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Research Article

ANTIBIOTIC SUSCEPTIBILITY IN BIOFILMS OF *MYCOBACTERIUM SMEGMATIS*

Tarun Kumar Sachan and Virendra Kumar*

National JALMA institute for leprosy and other mycobacterial Diseases, (ICMR) Agra-282001, India
Corresponding author's email: vksjalma@gmail.com

Abstract

In the present article we observed the quantification and morphological, ultrastructural features of biofilms of fast growing clinical isolates *M. smegmatis* in presence of first line antibacterial drug streptomycin, isoniazid rifampicin, ethambutol and pyrazinamide. Biofilm of *M. smegmatis* was found to be unaffected at concentration of drugs that inhibited growth of planktonic bacilli. Thus, the biofilm growth modus appears to be a strategy for replicating bacilli to evade the trap of antibacterials. Planktonic and biofilm cells had similar intrinsic antibiotic susceptibility. Electron microscopy revealed that control (no drug) biofilms consisted primarily of bacterial clusters and fibrillar elements. The extracellular polymeric substance (EPS) material was less abundant in antibiotic-treated than in control biofilms because in the presence of high antibiotic concentrations at MIC level. The study is explored that the effect of drug on biofilm is time dependent means if the drugs were added at initial phase of biofilm, significant inhibitory effect were observed.

Keywords: *M. tuberculosis*; *M. smegmatis*; *M. fortuitum*; *M. avium*; ECM; SEM; Ruthenium Red (RR)

Introduction

Biofilms are populations of micro-organisms growing on a surface that are surrounded by a complex extracellular polymeric substance (EPS) composed of proteins, glycoproteins, glycolipids, polysaccharides, mycolic acid and extracellular DNA (Flemming *et al.*, 2007). Reduced susceptibility of biofilm bacteria to antimicrobial agents is a crucial problem for treatment of diseases (Costerton, *et al.*, 1999; Mah, and O'Toole, 2001). It has been estimated that 65% of microbial infections are associated with biofilm (Potera, 1999) cells are 100 to 1,000 times more resistant to antimicrobial agents than planktonic bacterial cells (Costerton, *et al.*, 1999; Mah, and O'Toole, 2001). Many infectious diseases are based on bacterial growth as a biofilm (Stoodley, *et al.*, 2004). These infections are dissimilar and include colitis, vaginitis, urethritis, conjunctivitis, otitis, dental infections, biliary tract infections, prostatitis, osteomyelitis, burn, wound infections, endocarditis, lung infections in cystic fibrosis (Fux, *et al.*, 2003). Many species of mycobacteria form structured biofilm communities such as *M. avium*. *M. intracellulare*, *Mycobacterium avium* subsp. *paratuberculosis*, (Beumer, *et al.*, 2010), *M. fortuitum* (Stoodley and Scott, 1998) *M. smegmatis*, *M. gordonae*, *M. abscessus*, *M. septicum* and *M. gilvum*. (Korber, *et al.*, 1989), Ojha, *et al.*, 2008) reported biofilm formation in *M.*

tuberculosis H37 Rv. Most of the mycobacteria which produce biofilm are called NTM and these organisms may cause localized pulmonary disease, adenitis, soft tissue infections, infections of joints/bones, bursae, skin ulcers and generalized disease in individuals like leukaemia, transplant patients *etc* (Katoch, 2004). *Mycobacterium smegmatis* are opportunistic pathogens affecting people with underlying immune dysfunction or chronic disease (Falkinham, 1996). Bacteria within a biofilm often are more antibiotic resistant than are planktonic (free-living) bacteria (Parsek and Singh 2003) and biofilm infections are recalcitrant to antibiotic therapy. Unfortunately, there are no universally accepted methods for studying the antibiotic susceptibility of bacteria in biofilms. (Lewis, 2007) pointed out that although antibiotic resistance typically is defined as the ability of planktonic bacteria to grow in the presence of antibiotic concentrations above the minimum inhibitory concentration (MIC), most biofilm susceptibility studies assess antibiotic-mediated killing of biofilm-associated bacteria, rather than bacterial growth. This is important because clinical microbiology laboratories report the antibiotic susceptibility of actively growing planktonic cells. Infectious biofilms are recalcitrant to antimicrobial therapy, but the mechanism(s) responsible for the greater resistance are unclear. Experiments were designed to clarify the association between antibiotic resistance and biofilm ultrastructure.

Materials and Method

(I) Antibiotic susceptibilities of planktonic and biofilm-associated *M. smegmatis*

(i) Drug Efficacy by REMA plate method for planktonic cells of *M. smegmatis*

To determine the MIC (Minimal Inhibitory Concentration) of first line drug as Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide for the *M. smegmatis*, the REMA (Resazurin Microtiter Assay Plate) method used described by (Palomino *et al.*, 2002; Martin *et al.*, 2003). Briefly, 100 µl volume of Middlebrook 7H9 broth (Difco, USA) was dispensed in each well of a 96-well culture plate (Nunc, Denmark). Two fold increasing concentrations of selected drugs 1-256 µg/ml Streptomycin, 0.0156 to 4 µg/ml Isoniazid, 0.25 to 64 µg/ml of Rifampicin, 0.093 to 24 µg/ml Ethambutol, 0.78 to 200 µg/ml Pyrazinamide were taken for *M. smegmatis*. Perimeter wells of the plate were filled with sterile water to avoid dehydration of medium during incubation. Growth from LJ slope was scrapped into autoclaved beaded bijou bottle containing 400µl 7H9 media with OADC growth supplement & vortexes for 1 min or till no clumps observed and increasing the volume approximate 4 ml. Then standard bacterial suspension of no. 1 McFarland standard was prepared and diluted 1:10 in 7H9 broth; 100 µl inoculum was used to inoculate each well of the plate. A growth control containing no drug (positive control) and a sterile control without inoculum (negative control) were also included for each isolate. Plates were sealed and incubated at 37°C for one day. Twenty-five micro litres of 0.02% resazurin (Sigma) solution was added to each well, plate were re-incubated for an additional 1 days. A change in colour from blue to pink indicated the growth of bacteria, and the MIC was read as the minimum selected drug concentration that prevented the colour change in resazurin solution.

(ii) Drug Efficacy in Biofilm Cells of *M. smegmatis*

The cultures of *M. smegmatis* were grown in Sauton's media. Biofilm cultures were grown in the 200-µl dishes of 96-well, U-bottom, polyvinyl chloride plates (Axygen Cat. No.2797) with lids (Axygen-Cat. No-1179.GEN-MTP-L-S) as described by (Teng and Dick 2003) with two modifications. (i) To generate a biofilm growth from start to stationary phase within one day at 37°C incubation (ii) To enhance reproducibility of the growth inoculation was done with an exponentially growing pre-culture of defined cell density (rather than tooth-picking from a colony). Briefly, exponential phase pre-cultures (with 0.05% Tween 80, to prevent clumping) were grown overnight in tissue culture flasks (1-MC Farland Reagent), washed in Sauton's medium to remove Tween-80 and resuspended in Sauton's medium. The washed pre-culture was diluted to at 0.5 Mcfarland and 100µl aliquots (containing 10⁷ cfu) were

grown in dishes. streptomycin, isoniazid, rifampicin, ethambutol, pyrazinimide from Sigma. Stock solutions were made in water. Two fold increasing concentrations of selected drugs 2-512 µg/ml streptomycin, 0.125 to 32 µg/ml isoniazid, 0.125 to 32 µg/ml of rifampicin, 1.5 µg/ml to 384 µg/ml ethambutol, 12.5 µg/ml to 1600 µg/ml pyrazinamide were added in wells. Biofilm growth was monitored via crystal violet staining of the cell material. The wells were rinsed twice with water, and 120 µl of a 1% solution of crystal violet was added. Plates were incubated at room temperature for 30 min and rinsed with water three times. Quantification of biofilm formation was performed by extracting the biofilm-associated crystal violet with ethanol. 200 µl ethanol was added per dish for 10 minutes and the contents of eight dishes were pooled for measuring absorbance of crystal violet at 570 nm.

Scanning Electron Microscopy (SEM)

The mycobacterial biofilm developed in microtiter dish as described above were fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. The samples were rinsed once in the same buffer and dehydrated by increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%). The samples were dried in a fume hood and fixed on to stubs with conductive self-adhesive carbon tapes, coated with gold film sputtering and used for analysis with SEM. Samples were viewed with a SEM (S3000- N) (Hitachi High Technologies Japan, Inc.,) operated at 2.5 kV. Each biofilm was examined for at least 45 min. Results are based on >500 images from 21 samples, each processed in duplicate.

Statistical Analysis

Data (Mean±SD value) showing the effect of the different selected drugs i.e. on biofilm formation were compared. The data were analysed by Student's t test with Welch's correction at 5% level of significance and was also tested with a nonparametric test where it was required. The data were plotted and analyzed with GraphPad Prism 5 software (Graph Pad Software, San Diego, CA).

Results

The clinical isolates of *M. smegmatis* were taken for study of drug susceptibility from Mycobacterial Repository centre of our institute and characterize by biochemically and molecularly. The capacity of each strain to form a biofilm was compared with that of the confluent biofilm-forming *M. smegmatis* MC²155 control by analysing the absorbance of the crystal violet stain obtained for each biofilm. Each isolate to be assigned a percentage value depending on the proportion of biofilm and biomass which was able to establish after 7 days in comparison with the control (taken as 100%). Eight replicate wells were included for each isolate of each biofilm assay which was carried out three times.

(i) Drug Susceptibility Testing against *M. smegmatis* for selected drugs (Planktonic cells)

In this study the selected mycobacteria inhibited at different concentration of drug such as the MIC of streptomycin for *M. smegmatis* was 4 µg/ml and the. The MIC of isoniazid for *M. smegmatis* was 4 µg/ml. The inhibitory effect of rifampicin was at concentration 8 µg/ml for *M. smegmatis* respectively. Ethambutol also exhibits the inhibitory effect for *M. smegmatis* at 6µg/ml and for. The inhibitory effect of pyrazinamide was 100µg/ml for *M. smegmatis*

**(ii) Drug resistance in biofilm cells in fast grower
Drug efficacy for biofilm of *M. smegmatis***

The drug response curves for *M. smegmatis* biofilm cultures shown in demonstrate the strong inhibitory effect of isoniazid on suspension cultures, independent of the age of the exponentially growing culture shows the bactericidal effect of streptomycin incubated overnight with intact *M.*

smegmatis biofilms (rather than dispersed biofilm cells. Control (no drug) biofilms contained 10⁷ viable *M. smegmatis*, and overnight incubation with streptomycin. The MIC for isoniazid was about 4 µg /ml for planktonic cell culture in contrast, little or no inhibitory effect on biofilm growth was observed when isoniazid was added at 4µg/ ml. The MIC for biofilm growth was found to be as high as 32 µg/ ml, i.e. approximate eight times the MIC for planktonic growth Streptomycin decreased the biomass of *M. smegmatis* biofilms, as measured by the crystal violet assay. Fig. 1.

Fig. 2 shows the effect of streptomycin on the viability, metabolic capacity, and biomass of undisturbed *M. smegmatis* biofilms. Again, substantial numbers of cells survived treatment with streptomycin concentrations as the MIC for streptomycin was 4 µg/ ml for planktonic cells and for biofilm cells the MIC was 64 µg/ ml. statistically significant differences were found for streptomycin at concentration 128 µg/ ml

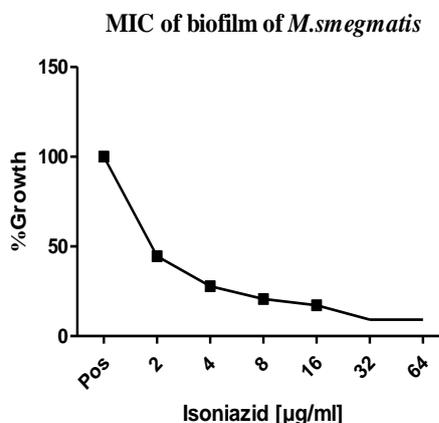
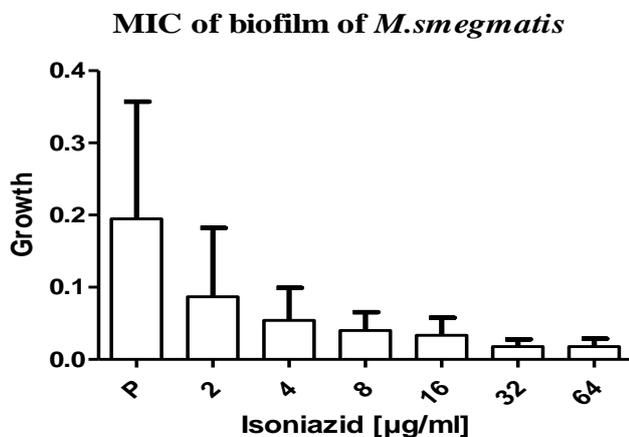


Fig. 1: Shows effect of isoniazid on *M. smegmatis* biofilm

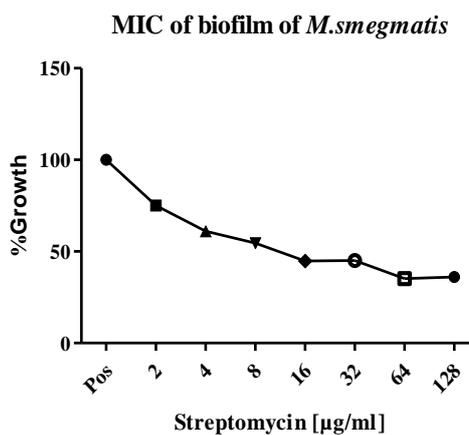
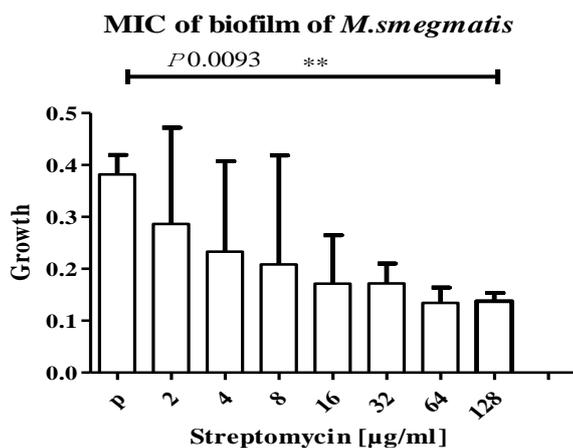


Fig. 2: Shows effect of streptomycin on *M. smegmatis* biofilm

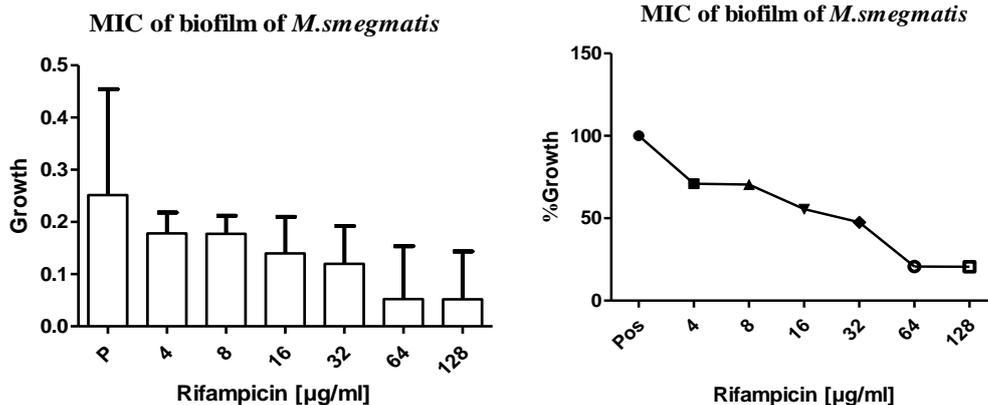


Fig. 3: Shows effect of Rifampicin on *M. smegmatis* biofilm

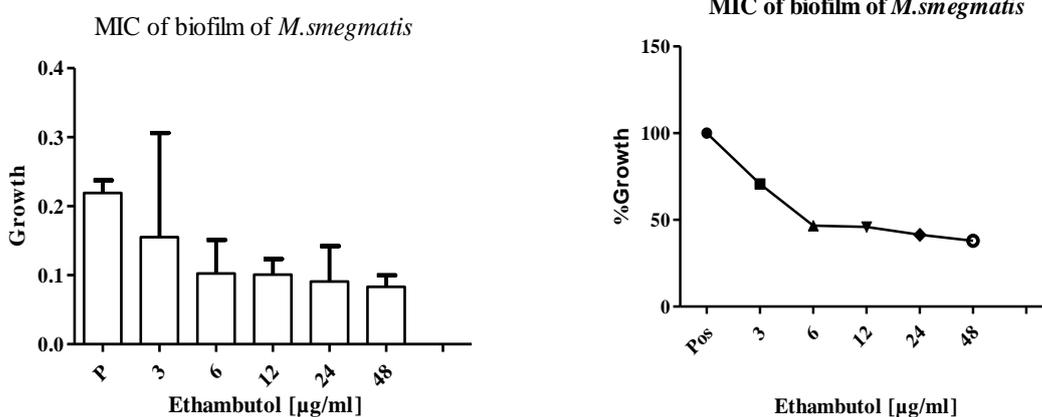


Fig. 4: Shows effect of ethambutol on *M. smegmatis* biofilm

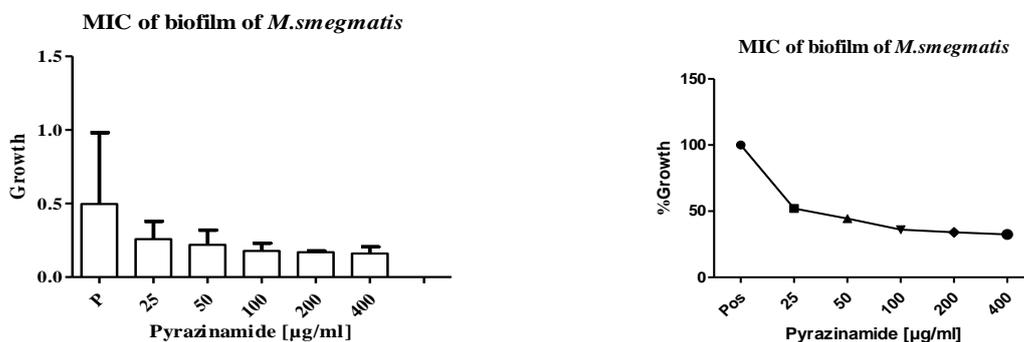


Fig. 5: Shows effect of pyrazinamide on *M. smegmatis*

Fig. 3 Rifampicin also shows strong inhibitory effect for planktonic cells at concentration 8 $\mu\text{g/ml}$ and for biofilm cells the inhibitory effect was 64 $\mu\text{g/ml}$.

Fig. 4, 5 Ethambutol and Pyrazinamide showed inhibitory effect for biofilm cells at concentration 48 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$ but for planktonic cells the effect was at concentration 6 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$.

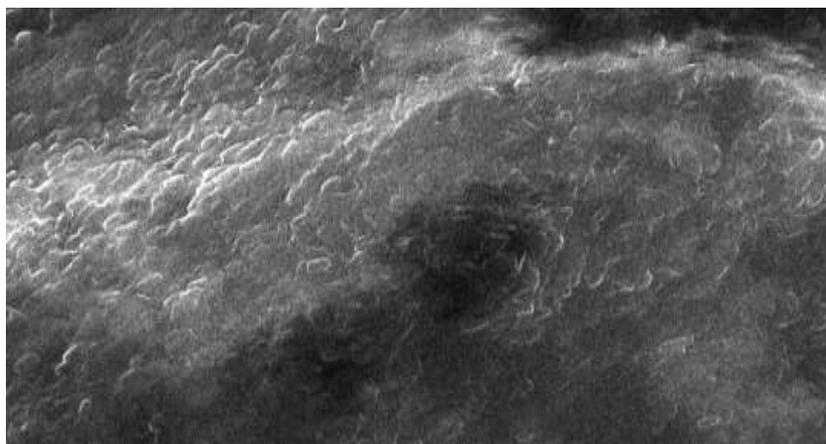


Fig. 6: Scanning electron micrographs of control (no drug) *M. smegmatis* MC² biofilms cultivated two days on Sauton's media. Low-magnification view of *M. smegmatis* biofilm showing bacilli primarily between individual sutures(asterisks), with occasional areas of dense matrix material (arrows). More clearly showing bacilli embedded in matrix material. More clearly showing bacilli enmeshed in fibrillar strands. Scale bars: 10 micrometers.

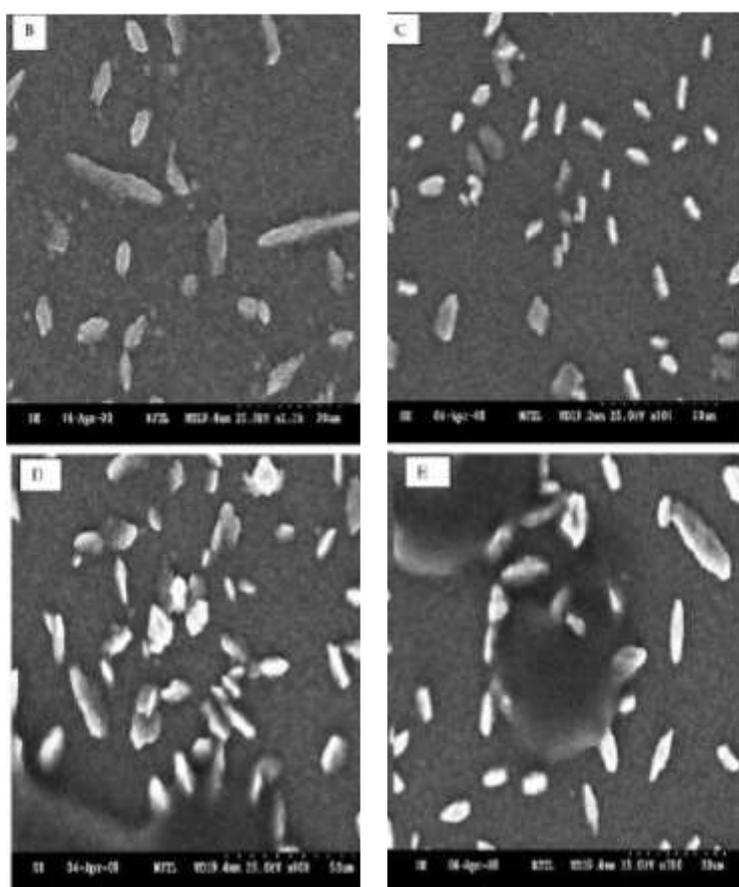


Fig. 7: Scanning electron micrographs of *M. smegmatis* 24 h on Sauton's media followed by overnight incubation with (A) Streptomycin 0.125 micrograms/mL (B) isoniazid (C) Rifampicin (D) Ethambutol. Ruffled matrix material and bacillus elements in clumps along individual silk threads, with higher magnification of the inset (B) showing these structures more clearly. Asterisk highlights a depression in the ruffled matrix similar to the size of a mycobacterial cell. (C) Higher magnification view of ruffled matrix, with asterisks highlighting circular depressions similar to size of mycobacterial allowing reasonable contraction of the hollowed circular material. Scale bars: B= 25 micrometers; C=5 micrometers; D= 0.5 micrometers.

Discussion

Although decades of research on antibiotic resistance in bacteria, a complete understanding of biofilm-specific

antibiotic resistance is lacking. When bacteria are presented with a surface and adequate nutrients, they grow within complex communities, called biofilms, which display an increased resistance to antimicrobial agents (Costerton *et*

al.,1995; Hoiby *et al.*,2010). Given the heterogeneous nature of biofilms (Stewart and Franklin 2008), it is likely that multiple mechanisms of resistance and/or tolerance act together to provide an overall high level of protection against natural and synthetic antimicrobial agents. Several studies, however, are beginning to address the complexity of biofilm-specific antibiotic resistance. For instance, components of the biofilm matrix, which consists of polysaccharides, DNA, and proteins (Mann and Wozniak 2012) can contribute to antibiotic resistance in biofilms. The extracellular DNA chelates cations and induces the expression of a cationic antimicrobial resistance operon (21). Furthermore, the presence of “persister” cells in a biofilm can contribute to the survival of biofilms. In our study the antimicrobials agent were used rifampicin, streptomycin, isoniazid, ethambutol and pyrazinamide for the study of antimicrobials resistance on mycobacterial biofilm. The bacteria developed different three dimensional ultrastructure for resistance of antibacterial drugs. The biofilms of *M. smegmatis* were more abundant, larger, and thicker in nature, and generally comprised a single morphotype, mostly rod shaped encased in a thick covering of EPS interspersed with channels. The tendency of the bacilli to become arranged together into linear cord-like formations was apparent. Ultrastructural analysis also revealed irregular and smooth crystalline structures, which appeared to be calcifications of biofilm material or the formation of mushroom shaped structures. Other studies also given the same evidence such as in *M. fortuitum* biofilm revealed the cell clusters exhibited heterogeneous morphology with a mycelial-like and pleomorphic cell structures. At higher magnification, mycobacteria of different sizes ranging from short curved rods to longer branching rods were evident. The efficacy of first line drug in biofilm formation was very dissimilar and depended on chemical composition of biofilm of mycobacteria. However, knowledge about the diverse components of extracellular matrix is still very limited, with almost nothing being known about the extracellular matrix of mycobacteria. In contrast to most bacteria which produce robust biofilms, mycobacteria do not produce exopolysaccharides. Given the important role of short-chain mycolic acids in developing the architecture of *M. smegmatis* biofilms. Bactericidal effect of first line drug was different for biofilm growth. Rifampicin (RMP) inhibits the crucial *rpoB* gene product β -subunit of DNA-dependent RNA polymerase activity of bacterial but not of mammalian origin, performing early in transcription. It binds to the β subunit, close to the RNA/DNA channel, and physically blocks the elongation of the growing RNA chain after 2 - 3 nucleotides have been added. RMP is one of the most potent anti-tuberculosis drugs. RMP is bactericidal for *M. tuberculosis*, and is active against both growing and stationary phase bacilli. The streptomycin works as a RNA level and inhibited protein synthesis. Additionally,

isoniazid (INH) inhibits InhA, a nicotinamide adenine dinucleotide (NADH)-specific enoyl-acyl carrier protein (ACP) reductase involved in fatty acid synthesis, resulting in loss of acid-fastness, probably as a result of the inhibition of the synthesis of mycolic acids, the long-chained found in the cell wall. Both INH and ETH are prodrugs that are activated by the catalase-peroxidase KatG (Mitchison, 2005) monooxygenase EthA, respectively, and they both target the NADH-dependent enoyl-ACP reductase InhA (Mitchison, 2005) inhibited the mycolic acid synthesis. Isoniazid shows specific activity of *M. smegmatis* biofilm cell growths and also destroys mycolic acid. As we have previously reported the component of biofilm is mycolic acid and glycopeptidolipid and isoniazid is a potent inhibitor of cell wall mycolic acid and other multiple effects on DNA, lipids, carbohydrates and NAD metabolism. PZA is an important first-line drug used along with INH and RMP and plays an essential role in TB treatment because it kills a population of latent bacilli in an acidic pH environment in lesions which is not killed by other drugs.

The work reported here observed that the effect of drug on biofilm is time dependent. Means if the drugs were added at the initial phase of biofilm, significant inhibitory effects were observed. Clarithromycin, when added at day 0 or 4 after bacterial seeding on PVC plates, significantly inhibited the formation of *M. avium* biofilm. Treatment at day 7 had no significant effect on the course of biofilm formation.

We demonstrate here for the first time that biofilm cultures of a Mycobacterium are capable of growing at higher drug concentrations (i.e. have higher MICs) than suspension cultures: the MIC of selected drugs concentration that inhibited exponentially growing biofilm was found to be higher than the MIC for planktonic culture.

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Conflict of interest statement: None

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