, $C_{24}H_{50}$, $C_{22}H_{39}N$, $C_{14}H_{23}BrO$, $C_{11}H_{24}O_2$ and $C_{14}H_{30}S$ were found in the sera of the infected at a level of 0.2%, 0.57%, 0.69%, 1.87%, 2.54%, 5.22%, 4.6%, 1.85%, 1.07% and 1.1%, respectively (Table 3 and Table 5). We identified the lipid biomarkers of *Mycobacterial tuberculosis* and further characterized its distribution in the circulation in the selected tb positive sera sample.

Table 1 Mass spectrometric characteristics of Lipid Biomarkers of *Tuberculosis*

S.No	Molecular formula	Molecular weight	Database Hit	Mass spectrum figures
1	$C_{17}H_{34}$	238	1-Heptadecene	1a,b
2	$C_{21}H_{32}O_6$	540	Hexopyranose	2a-d
3	$C_{34}H_{70}$	478	n-Tetratriacontane	3a-d
4	$C_{29}H_{60}$	408	n-Nonacosane	4a-d
5	$C_{34}H_{90}$	618	Tetratetracontane	5a-d
6	$C_{22}H_{39}N$	317	4-Hexadecylaniline	6a-d
7	$C_{18}H_{44}O_5$	452	Mannopyranose	7a,b

Table 2 Lipid Biomarkers of tuberculosis in Biological specimens.

Molecule	Positive sera	<i>M. tuberculosis</i> cell extract	Negative sera
$C_{17}H_{34}$	+	-	-
$C_{21}H_{32}O_6$	+	-	-
$C_{34}H_{70}$	+	+	-
$C_{29}H_{60}$	+	+	-
$C_{34}H_{90}$	+	+	-
$C_{22}H_{39}N$	+	+	-
$C_{18}H_{44}O_5$	+	-	-

Table 3 GCMS detection of Novel Biomarkers of tuberculosis under Split and Splitless mode of sample Injection.

Lipids identified	Split mode	Splitless mode of injections
$C_{17}H_{34}$	+	
$C_{21}H_{32}O_6$	+	
$C_{29}H_{60}$	+	+
$C_{34}H_{70}$	+	+
$C_{44}H_{90}$	+	+
$C_{14}H_{23}BrO$		+
$C_{11}H_{24}O_2$		+
$C_{18}H_{44}O_5$		+
$C_{14}H_{30}O_3S$		+
$C_{24}H_{39}N$		+

Table 4 GCMS detection of High abundant and low abundant novel lipid biomarkers in split injection mode.

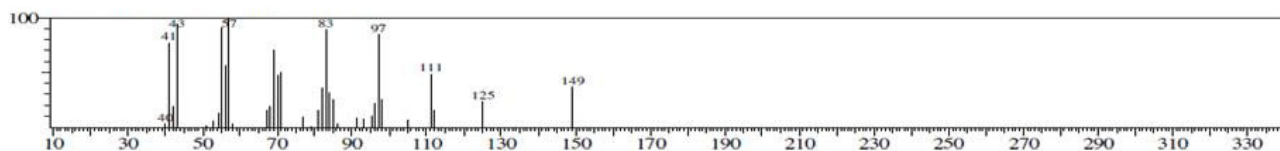
Lipids identified	High abundance lipids	Low abundance lipids
$C_{17}H_{34}$	-	1.46%
$C_{21}H_{32}O_6$	-	0.76%
$C_{29}H_{60}$	54.76%	-
$C_{34}H_{70}$	-	2.98%
$C_{44}H_{90}$	29.15%	-

The lipids constituting less than 5% of the total lipid were included in low abundance lipids and the lipids constituting above 5% of the total lipids were high abundance lipids.

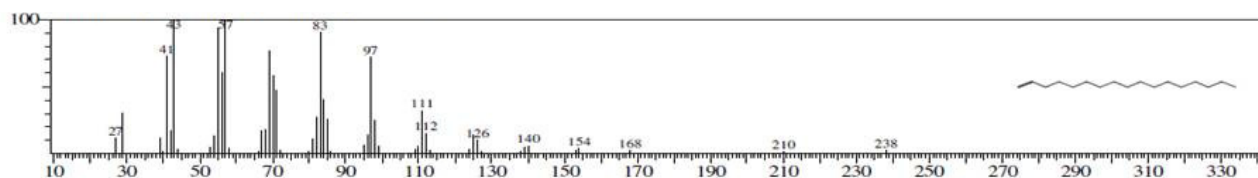
Table 5 GCMS detection of High abundant and low abundant novel lipid biomarkers in splitless injection mode.

Lipids identified	High abundance lipids	Low abundance lipids
$C_{29}H_{60}$	45.14%	-
$C_{34}H_{70}$	11.22%	-
$C_{44}H_{90}$	7.12%	-
$C_{14}H_{23}BrO$	-	1.85%
$C_{11}H_{24}O_2$	-	1.07%
$C_{18}H_{44}O_5$	-	0.69%
$C_{14}H_{30}O_3S$	-	1.11%
$C_{22}H_{39}N$	-	4.66%

The lipids constituting less than 5% of the total lipid were included in low abundance lipids and the lipids constituting above 5% of the total lipids were high abundance lipids.

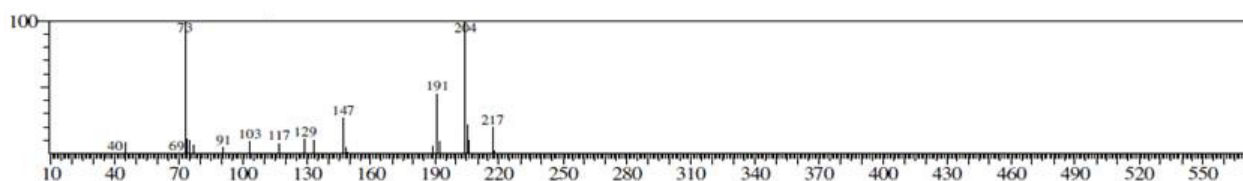


1a

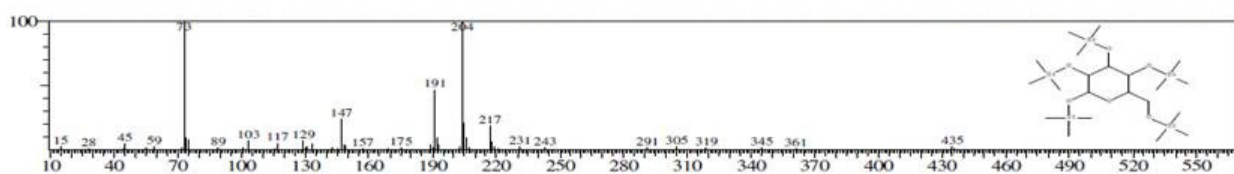


1b

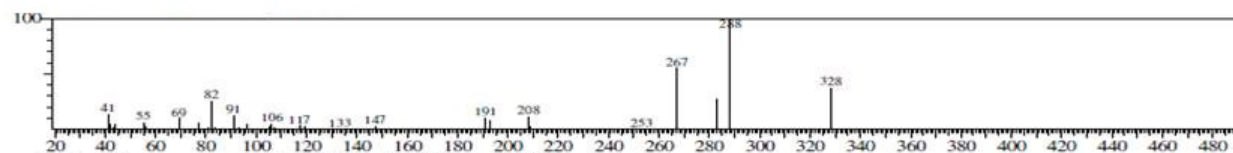
Figure 1a and 1b. Mass spectral similarity of lipid molecule M1 with Mass spectral library NIST08. The molecule had a retention of 10.183min and had 35 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C₁₇H₃₄. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 238.



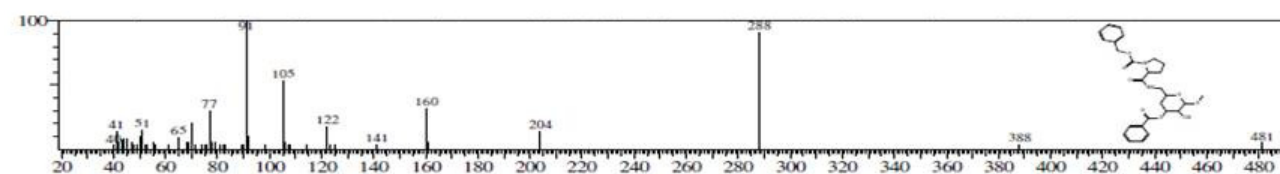
2a



2b



2c



2d

Figure 2a and 2b. Mass spectral similarity of lipid molecule M2 with Mass spectral library Wiley 8. The molecule had retention of 10.275min and had 28 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C₂₁H₅₂O₆. The spectrum showed a base peak at m/z 204 and had a molecular weight of 540.

Figure 2c and 2d. Mass spectral similarity of lipid molecule M2 with Mass spectral library Nist08. The molecule had a retention of 15.4 min and had 33 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C₂₇H₃₂N₂O₇. The spectrum showed a basepeak at m/z 288 and had a molecular weight of 528.

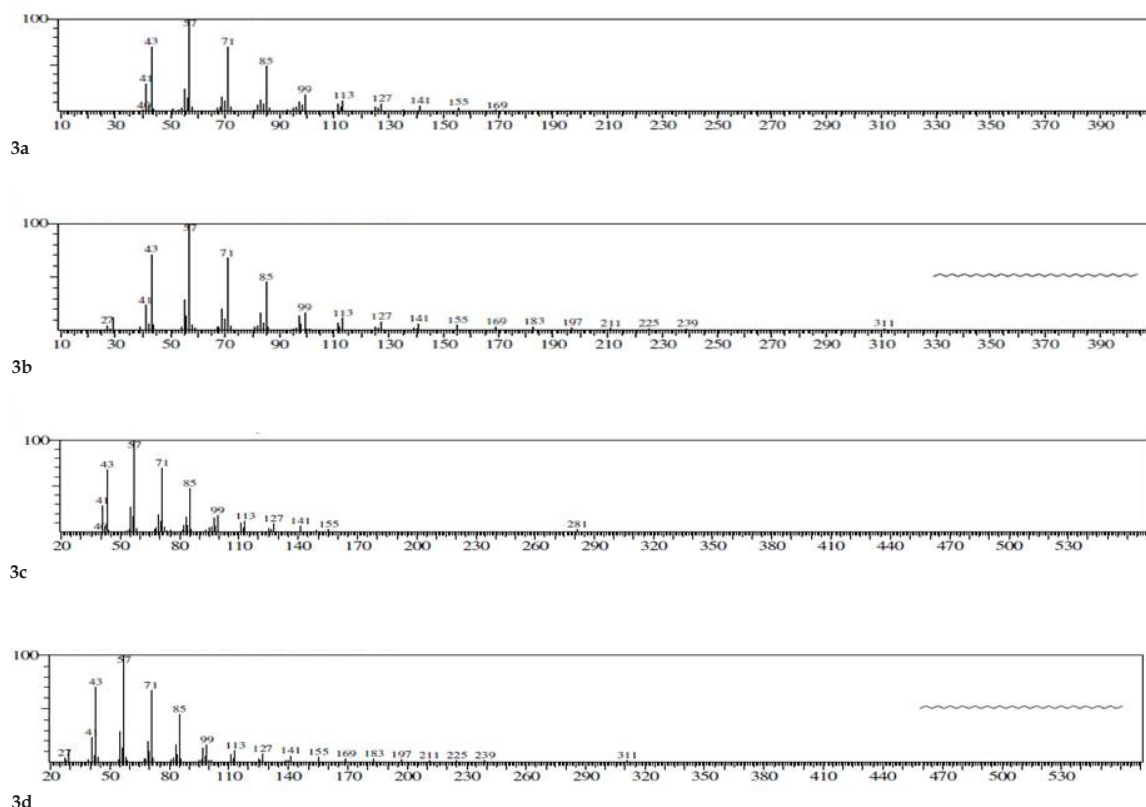


Figure 3a and 3b. Mass spectral similarity of lipid molecule M3 with Mass spectral library Nist08. The molecule had retention of 11.57min and had 44 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C₃₄H₇₀. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 478.

Figure 3c and 3d Mass spectral similarity of lipid molecule M3 with Mass spectral library Nist08. The molecule had retention of 12.8 min and had 45 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C₃₄H₇₀. The spectrum showed a basepeak at

DISCUSSION

Tuberculosis (TB) remains one of the world's deadliest communicable diseases with 9.6 million people fell ill and 1.5 million died in 2014 from the disease^[12].

In 2013, about 64% of the estimated 9 million people who developed TB were notified as newly diagnosed cases. This is estimated to have left about 3 million cases that were either not diagnosed, or diagnosed, but not reported to national TB programmes (NTPs)^[12]. The present investigation relates to the identification of few lipid molecules of *Mycobacterium* sp. origin in the sera specimen of the tuberculosis infected cases. The metabolomics based approach was used to characterize and identify the biomarkers from sera specimen.

Earlier studies revealed that Gas chromatography coupled with mass spectrometry (GC/MS) could offer a more rapid test for the diagnosis of TB by detecting lipid markers of *Mycobacterium tuberculosis* in biological matrices like sputum, bone, and tissue. The present study showed the presence of long carbon chain molecules in the sera of infected subjects, however, none of these molecules have been reported in the sera or any other tissue specimen. Tuberculostearic acid (TBSA) lipid was widely studied as biomarker from culture and sputum specimens and other members of actinomycetes^[13-20].

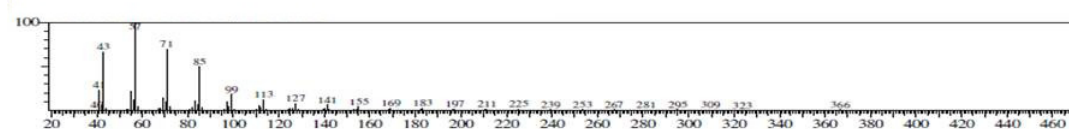
The other long chain carbon molecules detected in the tuberculosis positive sera was C₃₄H₇₀, C₂₉H₆₀, and C₃₄H₉₀. The presence of these molecules could be attributed to presence of PDIMs. Gas chromatographic-mass spectrometric (GC-MS) analysis has proven to be successful for the TB diagnosis by detecting *M. tuberculosis* PDIMs biomarkers in sputum^[9,21,22]. This PDIM has an exceptionally low polarity hence it is an advantage to separate these molecules

from other host specific lipids. The lipid extract of TB positive sera showed the presence of hexopyranose units. The mannopyranose units were also detected in the sera of tuberculosis infected cases. In *Mycobacterium*, the lipoarabinomannan structure is composed of mannopyranosyl branches^[22,23]. The presence of mannopyranose unit in the sera confirms the presence of *Mycobacterium* sp in the host. These lipids mainly produce the mycocerosic acid methyl esters C₂₉, C₃₀ and C₃₂, with molecular masses of 452, 466, and 494 Da, respectively^[24]. It was identified from the previous research that drug resistance has close influence on the PDIM biosynthesis^[25,26]. The rifampicin mutation was found to have up regulation in the polyketide synthase genes which are involved in the phenolphthiocerol biosynthesis^[27]. The present study identifies the PDIM lipids as high abundant biomarkers. These high abundant biomarkers are easily detected even at split mode of analysis.

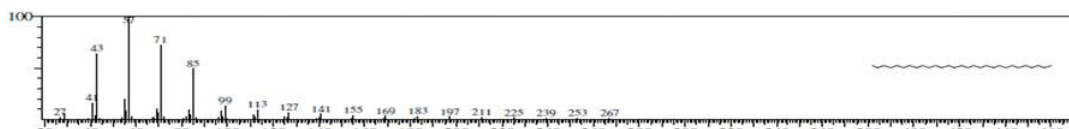
The splitless mode of detection identifies biomarkers like C₁₄H₂₃BrO, C₁₁H₂₄O₂, C₁₈H₄₄O₅, C₁₄H₃₀O₃S, C₂₄H₃₉N in the sera of infected cases. The lipids are high volatile and detected as low abundant molecules in the splitless mode of analysis.

There are no reports available on these low abundant biomarkers in the circulation of tuberculosis infected cases.

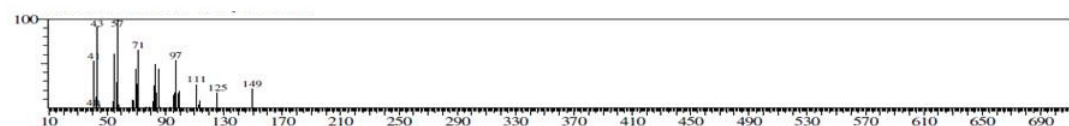
The overall study concludes the presence of several biomarkers in the circulation of tuberculosis infections. These biomarkers are the truncated fragments of larger cell lipid rafts. Based on the availability of these lipids in the specimen, they have been further characterized as high abundant and low abundant lipids. The presence of these lipids can also be correlated with the state of infection like active infection and drug resistance. Hence these biomarkers could be explored further at various other diagnostic platform for detection of tuberculosis infection.



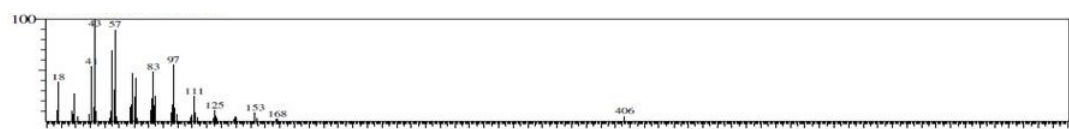
4a



4b



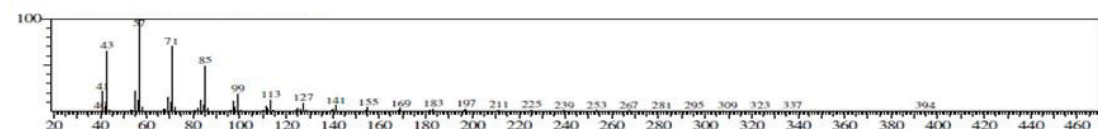
4c



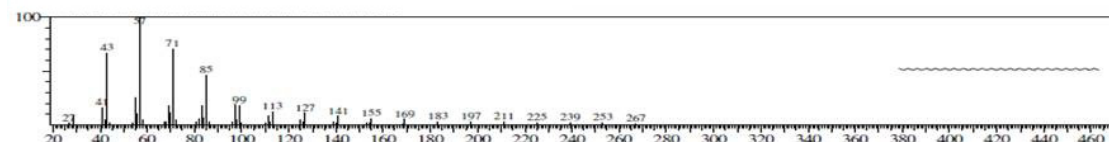
4d

Figure 4a and 4b Mass spectral similarity of lipid molecule M4 with Mass spectral library Wiley8. The molecule had retention of 13.82min and had 84 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C₂₉H₆₀. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 408.

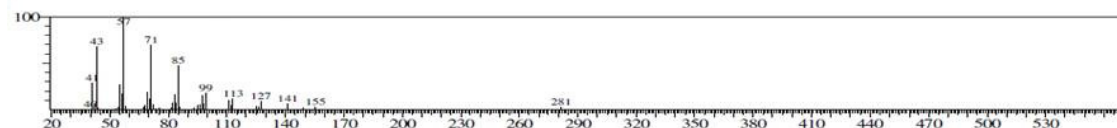
Figure 4c and 4d Mass spectral similarity of lipid molecule M4 with Mass spectral library Wiley 8. The molecule had retention of 11.61 min and had 39 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C₂₉H₆₀O. The spectrum showed a basepeak at m/z 57.10 and had a molecular weight of 424.



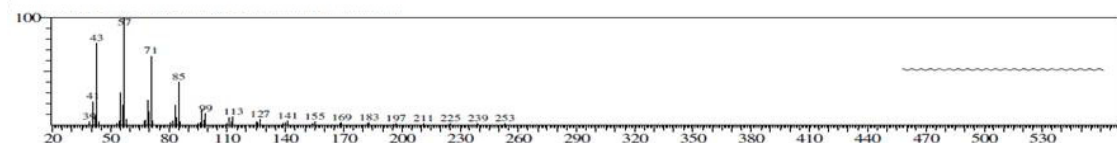
5a



5b



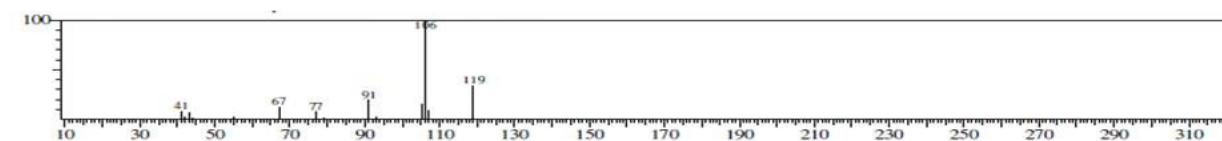
5c



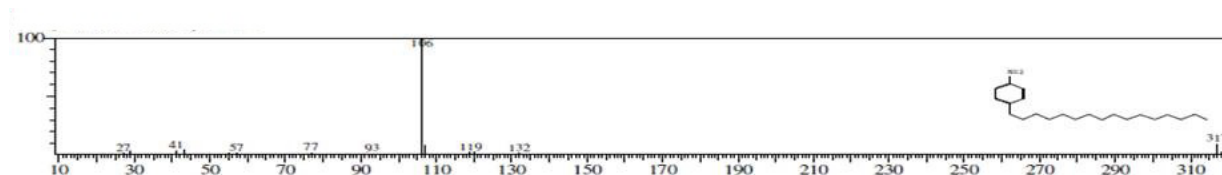
5d

Figure 5a and 5b. Mass spectral similarity of lipid molecule M5 with Mass spectral library Nist08. The molecule had retention of 14.8min and had 91 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C₄₄H₉₀. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 618.

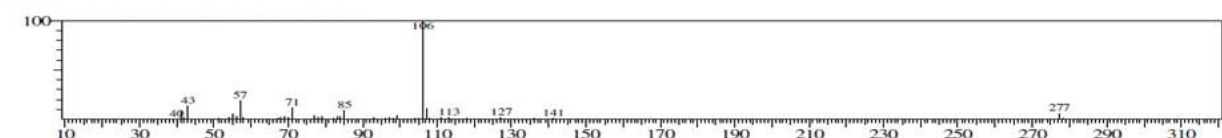
Figure 5c and 5d Mass spectral similarity of lipid molecule M5 with Mass spectral library Nist0 8. The molecule had retention of 12.8 min and had 45 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C₄₄H₉₀. The spectrum showed a basepeak at m/z 57.10 and had a molecular weight of 618.



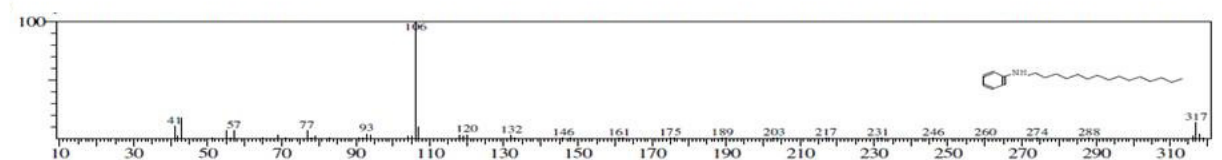
6a



6b



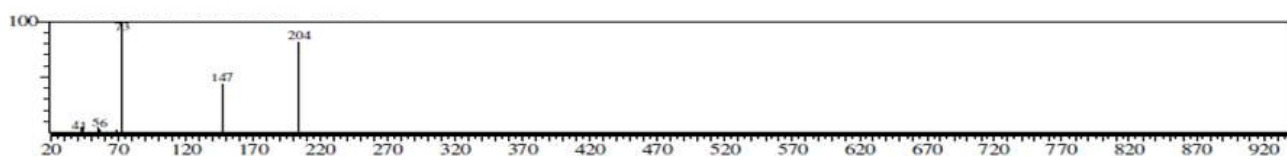
6c



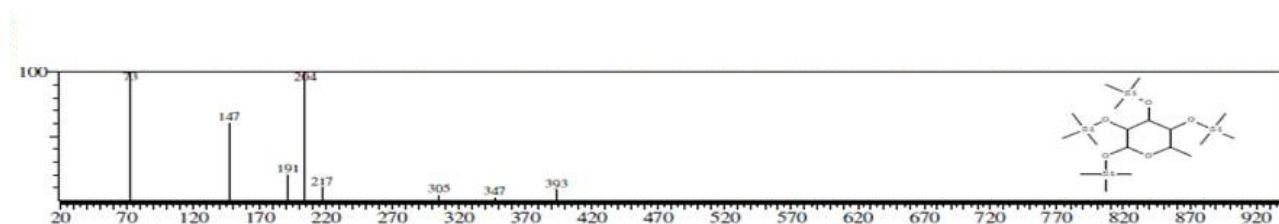
6d

Figure 6a and 6b. Mass spectral similarity of lipid molecule M6 with Mass spectral library Nist08. The molecule had retention of 13.05min and had 15 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C22H39N. The spectrum showed a base peak at m/z 106.10 and had a molecular weight of 317.

Figure 6c and 6d Mass spectral similarity of lipid molecule M6 with Mass spectral library Wiley8. The molecule had retention of 12.76 min and had 55 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C44H90. The spectrum showed a basepeak at m/z 106.10 and had a molecular weight of 317.



7a



7b

Figure 7a and 7b. Mass spectral similarity of lipid molecule M7 with Mass spectral library Nist08. The molecule had retention of 9.5min and had 11 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C18H44O5. The spectrum showed a base peak at m/z 73.05 and had a molecular weight of 452.

ACKNOWLEDGEMENT

The authors are thankful to Grand Challenges Canada (S4 025301) for financial support under bold ideas for humanity, and Vikrant Institute of Technology & Management, Gwalior, India for partial financial support. The authors also thank AIRF facility, JNU campus, New Delhi, India for GC-MS analysis.

CONFLICT OF INTERESTS

The authors declare that they do not have conflict of interests.

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