Comparative lipidomics of drug sensitive and resistant *Mycobacterium tuberculosis* reveals altered lipid imprints

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Abstract Lipids are most adaptable molecules that acclimatize to the development of multidrug resistance (MDR). The precise molecular mechanism of this acclimatization achieved in *Mycobacterium tuberculosis* (MTB) remains elusive. Although lipids of MTB have been characterized to some details, a comparable resource does not exist between drug sensitive (DS) and resistant (DR) strains of MTB. Here, by employing high-throughput mass spectrometry-based lipidomic approach, we attempted to analyze the differential lipidome profile of DS and DR MTB clinical isolates. We analyzed three major classes of lipids viz fatty acyls, glycerophospholipids and glycerolipids and their respective subclasses. Notably, we observed differential fatty acyls and glycerophospholipids as evident from increased mycolic acids phosphatidylinositol mannosides, phosphatidylinositol, cardiolipin and triacylglycerides abundance, respectively, which are crucial for MTB virulence and pathogenicity. Considering the fact that 30% of the MTB genome codes for lipid, this comprehensive lipidomic approach unravels extensive lipid alterations in DS and DR that will serve as a resource for identifying

biomarkers aimed at disrupting the functions of MTB lipids responsible for MDR acquisition in MTB.

Keywords Lipid · Mass spectrometry · Lipidome · *Mycobacterium tuberculosis* · MDR · Mycolic acid

Introduction

In recent times, the emerging cases of multi-drug resistance (MDR) tuberculosis (TB) have mainly been attributed to prolonged and extravagant usage of antitubercular drugs. This has raised serious concern for the effective treatment against MDR-TB (Kumar et al. 2014). Although the major factors contributing towards the development of MDR in *Mycobacterium tuberculosis* (MTB) are fairly well established, MDR being a multifactorial phenomenon, novel players responsible for MDR cannot be ruled out. Reportedly, lipids due to the advancement of mass spectrometry-based technology are better explored in recent years. Lipidomics approach which has recently emerged out has revealed novel lipids to play crucial roles in drug resistance of MTB. For instance, high-throughput lipidomics analysis confirms regulation of virulent lipids in MTB via metabolic coupling (Jain et al. 2007). Similarly, lipidome profiling has revealed relationship among iron acquisition, phospholipid homeostasis and virulence of MTB (Madigan et al. 2015). Even role of arabinomannan glycolipids in biofilm formation and sliding motility of MTB was unraveled through lipid profiling only (Syal et al. 2016). The specific links between rifampicin resistance and lipid factors to provide diagnostic and therapeutic targets were also accomplished by lipidomics technology (Lahiri et al. 2016). In fact, a spatiotemporally distinct and metabolically active membrane domain involved in cell envelope

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biogenesis was discovered by high-throughput lipidomics approach. Thus, the high-resolution lipidomic technologies have enabled to address diverse significant biological questions related to MTB pathogenesis. Apparently, lipids significantly contribute in acquiring drug resistance in MTB. For instance, electron microscopy has enabled to reveal differences in cell wall thickness between drug resistant and non-resistant MTB strains (Velayati et al. 2009). However, exact changes in the lipid pattern and metabolism that occur during this drug resistance are not fully deciphered.

Previously (Pal et al. 2015) we have reported the overview of whole cell lipids in a broad manner with changes in all six classes of lipids present in MTB viz. fatty acyl (FAs), glycerolipids (GLs), glycerophospholipids (GPLs), polyketides (PKs), prenols (PRLs) and sulpholipids (SLC). The present study is further insight where we have performed analysis beyond the classes of lipids. In this study, differential pattern of molecular species has also been studied between DS and DR strains which gives a detailed understanding as to how membrane remodeling occurs. The present study aimed to add to the existing literature and describe the changes in lipid classes and subclasses of drug sensitive (DS) MTB strain with drug resistant (DR) MTB strain which reflect the changes of lipid composition acquired during MDR development. Using high-throughput mass spectrometry (MS)-based approach; we analyzed three major classes viz. fatty acyls (FA), glycerophospholipids (GPL) and glycerolipids (GL) and its subclasses to comprehend alterations in lipidome profile of DS and DR strains of MTB.

Materials and methods

Middlebrook broth 7H9, Middlebrook agar, OADC and ADC (Difco), potassium chloride (KCl), glycerol, DMSO, chloroform, hexane, ethyl acetate, iodine, phosphomolybdic acid (Fisher Scientific), methanol (Merck), Teflon capping glass vials (Borosil), Pasteur pipette and Whatman No. 1 filter paper (Sigma-Aldrich), TLC silica gel 60 plate (Merck Millipore), ethanol.

Culture conditions

Clinical isolates of MTB-sensitive (DS) and -resistant strain (DR) (sensitive and MDR, respectively) were used for the study. DS refers to MTB clinical isolate which is sensitive to anti-TB first line drugs, i.e., Isoniazid (INH), Ethambutol (EMB), Rifampicin (RIF) and Streptomycin (STM). Similarly, DR refers to the MTB clinical isolate which was resistant to drugs RIF, INH and STM. Both the strains obtained from Division of Clinical Microbiology

and Molecular Medicine, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India. DS and DR were inoculated with starter culture of 0.1 O.D₆₀₀ in a screw cap flasks containing 10 mL of Middlebrook 7H9 broth supplemented with 10% albumin/dextrose/catalase (BD Difco), 0.2% glycerol (Fischer Scientific) and 0.05% tween-80 (Himedia) in 100 mL flasks (Schott Duran). The flasks were placed at 37 °C for 2 weeks with agitation.

Extraction of total lipids

Cells of DS and DR strains at exponential phase with 1.2 O.D₆₀₀ were used for lipid extraction by method as described elsewhere with slight modifications (Slayden and Barry 2001; Folch et al. 1957). Briefly, the DS and DR cells were harvested at 10,000 rpm for 10 min. Cells were homogenized in aqueous solution for 3 min and suspended in CHCl₃ and CH₃OH in ratio of (1:2). Cells were then shaken well and centrifuged at 2000 rpm at 4 °C for 10–15 min. Supernatant was transferred to another glass vial and remaining CHCl₃ was added and filtered through Whatman No. 1 filter paper. The extract was then washed with 0.88% KCl to remove the non lipid contamination. Two layer was formed the lower dense layer of chloroform containing lipid was taken by glass Pasteur pipette in 5 mL glass vial having Teflon capping. The vials are stored at –20 °C until further analysis.

Thin layer chromatography

Total cellular lipid was subjected to TLC and two dimensional thin-layer chromatography (2-D TLC) on silica gel-G plates in a variety of solvent systems to resolve lipids of various polarities. For fatty acid methyl ester (FAME) and mycolic acid methyl ester (MAME) solvent used was hexane/ethyl acetate (19:1, v/v) as previously described (Slayden and Barry 2001). For 2-D TLC solvents used for developing TDM (trehalose dimycolate) in single dimension (1-D) were Chloroform/methanol/water (100:14:0.8) (v/v/v) and for second dimension Chloroform/acetone/methanol/water (50:60:2.5:3) (v/v/v/v) as described previously (Slayden and Barry 2001).

Ultra performance liquid chromatography-electrospray ionization and mass spectrometry (UPLC-ESI-MS)

The qualitative analysis of the samples was performed on triple quadrupole tandem mass spectrometer ACQ-TQD#QBB1152. LC was done on a C18 column (100 mm × 3 mm, 2.6 µm) and elution was done for 30 min, using 5% isopropanol, 90% methanol and 5%

ammonium acetate (5 mM, pH 6.5), at a flow rate, 0.1 mL/min. The source temperature was 120 °C, the desolvation temperature was 350 °C and the cone voltage was set at 40 V. The sample was introduced using an autosampler with 1 µL of sample injection volume. Capillary voltage was set to 3.50 kV. The data were recorded in the mass range, m/z 200–2000 and processing was performed with the Masslynx software, where each chromatogram was smoothed and the background was subtracted. The analysis of the acquired data was performed by mass spectrometry-based lipid(ome) Analyzer and molecular platform (MS-LAMP) software (Sabareesh and Singh 2013; Layre et al. 2014). This software is a graphical user interface (GUI) standalone programme built using Perl::Tk. It is a combination of MTB lipid database (www.mrl.colostate.edu) and lipid metabolites and pathways strategy consortium (LIPID MAPS; www.lipidmaps.org). The most probable adduct was recognized to be $[M - H]^+$ and 0.5 and 0.25 window ranges was used for analysis. Further, it needs to be noted that the data herein are analyzed qualitatively only. All the experiments were performed in triplicates to ensure reproducibility and accuracy.

Results and discussion

Global lipidome screening of DS and DR strains

The present study attempts to reach more closely to the actual composition of the DS and DR MTB major lipid classes (Table 1). The analysis of lipid subclasses was performed by considering only lower window ranges available in MS-LAMP, i.e., 0.5 and 0.25 to avoid non

specific identification. Thus, the output generated through this software provides a global illustration of various lipid categories present in MTB facilitating differential analysis of DS and DR MTB lipid profile. Figure 1 shows the representative mass spectra obtained by UPLC–MS of DS and DR MTB strains along with total mass spectra shown in Fig. S1 and S2. The global lipid profile illustrated the relative variation in abundance of major classes FAs, GPLs and GLs between DS and DR strains. Our results depict that total number of lipids observed in DS and DR strains at WR 0.5 and 0.25 possesses considerable compositional differences (Fig. 2). We further performed m/z survey based on the data obtained from LC-MS m/z peaks and recognized the maximum and minimum ranges of m/z covered by each of the three major classes of MTB lipids. The observed m/z values show that each of the three classes has varied minimum and maximum ranges (Table 2). Therefore, these results gave an impression that composition of DR MTB lipids is different from DS MTB lipids.

FA abundance is enhanced in DR strain

The importance of lipids in the physiology of MTB rendering the development of MDR has already been realized (Sartain et al. 2011), but dissection and evaluation of lipid metabolome between DS and DR MTB is rudimentary. FAs are one of the most significant and prominent class of lipids in MTB governing pathogenesis. To highlight the changes between DS and DR MTB strains, initially we performed the TLC for MAME and FAME family and found that DR strain has significantly intense spots on TLC plate as compared to DS for MAME family (Fig. 3a), which indicates that abundance of FAs are more in DR

Table 1 Major lipid classes and subclasses analyzed in this study

Class	Subclass	Structures
Fatty acyls (FA)	Alpha-MA, DIMA, DIMB, Glycosylated phthidiolone dimycocerosate, GMM Alpha-MA, GMM (keto-MA, GMM (methoxy-MA), Hydroxyphthioceranic acid, Keto-MA, Methoxy-MA, Mycocosic acid, Mycocosoci acid, Mycolipanic acid, Mycolipenic acid, Mycosanoic acid, PGL-tb, Phthioceranic acid, TMM (Alpha-MA), TMM (keto-MA), TMM (Methoxy-MA)
Glycerophospholipids (GPL)	Ac1PIM1, Ac1PIM2, Ac1PIM3, Ac1PIM4, Ac1PIM5, Ac2PIM2, Ac2PIM3, Ac2PIM4, CL, Lyso-PE, Lyso-PG, Lyso-PI, Lyso-PIM1, Lyso-PIM2, Lyso-PIM3, Lyso-PIM4, Lyso-PIM5, Lyso-PIM6, PE, PG, PI, PIM1, PIM2, PIM3, PIM4, PIM5, PIM6.
Glycerolipids (GL)	MG (RCO2H), DG (R1CO2H+R2CO2H), TG (R1CO2H+R2CO2H+R3CO2H)

Fig. 1 Chromatogram. Representative mass spectra obtained by UPLC–MS of DS and DR MTB strains

Fig. 2 Total lipid composition of major classes (FAs, GPLs and GLs) in DS and DR strains of MTB. Data is shown by heat map representing total number of lipid moieties present at WR 0.5 and 0.25, respectively. Color bar depicts increasing number of lipid abundance from yellow to black

strain, contrary for FAME no such differences were observed. After this, we run 2D TLC of DS and DR MTB strains and observed intense pattern of spots for TDM which further strengthen the notion that FAs content is higher in DR MTB strain as compared to DS (Fig. 3b). Interestingly, we could observe some unidentified spots belonging to unknown lipids which we expect to be new lipids; however, further validation is needed. Next, we analyzed the FA class using MS-LAMP, where major FAs subclasses were studied. The most abundant and hallmark lipids of MTB, i.e., alpha-mycolic acid (MA), Keto-MA, methoxy-MA, glucose monomycolates (GMM) alpha-MA, GMM (keto-MA), GMM (methoxy-MA), trehalose monomycolates (TMM) alpha-MA, TMM (keto-MA), TMM

(methoxy-MA) and other species like phthiocerol dimycocerosates (DIM) DIMA, DIMB, glycosylated phthiodiolone dimycocerosate, hydroxyphthioceranic acid, mycocerosic acid, mycocersoci acid, mycolipanic acid, mycolipenic acid, mycosanoic acid, phenolic glycolipid (PGL-tb) and Phthioceranic acid were analyzed. The MS-LAMP having WR 0.5 was used for primary analysis. Through this WR, we have identified unique 5 and 8 m/z in DS and DR MTB strains, respectively. Additional 2 m/z were found to be common in both DS and DR MTB strains as represented by Venn diagram (Fig. 4a). These five identified m/z of DS MTB strain correspond to seven lipid species and eight identified m/z of DR MTB strain correspond to 13 lipid species represented by bar graph

Table 2 Maximum and minimum m/z ranges from chromatogram

	Sensitive			Resistant	
	Minimum	Maximum		Minimum	Maximum
WR = 0.5					
FAs	427	1798	FAs	580	1534
GPLs	587	1798	GPLs	328	1212
GLs	357	1156	GLs	1802	–
WR = 0.25					
FAs	1156	–	FAs	121	1272
GPLs	1156	1798	GPLs	1212	1802
GLs	1156	–	GLs	822	1212

Coverage of maximum and minimum m/z ranges of DS and DR lipid classes at WR 0.5 and WR 0.25, respectively

Fig. 3 a TLC of MAME and FAME of total lipids in DS and DR. b 2D TLC of total lipids in DS and DR

(Fig. 4a). The mass spectra of DR possess more m/z values than DS, therefore identified proportionally more number of lipid species.

It has been well documented that MA which is a subclass of FAs is known to be important for the growth of MTB, crucial for the development of drug resistance, alters the membrane permeability of MTB cell envelope

(Gebhardt et al. 2007; Forrellad et al. 2013). Our data have shown enhanced TDM, a class of MA in DR MTB strain which is important for bacilli survival in foamy alveolar macrophages (Bailo et al. 2015). TDM is also responsible for inhibiting the phagosome–lysosome interaction which contributes in lesions formation and caseating granulomas formation (Rajni et al. 2011). Apart from the enhanced MA

Fig. 4 Changes in FA composition of DS and DR strains of MTB. **a** Venn diagram showing the number of m/z values which were common and unique at WR 0.5. Lower panel (bar graph) depicts number of lipid moieties identified from m/z values observed by Venn

diagram at WR 0.5. **b** Venn diagram showing the number of m/z values which were common and unique at WR 0.25. **b** Lower panel (bar graph) depicts number of lipid moieties identified from m/z values observed by Venn diagram at WR 0.25

in DR MTB strain, we also observed increase in DIM-B (Fig. 4a). DIMs are known to contribute to the permeability of MTB membrane as well (Camacho et al. 2001). WhiB3, a member of intracellular iron-sulfur (Fe-S) cluster proteins family has appeared as a redox sensor and effector molecule regulating various characteristics of virulence in MTB (Saini et al. 2012)). It has been suggested by Forrellad et al. that mutation in whiB3 leads to modification in the production of lipids like methyl-branched lipids, PDIMs and triacylglycerol (TAG). Probably it is mainly due to the lack of WhiB3-mediated regulation of complex lipids that MTB exploits to inhibit immune responses of the host. Therefore, it can be hypothesized that WhiB3 alteration can over produce the DIMs which also support our present study where DIMs were found to be enhanced in DR in comparison to DS. However, no major differences were observed in phthioceranic acid and hydroxyphthioceranic acid. To further narrow down our search, we have selected WR 0.25 of MS-LAMP for analysis. Through this WR, we have identified unique 1 and 3 m/z in DS and DR MTB strains, respectively. Interestingly, no common m/z was recognized as shown by Venn diagram (Fig. 4b). This single m/z of DS MTB strain corresponds to single lipid species and 3 m/z of DR MTB strain corresponds to three lipid species as represented by bar graph (Fig. 4b). Hence, specific lipid molecules were

distinctively present in DS and DR MTB strains when analyzed with WR 0.25. We observed that only mycolic acids GMM (alpha-MA), GMM (methoxy-MA) and DIM-B were enhanced in DR while other subclasses of MA were absent in DR MTB strain; however, only methoxy-MA was present in DS MTB strain (Fig. 4b). Considering the fact that MA have significant roles in the pathogenicity and resistance of MTB, the elevated MA in DR MTB strain could be linked to the resistant phenotype of MTB. *InhA* is an essential gene involves in the biosynthesis of mycolic acids (MA) and a key step for the elongation of fatty acid system-II (FAS-II). Due to its involvement in the biosynthesis of MA, it is the primary target for anti-TB drug like INH. (Shaw et al. 2017) Thus over-expression of *inhA* gene which directly correlates with enhanced biosynthesis of MA in the DR strain cannot be ruled out. Moreover, it is well established fact that MTB depends on FA as a preferred energy source during infection (Sinsimer et al. 2008) hence could be another reason for enhanced FA in DR.

GPL abundance is enhanced in DR strain

Next, we analyzed another important class GPLs at subclass level that appeared to have significant role in drug resistance. GPL subclass includes cardiolipin (CL),

phosphatidyl ethanolamine (PE), phosphatidylinositol (PI), phosphatidyl glycerol (PG), LysoPI and LysoPG and phosphatidylinositol mannoside (PIMs). The MS-LAMP having WR 0.5 was used for primary analysis. Through this WR, we have identified unique 7 and 16 m/z in DS and DR MTB strains, respectively. Additional 3 m/z were found to be common in both DS and DR MTB strains as represented by Venn diagram (Fig. 5a). These seven identified m/z of DS MTB strain correspond to 15 lipid species and 16 identified m/z of DR MTB strain correspond to 27 lipid species as represented by bar graph (Fig. 5a). The GPL subclass moieties CL, PE, PG, PI, Lyso-PG were found to be elevated in DR MTB strain (Fig. 5a). CL is one of the most abundant phospholipids in mycobacteria typically required for pathogenesis (Paul et al. 2013). Owing to their antigenic, serological and physiological attributes, GPLs are of clinical relevance. Moreover, it is already known that virulent strains of MTB possess higher GPLs (Paul et al. 2013).

MTB synthesize PIMs that are again a subclass of GPLs and important component of the cell envelope (Guerin et al. 2010). Polar PIM species can also serve as membrane anchors for lipomannan (LM) and lipoarabinomannan (LAM). Extensive genetic and biochemical studies have demonstrated that the synthesis of PIMs occurs linearly in mycobacteria with PI as the starting substrate (Haite et al.

2005). PI being the anchor for the PIMs is substrate for heavy mannosylation to form LMs and subsequently produce LAMs (Kovacevic et al. 2006; Ridell et al. 1992). In our study when PIMs were analyzed it was found that acylated phosphatidylinositol mannosides Ac1PIM1, Ac1PIM2, Ac1PIM3 were elevated in DR MTB strain. However, no change in Ac1PIM4 and Ac2PIM2 could be detected in DR MTB strain. Interestingly in Ac2PIM3, and PIM2 were found to be present but reduced number in DR MTB strain. Notably, Lyso-PIM2 and PIM6 was found to be completely missing in DR MTB strain (Fig. 5a). Recent study showed that PIM is required for the viability in vitro and also play a vital role in cell envelop permeability, integrity, cell division and septa formation (Jackson 2014).

While analyzing the GPL subclass changes through WR 0.25 interestingly we have found total 4 m/z that were unique to DS MTB strain whereas 9 m/z values were unique for DR MTB strain and no commonality have been observed as shown in Venn diagram (Fig. 5b). These 4 m/z values correspond to eight lipid species and 9 m/z values correspond to 15 lipid species as shown by bar graph (Fig. 5b). Further going into the details, we could observe differences in CL and PIMs as well. CL was significantly enhanced in DR MTB strains which may be due to over-expression of PgsA2 gene which encodes CL synthesis enzyme (Jackson et al. 2000). Similarly PE synthesis

Fig. 5 Changes in GPL composition of DS and DR strains of MTB. **a** Venn diagram showing the number of m/z values which were common and unique at WR 0.5. **a** Lower panel (bar graph) depicts number of lipid moieties identified from m/z values observed by Venn

diagram at WR 0.5. **b** Venn diagram showing the number of m/z values which were common and unique at WR 0.25. **b** Lower panel (bar graph) depicts number of lipid moieties identified from m/z values observed by Venn diagram at WR 0.25

Fig. 6 Changes in GL composition of DS and DR strains of MTB. **a** Venn diagram showing the number of m/z values which were common and unique at WR 0.5. **a** Lower panel (bar graph) depicts number of lipid moieties identified from m/z values observed by Venn

diagram at WR 0.5. **b** Venn diagram showing the number of m/z values which were common and unique at WR 0.25. **b** Lower panel (bar graph) depicts number of lipid moieties identified from m/z values observed by Venn diagram at WR 0.25

includes, PS synthetase and PS decarboxylase enzymes which is encoded by Genes (pssA, Rv0436c) and (psd, Rv0437c) (Jackson et al. 2000). However, there is no experimental evidence demonstrating the identities of the genes but considering our results hypothetically we can assume overexpression in any of these genes leading to more PE production in DR strain. PimA, B and C are the genes responsible for the synthesis of LAM from PI to PIM, PIM to LM and LM to LAM. Over-expression of PIMs gene resulting in the synthesis of higher PIMs as observed in our study could be a possibility (Mishra et al. 2011). Similarly, Ac1PIM1, Ac1PIM2, Ac1PIM3 was found to be enhanced DR strain where as for Ac1PIM4 and Ac2PIM2 no changes were observed which was also similar to the finding from WR 0.5 analysis. We also observed depletion Ac1PIM3 and PIM6 in DR MTB strain with both the WRs (Fig. 5b).

GL showed variations at species level in DR

Drug resistance-induced lipid remodeling was further evident from the fact that GLs were also found to be altered. GL were mainly classified into three subclasses, i.e., monoacylglycerides (MG), diacylglycerides (DG) and triacylglycerides (TG). GLs are primarily used as stored energy source and lipase is an enzyme encoded by

Rv3097 in MTB that catabolizes TG under starvation. Deletion of this enzyme fails to utilize stored TGs under stress conditions (Sinsimer et al. 2008). After getting difference in FAs and GPLs, we further explored GLs to analyze the variation in GL subclasses. For this, we first examined the data at WR 0.5 and found total 1 m/z which was unique to DS strain whereas 11 m/z values were unique to DR strain. Excluding these unique m/z values, there are 4 m/z values which were common in both DS and DR as depicted in Venn diagram (Fig. 6a). This 1 m/z values corresponds to 1 MG, 3 DG and 1TG in DS whereas 11 m/z in DR corresponds to 3 MG, 7 DG and 6 TG lipid species, respectively as shown by bar graph (Fig. 6a). Thus, we observed marked increase in MG, DG and TG in DR MTB strain. Next, we determined the commonalities and uniqueness of lipid moieties in GLs between of DR and DS through Venn diagram at WR 0.25. We found only 1 m/z value to be unique to DS MTB strain, whereas 6 m/z values are unique to DR MTB strain (Fig. 6b). However, under stringent conditions at WR 0.25, MG was not detected, no change in DG but appreciable increase in TG in DR strain (Fig. 6b). This 1 m/z corresponds to 1 DG lipid species in DS and 6 m/z values corresponds to 1DG with 6 TG lipid species in DR MTB strain, respectively, as shown by bar graph (Fig. 6b). It is evident from lipidome analysis that

reshuffling in terms of lipid species was predominant in DR strain. The alteration in lipid species at class and subclass level could be attributed to the fact that lipid play crucial role in acquisition of drug resistance.

Conclusion

The cell envelope of MTB contains one of the most sophisticated and exquisite compositions of biologically active lipids. These are essential for mycobacterial viability, pathogenicity and resistance which play active role in modulating the host immune response. This study through an interrogation of the DS and DR MTB lipids analyzed the lipid makeup of MTB clinical isolates. Here, we confirmed that DR MTB isolates have distinct lipid imprints from DS strains. MTB lipids are potential candidates for diagnostic and therapeutic biomarkers owing to their limited structural similarity with human lipids. Since lipid compositional changes are significant for the sustenance of MDR and this study points towards compositional variation in resistant strain, further intricate studies are warranted for its implication in biomarker(s) identification.

Availability of data and materials

All the relevant data are within the paper and its supporting information file (Table S1 and S2).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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