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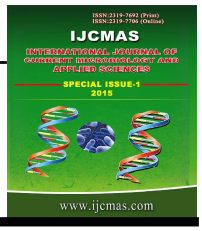

Original Research Article

Comparative Lipidome Profile of Sensitive and Resistant *Mycobacterium tuberculosis* Strain

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K e y w o r d s

Lipid,
MDR,
MS,
lipidome,
Mycobacterium tuberculosis

A B S T R A C T

The role of lipids in development of Multidrug Resistance (MDR) in *Mycobacterium tuberculosis* is emerging as new tool in current scenario but mechanisms to acquire MDR and exactly how lipid molecules govern the MDR phenotype is still poorly understood. In the present study, we have used a high-throughput mass spectrometry-based approach and compared the lipidome profile of $H_{37}Rv$ and MDR-MTB strains. Although there were some significant commonalities in the lipid profiles of this pair, we have identified six major classes of lipids viz. Fatty acids (FA), glyerolipids (GL), glycerophospholipids (GPL), prenol (PL), polyketide (PK) and saccharolipids (SL) with critical differences between sensitive and resistant MTB strains. Taken together, the present lipidomic approach will serve as a resource for further quantitative validation so that assessment of various strategies aimed at disrupting the functions of MTB lipids and thereby MDR could be employed.

Introduction

Tuberculosis (TB) is a dreadful disease caused by the notorious pathogen *Mycobacterium tuberculosis* (MTB). The death toll caused by MTB is on the mount and mortality rate is exceeding 1.5 million deaths annually (He *et al.*, 2008) as also reported by global TB report in 2014. One of the reasons associated with the failure of current therapeutic regimes for TB is emergence of resistance against these drugs by the phenomenon of multidrug resistance (MDR) (Shah *et al.*, 2007; Zignol *et al.*, 2006; Matteelli et al., 2007). MDR is defined as the resistance acquired by the organism to more than one drug which share neither the common structure nor the common target. There are several mechanisms which contribute towards the development of MDR in MTB viz. over expression of drug efflux pumps, alteration in membrane permeability, drug modification and target alteration (Pal *et al.*, 2014). Therefore development of new strategies with new therapeutic targets is the

urgent need of hour and demand immediate attention.

One factor that is crucial for the enhanced intracellular survival of MTB is an unusually hydrophobic and multilayered protective cell envelope composed of typical lipids such as mycolic acid (MA) (Daffe and Draper, 1998). Moreover, over expression of drug efflux pumps conferring MDR being the most significant mechanism of MDR as stated above is also associated with the lipid structure and composition since they are localized within micro-domains of membrane known as 'Lipid rafts'. In the recent times there has been a considerable advancement in the awareness of lipid molecules possessing some unique biological roles that is distinct from their usual functions (Korf *et al.,* 2005). In fact, it is now recognized that lipids play substantial roles in functions such as cellular trafficking, signaling and immune system (Hameed *et al.,* 2013). Recent evidences have also provided a comprehensive amount of data regarding the role of lipids and many other lipid derivatives in establishment of MTB infections. Thus elucidating the role of lipids in drug resistance of MTB has emerged as one of the important strategies to dissect MDR regulatory circuits in many organisms including MTB. Fatty acids (FAs), such as MA, constitute a key component of the MTB cell envelope. This cell envelope is responsible for giving unique characteristics to mycobacteria, and also contains immunomodulatory molecules. Recently, high throughput lipidomics analysis revealed regulation of virulent lipids in MTB via metabolic coupling (Jain *et al.,* 2007). A new report demonstrated the role of lipid rafts aggregation in the MTB infection process thus highlighting the significance of membrane homeostasis (Fine-Coulson *et al.,* 2012).

MTB cell envelope lipid architecture and its integrity being an important determinant of the viability and progression of pathogen holds promise for the search of novel anti-tubercular drug targets within the array of its metabolic intermediates. In this study, considering the turn over role of lipid nowadays in the pathogenesis of MTB, we have explored the possibility of highlighting the differences in lipid profiles, if any, of MTB sensitive and resistant $H_{37}Rv$ strains. About 6 major categories of lipids viz. Fatty acids (FA), glycerolipids (GL), glycerophospholipids (GPL), prenol (PL), polyketid (PK) and saccharolipids (SL) in sensitive and resistant MTB strains was compared. We found that though both strains share a few commonalities, each strain shows a different pattern in regard to its lipid profile and warrants further quantitative validation to device various strategies aimed at dissecting the functions of MTB lipids.

Material and Methods

Microorganism

E. coli (DH5α) and clinical isolates of MTB sensitive and resistant strains ($H_{37}R_v$ and MDR respectively) were used for the study.

Culture Conditions

$H_{37}R_v$ and MDR clinical isolates was inoculated with starter culture of 0.1 $O.D_{600}$ 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 37ºC for 2 weeks with agitation.

Extraction of total lipid

Cells of $H_{37}R_v$ and MDR at exponential phase were used for lipid extraction by modified Folch method (Folch *et al.*, 1957). Briefly, the $H_{37}R_v$ and MDR cells were harvested at 10,000rpm for 10min. Cell was homogenized in aqueous solution for 3 minutes and suspended in $CHCl_3$ and CH_3OH in ratio of (1:2). Cells were shaken well and centrifuged at 2000 rpm at 4°C for 10–15 minutes. Supernatant was transferred to another glass vial and then remaining $CHCl_3$ 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 –20ºC until further analysis.

Thin layer chromatography

Thin layer plates were prepared using silica gel G (CDH, India). Preparation of silica gel G plates was achieved manually by dissolving silica gel G in distilled water in a ratio of (1:2) to form slurry. Slurry was poured on tilted glass plate to get uniform layer. Plates were activated by drying in an oven for 45 minutes at 110ºC. The lipid extract obtained from *E. coli*, $H_{37}R_v$ and MDR was loaded on TLC plate at a distance of 2 cm up from the plate end. Chloroform-methanol-water (65:25:4) was used for developing the plates. Developed chromatogram was dried at room temperature for 2 minutes and then exposed to iodine fumes generated by iodine crystal balls placed in glass chamber. For 2-D TLC solvents used for developing cord factor/TDM (trehalose dimycolate) in single dimension (1-D) were $CHCl_3$:CH_3OH: $(CH_3)_2CO$:CH_3COOH (90:10:6:1, V/V/V/V). For second dimension, solvents used were $CHCl_3$:CH_3OH:H_20 (90:10:1, V/V/V) (Fujiwara, 1997). Developed chromatogram was dried at room temperature for 2 minutes and then methanolic iodine was sprayed to locate the bands.

Ultra Performance Liquid Chromatography-Electrospray Ionization and Mass Spectrometry (UPLC-ESI-MS)

The samples were analyzed on "triple" quadrupole tandem mass spectrometer in polarity switching mode. LC has been done on a C18, 100X3, 2.6µm column through isocratic mode of elution for 30 minutes using 5% Isopropanol, 90% Methanol and 5% of 5 mM Ammonium acetate (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 energy was 40V. The sample was introduced using an autosampler with 1µL of sample injection volume. Data processing was performed by Masslynx software and the background was subtracted and the data were smoothed in each chromatogram. The data was obtained in ESI positive mode with scanning mass range 202 to 2000 Da. m/z values obtained were identified from MTB Lipidome present in Mass Spectrometry based Lipidome Analyzer and Molecular Platform (MS-LAMP) software as described elsewhere (Sabareesh and Singh, 2013).

Result and Discussion

Although the significance of lipids in the pathogenesis of MTB is already realized, the prevalence of TB in conjunction with drug resistance, leads to focused research which indicated MTB lipids carry immunogenic and virulence determining properties. The unique cell wall of Mycobacterium genus has the most complex bacterial lipidome and therefore encounters several ambiguities.

Plethora of evidences suggesting involvement of MTB lipids in establishment of infection compelled systematic study of MTB lipidome (Korf *et al.,* 2005; Jain *et al.,* 2007; Layre *et al.,* 2011). With the advancement of LC/MS based lipid profiling, new researches are coming up to detect and identify lipids from all major MTB lipid classes. Sartain *et al.* (2011) has created novel database (MTB LipidDB) that contains 2,512 lipid entities for accurate mass determination. Recent sophisticated advances in ESI-MS based techniques although made feasible the characterization of MTB lipid samples in more exhaustive manner, a systematic comparative lipidomics of related strains of the pathogen would provide information about the biomarkers and strategy to identify virulence factor (Layre *et al.,* 2011). In this study, we have adopted the available resources to provide a comparative analysis of the categories of MTB lipid and decipher the variations in the lipid profile of sensitive and resistant MTB strain.

The global lipidomic profile of MTB sensitive and resistant strain was performed through the direct comparison of their lipidomes. For lipidome analysis sensitive and drug resistant MTB cells were harvested in the exponential growth phase and their total lipids were extracted as described in Materials and Methods. Firstly, to validate the presence of lipids in the extract, a TLC was performed for the total lipids of sensitive and resistant MTB strains along with *E. coli* lipid extract which served as positive control. Our result demonstrates the presence of lipids in all the three samples in sensitive and resistant MTB strains and *E. coli* (Fig. 1A). Next, we confirmed the presence MTB specific lipids such as MA which is a hallmark of *Mycobacteria* genus. For this, we run a 2-D TLC with the solvents specific for MA. We observed that the MTB lipid extract in lane 2 shows the presence of spots of MA while lane 1 (*E. coli*, which served as negative control) shows no spot of MA (Fig. 1B). Our 2-D TLC showed a typical pattern depicting spots of methyl mycolates, TDM and SL as described elsewhere (Fujiwara, 1997). These observations suggest the clarity of the extracts for utilization of these samples for highly sophisticated UPLC-ESI-MS run.

The extracted lipids were further subjected to UPLC-ESI-MS by direct infusion of the lipid extracts. Amid of various available software tools, MS-LAMP by Sabareesh and Singh (2011) was selected for interpretation of ESI-MS data. This graphical user interface (GUI) provides a wide spectrum detection of lipids by ascribing as many signals (m/z values) as possible in a mass spectrum to one or more lipid(s). The data was analyzed in the positive-ion mode where the most common monoisotopic adducts are often $[M+H]^+$, $[M+Na]^+$, and $[M+NH_4]^+$ (Sartain *et al.,* 2011). Therefore all the three common possible adducts were included in the study to reach more closely to the actual molecular mass of the lipids. The analysis was performed by considering all the window ranges available in MS-LAMP i.e. 0.01, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0. However, only the window ranges 0.75 and 1.0 was selected for the further analysis to minimize the possibility of skipping any lipid moiety present in the sample. The output generated through this software provides a complete illustration of different lipid categories which facilitated comparative analysis of sensitive and drug resistant MTB lipids. We could determine the abundance of 6 major classes of lipid which displayed a comprehensive coverage of MTB lipid metabolic network (Fig. 2 and 3). Of note, the abundance of Phosphoglycerolipids (GP) out of all 6 major classes was drastically changed

between sensitive and resistant MTB lipids with all the three analyzed adducts in both the window ranges. Pertinent to mention here that GP has critical role in MTB cell wall functioning and interaction with host (Cao *et al.,* 2011). Furthermore, phosphatidylinositol mannosides (PIMs), an important pathogenic lipid involved in immunogenic response of MTB belongs to the GP category. We were also been able to find considerable differences in GL and FA categories between sensitive and resistant MTB lipids. MA sub classes Trehalose monomycolates (TMMs), Trehalose dimycolates (TDMs) and Phthiocerol dimycocerosates (DIMAs) belongs to FA category (Sartain, 2011). The PK, PR and SL categories remain relatively less changed, between sensitive and resistant MTB strains albeit their numbers were also in less amount. Since, MS-LAMP MTB lipidome, does not contain lipids in the categories of sterols and sphingolipids, therefore, these categories were also not detected neither in both the window ranges selected 0.75 and 1.0 nor in any of H^+, Na^+, and $NH4^+$ adducts.

Figure.1(A) Thin Layer Chromatography of total extracted lipid samples from *E. coli* (Lane 1) and of MTB strains $H_{37}Rv$ (Lane 2) and MDR (Lane 3). **1(B).** 2-D TLC of *E. coli* (Lane 1) and MTB (Lane 2) in presence of solvents as described in materials and methods

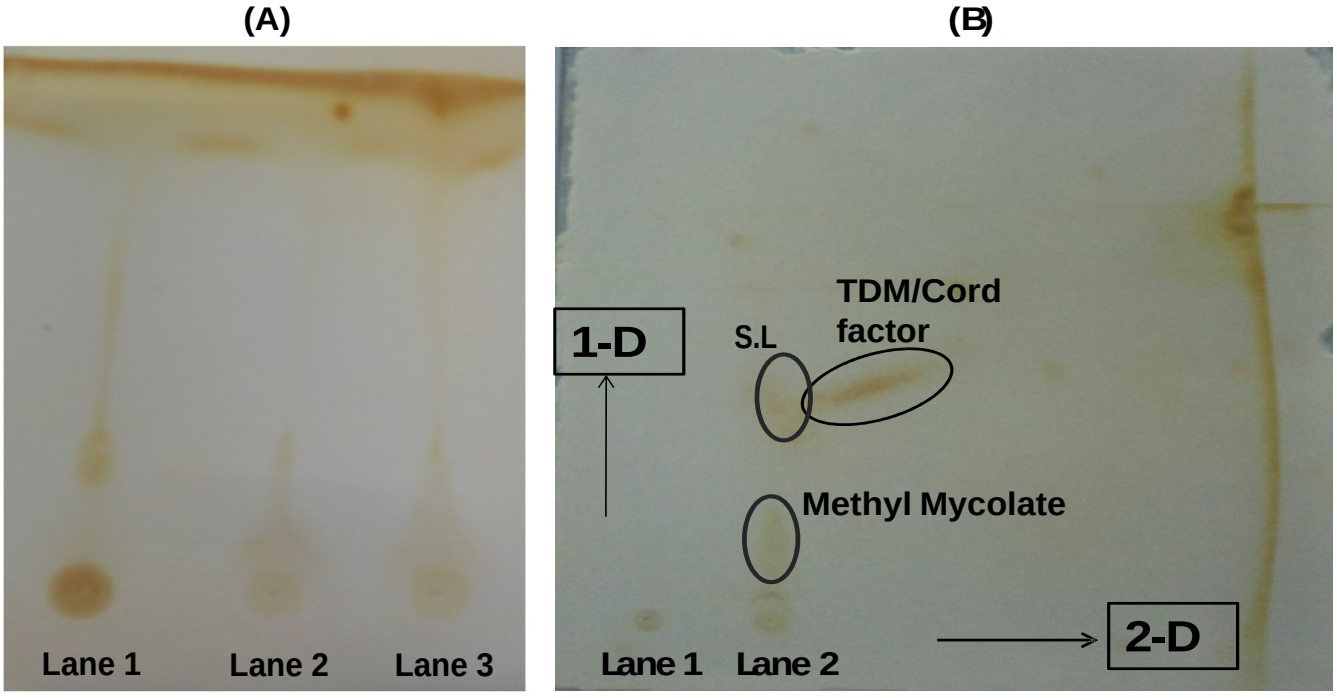


Figure.2 Bar graphs showing abundances of six categories of lipid between MTB $H_{37}Rv$ (black bar) and MDR (shaded bar) strains detected with window range 0.75 from MTB MS-LAMP software as described in materials and methods. Y-axis depicts the total number of lipid moieties in particular category of lipid and X-axis depicts the three positive monoisotropic adducts

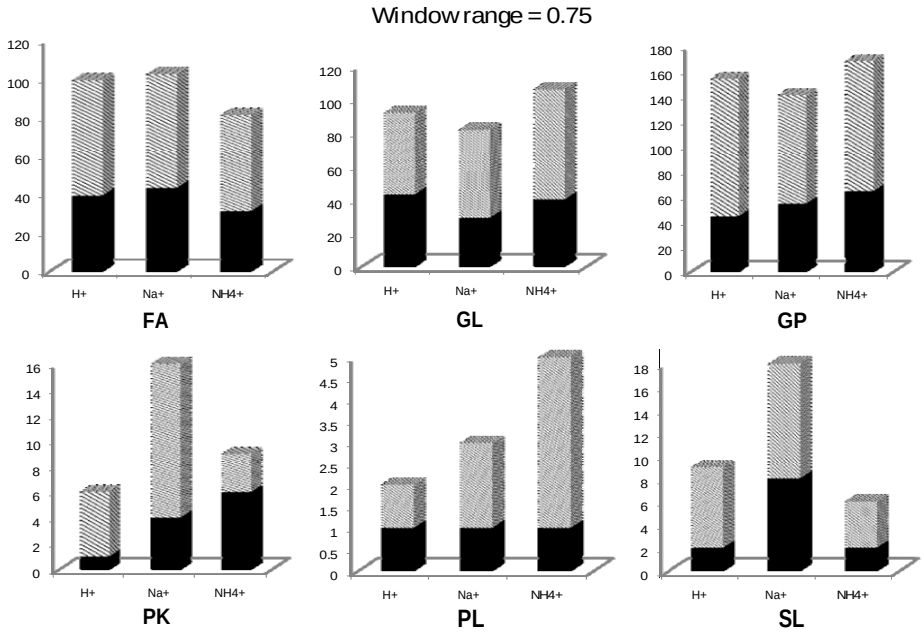


Figure.3 Bar graphs showing abundances of six categories of lipid between MTB $H_{37}Rv$ (black bar) and MDR (shaded bar) strains detected with window range 1.0 from MTB MS-LAMP software as described in materials and methods. Y-axis depicts the total number of lipid moieties in particular category of lipid and X-axis depicts the three positive monoisotropic adducts

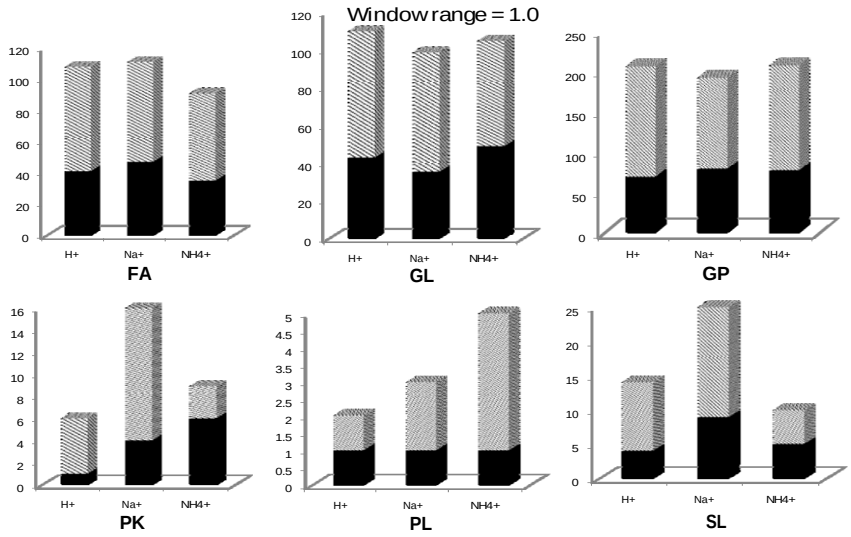


Figure.4 Venn Diagrams showing common and distinct patterns of six categories of lipid between MTB $H_{37}Rv$ (S) and MDR (R) strains detected with window range 0.75 and three positive monoisotropic adducts

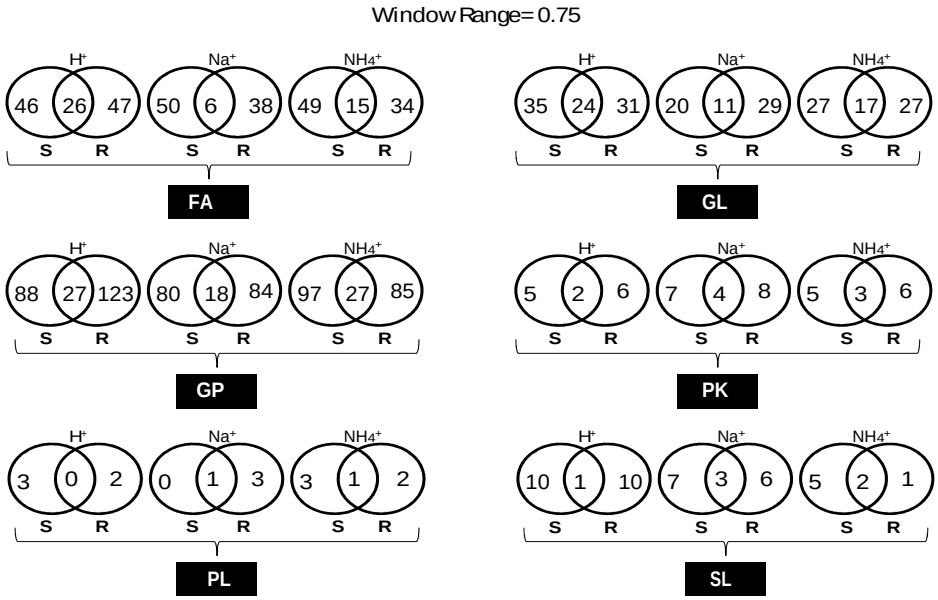
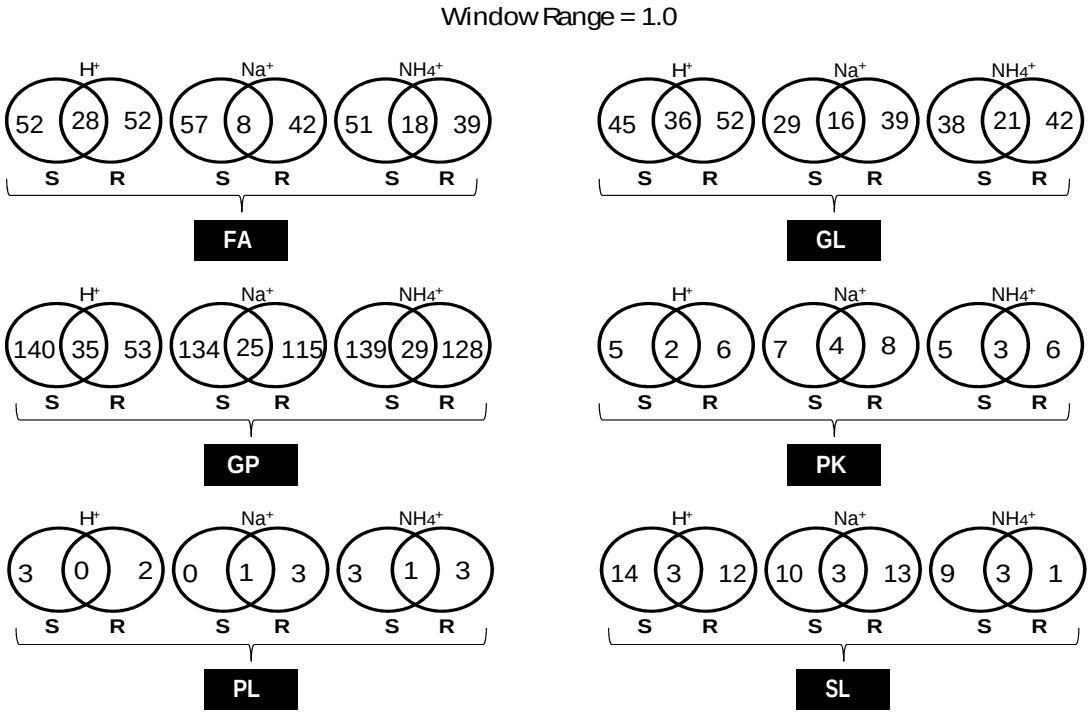


Figure.5 Venn Diagrams showing common and distinct patterns of six categories of lipid between MTB $H_{37}Rv$ (S) and MDR (R) strains detected with window range 1.0 and three positive monoisotropic adducts



The data obtained from the analysis also led us to further dissect the m/z values which were unique to sensitive and resistant MTB lipids with the help of MS-LAMP. This was again achieved taking into the consideration all the three positive ion mode adducts and both window ranges. We found a typical pattern of variation within the 6 major categories (Fig. 4 and 5). Particularly, in the FA category among all the three adducts, Na^+ adduct showed the maximum variation between the sensitive and resistant MTB lipids in both the window ranges, while the other two adduct have relatively high number of similar FAs. These observations suggest the need of further intricate study at subclass levels of the lipid categories.

In Conclusion, although from this comparative analysis no typical lipid composition or pattern emerges out which could be directly linked with the resistance in MTB or to pin point how and which lipid molecules will be involved in drug resistance, but one can safely conclude that the differences in the lipid profiles of sensitive and resistant strains do implicate a possible role of lipid composition in development of resistant phenotype in MTB. Therefore to determine the more precise correlation between lipids and drug resistance and with the advent of MS based high throughput technologies, extensive lipidomics studies should be implemented to have a closer look at molecular species which might provide deeper insights in future to substantiate the possible engagement of lipids moieties relative to sensitive and resistant MTB lipid profile. This study will be a step towards understanding the complexity of lipid environment in a systematic manner.

Author's contribution

Rahul Pal and Saif Hameed – Both authors contributed equally to this work.

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