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TOPOGRAPHIC IMAGING OF *MYCOBACTERIUM SMEGMATIS* CELLS SURFACE TREATED WITH ETHAMBUTOL AND RIFAMPICIN

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ABSTRACT

The mycobacterial cells were treated with 0.96 mg/ml of Ethambutol (EMB) and 0.64 mg/ml of Rifampicin (RIF) in Luria Bertani (LB) broth. The native and treated cells were serially diluted (10^{-1} to 10^{-30}), from the 10^{-25} dilution native cells and antibiotics treated cells were imaged using Atomic Force Microscopy (AFM). The normal surface roughness (R_{rms}) of the native cells was 0.353 nm, whereas the surface roughness for EMB treated cells was 3.14 nm. The RIF treated cells pores (width size: 19.86 nm) were created on the surface of the cell membrane. This paper provides a new finding on EMB was alone creates the roughness on the cell wall surface of *Mycobacterium smegmatis*. Interestingly we got the better image of after treatment of RIF was creates pores on the cell wall surface of *M. smegmatis.*

KEYWORDS: ***Mycobacterium smegmatis,*** **Ethambutol, Rifampicin, Roughness, Pores**

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INTRODUCTION

Mycobacterium tuberculosis is a gram-positive bacterium causing Tuberculosis (TB) in human and animals. *M. tuberculosis* as an opportunistic infection has a complex cell wall system. The World Health Organization (WHO) has estimated that the global burden of TB disease, that is the number of positive cases and mortality rates, ranges between 8.9 million to 9.9 million [1]. In the study presented here, we used a model microorganism, *M. smegmatis*, which is a non-pathogenic and rapidly growing; to investigate the efficiency of antibiotics drugs for the treatments of *M. tuberculosis* caused TB [2]. The highly complex mycobacterial cell wall plays a vital role in the growth and survival of the pathogen in the infected host, and in the efficiency of some of the most effective antimycobacterial drugs [3]. EMB and RIF are one of the first-line drugs recommended for the treatment of *M. tuberculosis* [4]. The Atomic Force Microscopy (AFM) has opened up a range of unprecedented changes for visualizing the microbial cells in their native environment [5]. In *in-situ* AFM analysis are permits to visualize the cells without using harsh chemical of physical treatment and another interest in the pharmacological background is the possibility to visualize the effect of antibacterial drugs on the cell surface [6]. Early investigations performed in air demonstrated the ability of AFM to visualize drug-induced alterations in the cell walls of *Escherichia coli* [7], Helicobacter *pylori* [8], *Staphylococcus aureus* [9]. In the present study, we have treated the mycobacterial cell wall with EMB and RIF to find the alteration in cell wall surface and compared with native cells with AFM technique.

MATERIALS AND METHODS

(i) Bacterial culture & Cell harvesting

Mycobacterium smegmatis (MTCC-993) was obtained from the Microbial type culture collection (MTCC), India. The cells were grown in Nutrient Broth (NB) (Himedia / Mumbai) and incubated at 37^0C for 24 hrs. After 24 hrs the culture was transferred into 25 ml of LB broth (Himedia / Mumbai). About 5 ml of the mycobacterial cells were centrifuged (by using Eltek RC4815S) at 10,000 rpm for 10 min at 4ºC. The supernatant was discarded, and the cell pellet was washed 3 times with sterilized deionized water. The cells were serially diluted in up to 10^{-30} test tubes (dilution starting from 10^{-1} to 10^{-30}). From 10^{-25} test tube 5 µl of the diluted culture was transferred onto a freshly cleaved mica surface and dried (BOD Incubator) at 20ºC for a few minutes. The culture was further desiccated under nitrogen gas. The sample mounted on the mica surface was pasted by adhesive carbon tape on the steel puck for topography imaging.

(ii) ***Ethambutol & Rifampicin***

M. smegmatis (MTCC- 993) cells was inoculated at (OD_{600}) of 0.005 in LB broth. The cells were grown under three conditions 1. Control (Absence of EMB and RIF). 2. The *M. smegmatis* culture was grown in LB broth with 0.96 mg/ml of Ethambutol (EMB). 3. The culture was grown in LB broth with 0.64 mg/ml of Rifampicin (RIF) antibiotics

(ii) Atomic force microscopy (AFM)

AFM images were recorded at room temperature; 25ºC in sterilized distilled water using an Innova SPM (Veeco Metrology Group, Santa Barbara, CA, USA). We used oxide-sharpened micro-fabricated Si_3N_4 cantilevers with spring constants of 0.01 N/m (Microlevers, Veeco Metrology Group, USA).

(iii) Cell wall isolation and fractionation of the polar lipids

According to Piddock L J V *et al.,* protocol we followed the isolation and purification of the cell walls. We slightly modified the protocol 1. The *M. smegmatis* cells were harvested from the stationary phase and the cells were centrifuged at 2,500 rpm/15 min; 2.The pellets were

separated and washed twice with phosphate buffered saline (PBS) and freeze-dried and 3. The A-polar lipids were extracted, and 10 mg of freeze-dried biomass was analyzed by TLC using a petroleum ether/acetone (96:4 v/v) solvent system and stained with iodine vapor [10].

(iv) Lowry's Method

The collected supernatant fractions of the samples were quantified for the protein content according to the standard protocol by Lowry's *et al*. (1958) [11].

RESULTS

The cell surface of *M. smegmatis* was visualized by AFM before and after treatment with EMB and RIF. The overall approach of the present study is represented in a schematic diagram (Fig. 1), which shows the modification of cell roughness and the pores created on the cellsurface that were imaged by AFM. The cells were serially diluted before being spread over the freshly cleaved mica surface for imaging (see the Materials and Methods section for description). Figure 2 represents the surface of native cells of 1 µm in size that were observed. A single colony of EMB treated *M. smegmatis* cell surface was imaged in an area 1 µm in size and the effect of EMB was indicated by a variation in the rms roughness (R_{rms} = 3.14 nm in a 500 nm × 500 nm area) compared with the native cells, having an rms roughness value of approximately 0.353 nm (Fig. 3). Here we found the remarkable changes in the RIF-treated single cells with an average size of 75.04 nm, and average pore size of 19.65 nm were visualized on the top of the cell wall surface of *M. smegmatis* (Fig. 4 (a-c)). The total protein content of the native *M. smegmatis* cell walls was 146.82 mg/5 ml and the total protein content in the EMB treated cells was 123.01 mg/5 ml, whereas a significant reduction of total protein content of 60.31 mg/5 ml was observed in EMB treated cells (Fig. 5). The average total lipid content of the migration positions: for the EMB-treated cell wall, the Rf value was 0.685, and the RIF treated cell wall Rf value was 0.575 compared with a native Rf value of 0.405 (Fig 6).

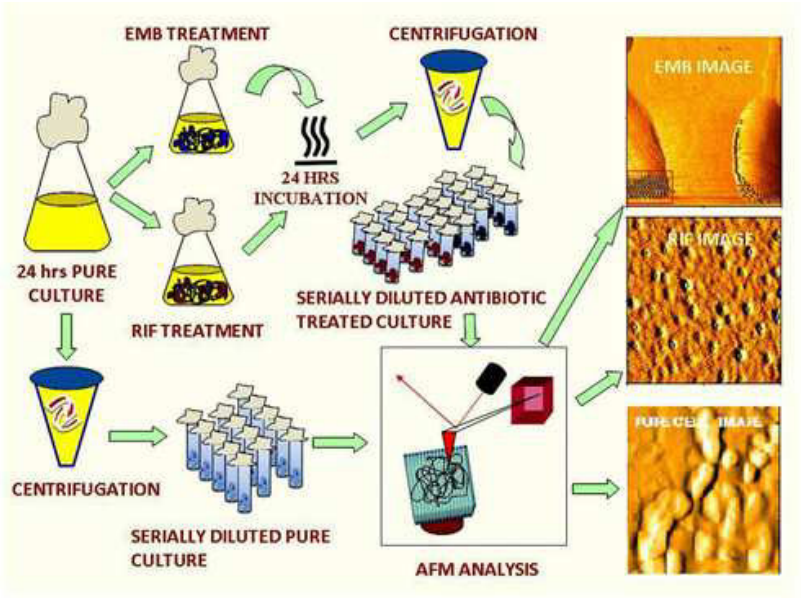

Figure 1
Schematic representation of M. smegmatis in AFM analysis.

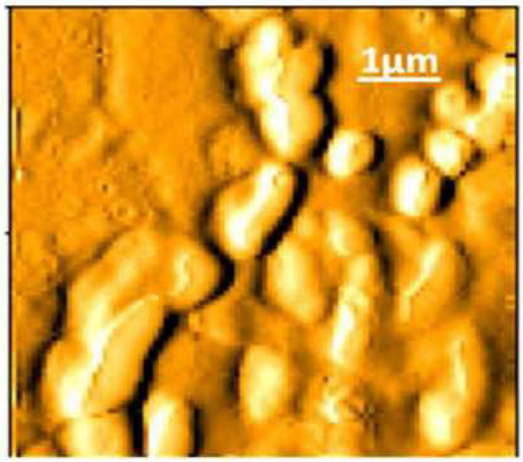

Figure 2
The homogeneous cell surface of native M. smegmatis.

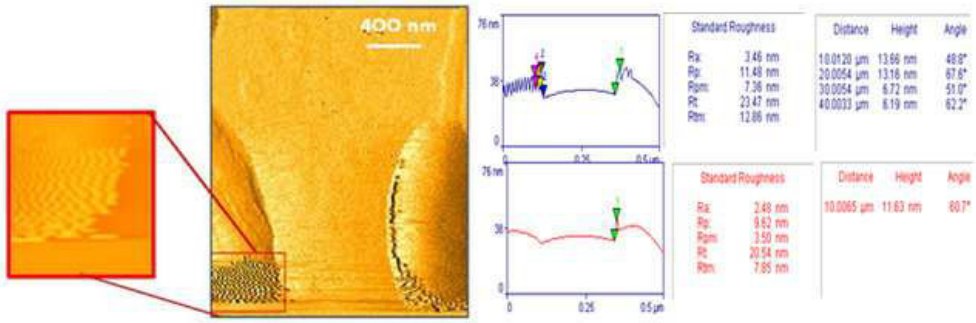

Figure 3
Roughness created on a single colony of M. smegmatis treated with Ethambutol.

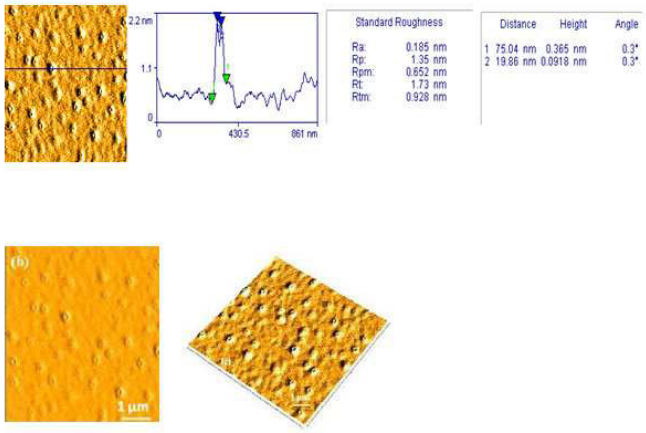

Figure 4
Height (a) and deflection (b) images of pores created on M. smegmatis by the treatment with Rifampicin (c) 3D view of pores on Mycobacterium smegmatis cell surface.

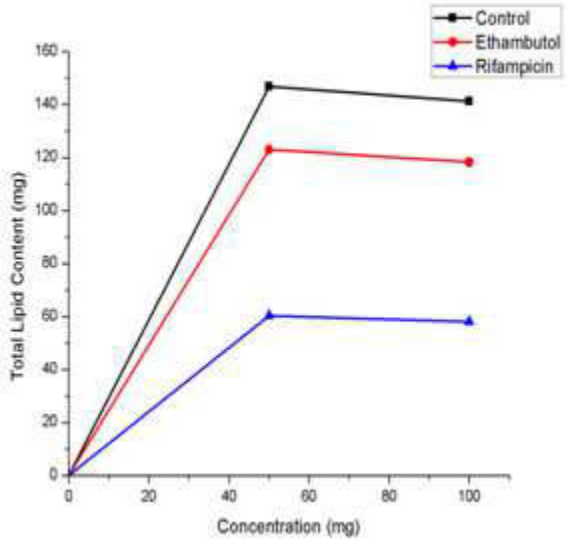

Figure 5
Total lipid content of M. smegmatis at different concentrations for native cells and Ethambutol- and Rifampicin-treated cells using the Lowry's method.

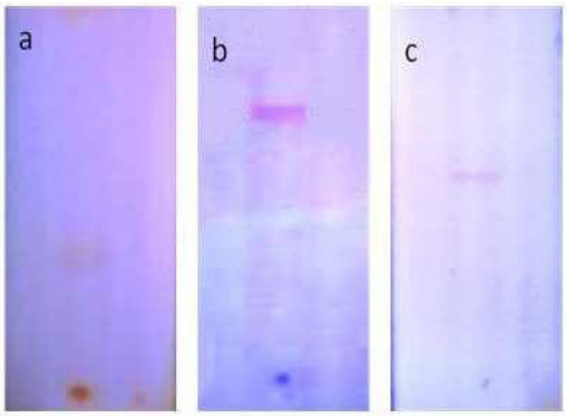

Figure 6
TLC of M. smegmatis lipids. Apolar lipids were extracted and dissolved in 0.2 ml dichloromethane. Samples (1 µl) of lipids isolated from stationary-phase native cells (a), Ethambutol-treated cells (b) and Rifampicin-treated cells (c) were applied to TLC plates and were developed in petroleum ether/acetone (96: 4, v/v), dried and stained with iodine vapor.

DISCUSSION

Generally antimycobacterial drugs are used to treat Tuberculosis and BCG vaccination to prevent the Tuberculosis diseases. The current aspects of TB infection and chemotherapy have not been determined. For example, Mode of action, Surface properties and mechanisms of resistance of the first line drugs (e.g. Ethambutol and Rifampicin) have not been fully understood for the past 35 years after their introduction. Normally the TB patients have

used first line drugs the combination of three antibiotics the mode of action is in a different ways. Ethambutol can cleave the surface cell wall of LAM and Rifampicin can inhibit the RNA molecules. The primary investigation into the alteration of cell wall surface treated with individual drugs such as Ethambutol and Rifampicin were imaged using Atomic Force Microscopy. Alsteens *et al* have reported that the treated *Mycobacterium semgamtis* cells were filtered with polycarbonate membrane and mechanically immobilized cells were imaged using AFM[12]. In this study we used the serial dilution method instead of using Polycarbonate membrane; from the 10^{-6} dilution one ml of the sample was transferred to the cleaved mica surface and imaged using AFM. From the 10^{-6} dilution more number of bacterial colonies were attached on the surface of mica, after that we increased the serial dilution rate starts from 10^{-1} to 10^{-30} dilution to minimize the number of colonies. From the 10^{-25} dilution we obtained single colonies of Mycobacterium cells and were attached on the surface of mica and the native *Mycobacterium smegmatis* cells were imaged using AFM. There have been several reports mentioned the effects of Ethambutol on the mycobacterial cell wall structure and the inhibition effect of Ethambutol on increasing anti-mycobacterial activity of the drugs [13 – 17] mainly proposed to act upon several cell wall components including the transfer of mycolic acids into the cell wall, and inhibition of the LAM [18]. The structural cell wall changes may lead to resistance of *Mycobacterium tuberculosis* [19]. The current data showed the effect of native cell Roughness root-mean square 0.353(R_{rms}: 0.353 nm) in 500 nm × 500 nm, and also similar to in *Saccharomyces cerevisiae* showed (0.34 ± 0.02nm (on a 400 nm x 400 nm height image)) which is very close to the native *Mycobacterium smegmatis* cell wall [20]. The Ethambutol treated cells significantly creates roughness on the cell wall surface, the Roughness root mean square R_{rms} = 3.14 nm in a 500 nm × 500 nm area, which may leads to the cleavage of Lipoarabinomanan (LAM) on the surface of cell wall and increase the cell wall permeability. The native cell Roughness root-mean-square (R_{rms}) value is 0.5 nm and that cells treated with EMB and Streptomycin (STR) lead to even larger R_{rms} values (3 nm to 4 nm). Therefore, the significant alteration of *Mycobacterium bovis* cell surface alteration was imaged in a superimposed layer of 8 ± 1 and 12 ± 1 nm using AFM and electron Microscopy [21 & 22]. In this study we found that the Rifampicin alone created the pores on the surface of *Mycobacterium smegmatis* cells were imaged using the Atomic Force Microscopy. Similarly the *E. coli* cells treated with visible light (Photo catalyst) can damaged the cell surface with the appearance of hole like structure were imaged by AFM [23]. The moderate changes in both the target mycolic acids and more pronounced modifications were found after EMB and Streptomycin (STR) treatments, which target arabinogalactans and protein synthesis, respectively [12]. EMB causes the disruption of the cell wall layer, which induces characteristic concentric striations of the assembly of arabinogalactan. Using AFM force spectroscopy to investigate the cell wall properties treated with EMB at various concentrations level [20]. In-vitro study the bacterial cells lipids contents was confirmed and the total lipids content per 1 mg of dried cells was 0.05 ± 0.006 µg for stationary-phase cells and 0.08 ± 0.007 µg for cells [24]. The treated *Mycobacterium smegmatis* cells lipid and protein contents were reduced due to the effect of drug molecule, this molecule inhibit and denature the protein and lipid content from the *Mycobacterium smegmatis* cell wall.

CONCLUSION

Our data suggested that the treatment of EMB alone creates roughness and significantly cleaves the surface of the cell wall and RIF alone were creates pores on the cell wall of *M. smegmatis*. These findings may lead to better understanding of activity of the drug molecules and this may help to improve the existing drugs molecules for the treatment of Tuberculosis.

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