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Drug Sensitivity Assay on Development of Biofilm in Non-Tuberculosis Mycobacteria

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Abstract

The bacteria enclosed within the biofilm are extremely resistant to antibiotic treatments; such resistance can be explained by hypotheses, not necessarily limited to the following ones. First, the EPS secreted by biofilm bacteria, acts as a physical/chemical barrier, thus preventing penetration by antibodies or many antibiotics. The EPS is negatively charged and functions as an ion-exchange resin which is capable of binding a large number of the antibiotic molecules that are attempting to reach the embedded biofilm cells. Second, embedded biofilm bacteria are generally not actively engaged in cell division, are smaller in size and less permeable to antibiotics. 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. The biofilm literature offers essentially three possible explanations for biofilm-associated drug resistance: (i) Slower biofilm growth, (ii) Decreased drug penetration into the biofim due to architecture and/or extracellular matrix of the biofilm, (iii) Resistance mechanisms expressed specifically in biofilm bacilli but not in planktonic cells. The generation times of biofilm and planktonic *M. smegmatis,* and *M. fortuitum* cultures were found to be similar. Isoniazid shows specific activity of *M. smegmatis* and *M .fortuitum* biofilm cell growths. The work reported here shows that the bacterium is furthermore capable of differentiating into a multicellular, surface-attached form that is resistant to growth inhibition by antimycobacterials.

Keywords: *Biofilm, Mycobacteria, SEM, Drug resistance, EPS.*

Introduction

Mycobacterium tuberculosis causes tuberculosis which is a major global health problem in 2011, there were 8.7 million new cases of active tuberculosis worldwide and 1.4 million deaths, plus 430,000 deaths among HIV-infected patients **(WHO, 2012).** While India is the second-most populous country in the world, have more new TB cases annually than any other country. In 2012, 2.3 million TB cases were observed in India **(RNTCP, 2013).** It has been estimated that there were 3,10,000 of multidrug-resistant tuberculosis (MDR) cases occurred and caused by organisms resistant to at least Isoniazid and Rifampacin, among patients in which tuberculosis was reported in 2012. More than 60% in these patients were from China, India, the Russian Federation, Pakistan, and South Africa (**WHO 2012)**. Species other than *M. tuberculosis* and *M. leprae* have been nominated “atypical mycobacteria” or “mycobacteria other than *M. tuberculosis*” (MOTT) in the past and are now called simply “non-tuberculous mycobacteria” (NTM**).** These have been caused pulmonary and non pulmonary infections (**Katoch, 2004**). In the immunocompromised persons the infections due to NTM have been observed to be an important cause of morbidity and mortality in western countries (**Wallace**

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***et al*., 1990).** In the natural world more than 99% of bacteria survive as biofilms (**Costerton *et al*., 1999)** and according to NIH report more then 65% of all human infections are associated with biofilms formation (**Spoering *et al*., 2001)**. Biofilm is a microbial derived sessile community of bacteria in which bacteria are attached to the substratum and produce an extracellular polymeric substance (EPS) **(Donlan and Costerton, 2002).** It is approximated that the majority of all medical infections are caused by bacterial biofilms that colonize either non-biological or biological surfaces (**Costerton *et al*., 1999; Hoiby *et al*., 2011; Romling *et al*., 2012**). Abiotic surfaces such as medical devices are usually infected by biofilms. Examples include intravenous, endotracheal, Hickman and dialysis catheters, prosthetic heart valves, orthopaedic devices, tissue fillers, cardiac pacemakers and cerebrospinal fluid shunts. The presence of an exopolysaccharide matrix can slow the flow of antibiotics. Slow growth undoubtedly contributes to resistance to killing by antimicrobials, multidrug resistance pumps represent a generalized resistance mechanism and have been considered as an additional candidate for a resistance mechanism. Scanning electron microscopy (SEM) is a rapid and suitable means of assessing the pattern of colonization as well as screening samples for major bacterial morphotypes (Samarananyake et al., 1996). Hence, SEM was used to study the detailed surface structure and configuration of the material, which may lead to an understanding of the patterns of biofilm formation on different appliances. However, many species are known to form biofilms, little is known about the genetic requirements, patterns of gene expression, or the nature of the extracellular matrix of mycobacteria. Therefore, the present study has been designed to record the extent to which a single change in growth condition affects the formation of a useful mono species biofilm, excluding possible variables such as interspecies interactions and communication which are often observed.

Materials and Methods:

(i) *Methods for development of biofilm:*

We were taken *Mycobacterium smegmatis* MC^2 155 and *M.fortuitum* for study of mycobacterial Biofilm. *M. smegmatis* and *M.fortuitum* was subcultured and characterized biochemically and molecularly. For Planktonic cell growth, *M. smegmatis M.fortuitum*was grown in Middlebrook's 7H9 broth supplemented with 0.05% Tween 80 and 2% glucose. For development of biofilm of *M. smegmatis, M.fortuitum*was grown in modified Sauton's medium (containing, gl^{-1}: ferric ammonium citrate 0.0167, L-aspargine 1.33, citric acid 0.66, magnesium sulphate 0.166, dipotassium hydrogen phosphate 0.287, sodium dihydrogen phosphate 0.633, sodium chloride 0.4, and glucose 2% w/v).One loopfull culture of *M. smegmatis, M.fortuitum*from the LJ slopes was scraped and suspended in 300 µl of Middlebrook's 7H9 media with Tween 80 in a dilution bottle and vortexed to break clumps. This suspension in dilution bottle was made up to 5 ml with Middlebrook's 7H9 medium and incubated at 37^0C up to 36-40 hrs (log phase). Culture from these bottles was centrifuged at 1467 RCF for 5 minutes at 4°C in eppendroff tubes. Subsequently, the Pellet was washed in PBS to remove the media and suspended in 200 µl Sauton's media containing 2% glucose. After this, 200 µl culture media was used to inoculate in 40 ml Sauton's media in tissue culture flask of 200 ml. The flasks were incubated in a humidified incubator and observed at different periods of intervals. For observing the surface pellicles, cultures were similarly inoculated and incubated in 30 ml Sauton's medium in 90 mm diameter polystyrene petri dishes.

(ii) *Electron Microscopy:*

Electron microscopes are scientific instruments that use beams of energetic electrons to examine objects on a very fine scale. Based on the design of optical microscopes, electron microscopes exploit the fact that fast moving electrons have a much smaller wavelength than visible light, which results in high-resolution images. Electron microscopes can routinely image at magnifications over 1,000,000×, compared to light microscopes which are limited to magnifications of the order of 2000×. Electron microscopes use an electron gun to generate the beam of energetic electrons. Whereas the light microscope uses glass lenses to magnify and focus images, the electron microscope uses magnetic lenses to magnify and focus images. Since electrons

cannot travel freely in air, electron microscopes are built into airtight metal tubes or “columns” and use vacuum pumps to remove all the air from within the microscope.

Biofilm was grown in tissue culture flasks as described above and fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for overnight at 4^0C. The samples were rinsed in the same buffer and dehydrated by increasing concentrations of ethanol (30 %, 70 %, and 90% and 100 %). The samples were coated with gold film sputtering and used for image analysis of *M.smegmatis* and *M.fortuitum* by Scanning Electron microscope (Hiachi S 3000 N).

***(iii) Drug Efficacy by REMA plate method for planktonic cells*:**

To determine the MIC (Minimal Inhibitory Concentration) of first line drug such as Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide the *M.fortuitum, M.smegmatis,*. Briefly, 100 µl volume of Middlebrook 7H9 broth **(**Difco, USA) was dispensed in each well of a 96-wellculture plate (Nunc, Denmark).

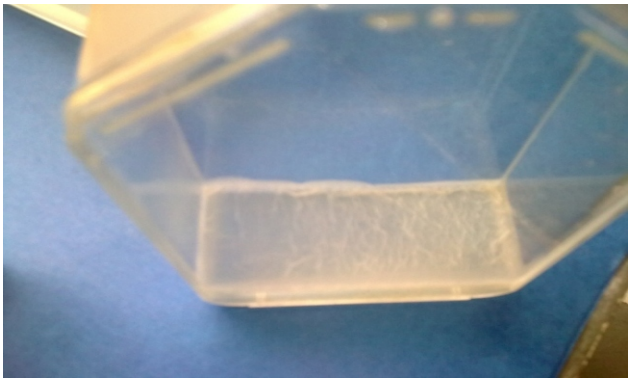


Fig. 1. Thin Biofilm of *M. smegmatis*

Fig. 2. Thick Biofilm of *M. fortuitum*

Two fold increasing concentrations of selected drugs 1-256µg/ml Streptomycin, 0.0156 to 4 µg/ml Isoniazid, 0.25 to 64 µg/ml of Rifampicin, 0.093 to 24µg/ml Ethambutol, 0.78 to 200µg/ml Pyrazinamide were taken for *M.smegmatis*, *M.fortuitum*, were prepared directly in the MB7H9 medium. Perimeter wells of the plate were filled with sterile water to avoid dehydration of medium during incubation. Growth from LJ slope was scrapped in to 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 inoculums was used to inoculate each well of the plate described by **(Palomino et al 2002, Martin et al 2003).** A growth control containing no drug (positive control) and a sterile control without inoculum (negative control) were also included for each isolate **(**Table 6). Plates were sealed and incubated at 37ºC for one week. Twenty-five micro

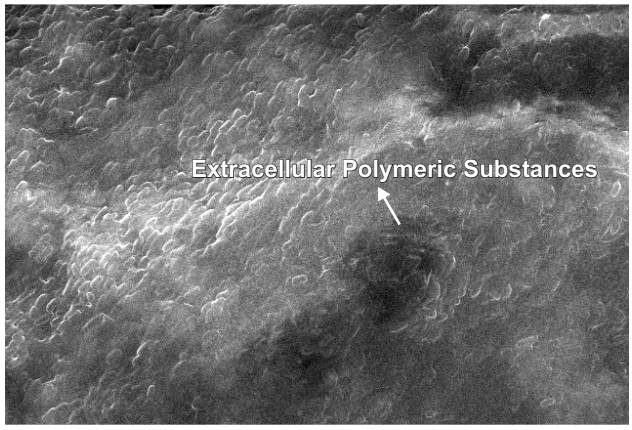


Fig. 3. Ultrastructure of biofilm of *M.smegmatis* at scale 20µM

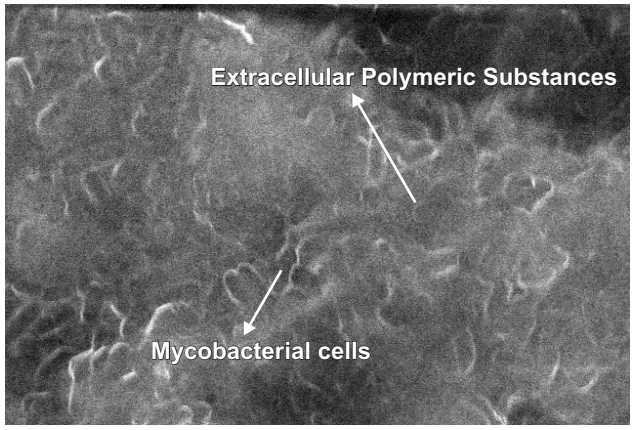


Fig. 4. Ultrastructure of biofilm of *M.smegmatis* at scale 10µM

Table 1. Different concentrations of drugs (µg/ml) for planktonic cells of *M.smegmatis, M.fortuitum.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W
B	S^{1}	S^{2}	S^{4}	S^{8}	S^{16}	S^{32}	S^{64}	S^{128}	S^{256}	GC	M	D/W
C	$I^{0.0156}$	$I^{0.0312}$	$I^{0.062}$	$I^{0.125}$	$I^{0.25}$	$I^{0.5}$	I^{1}	I^{2}	I^{4}	GC	M	D/W
D	$R^{0.25}$	$R^{0.5}$	R^{1}	R^{2}	R^{4}	R^{8}	R^{16}	R^{32}	R^{64}	GC	M	D/W
E	$E^{0.093}$	$E^{0.18}$	$E^{0.375}$	$E^{0.75}$	$E^{1.5}$	E^{3}	E^{6}	E^{12}	E^{24}	GC	M	D/W
F	$p^{0.78}$	$p^{1.56}$	$p^{3.125}$	$p^{6.25}$	$p^{12.5}$	p^{25}	p^{50}	p^{100}	p^{200}	GC	M	D/W
G										GC	M	D/W
H	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W

Table 2. Different concentrations of drugs for biofilm cells of *M. smegmatis,M. fortuitum*

	1	2	3	4	5	6	7	8	9	10	11	12
A	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W
B	D/W	S^{2}	S^{4}	S^{8}	S^{16}	S^{32}	S^{64}	S^{128}	S^{256}	S^{512}	GC	M
C	D/W	$I^{0.125}$	$I^{0.25}$	$I^{0.5}$	I^{1}	I^{2}	I^{4}	I^{8}	I^{16}	I^{32}	GC	M
D	D/W	$R^{0.125}$	$R^{0.25}$	$R^{0.5}$	R^{1}	R^{2}	R^{4}	R^{8}	R^{16}	R^{32}	GC	M
E	D/W	$E^{1.5}$	E^{3}	E^{6}	E^{12}	E^{24}	E^{48}	E^{96}	E^{192}	E^{384}	GC	M
F	D/W	$P^{12.5}$	P^{25}	P^{50}	P^{100}	P^{200}	P^{400}	P^{800}	P^{1600}	D/W	GC	M
G											GC	M
H	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W	D/W

litres of 0.02% resazurin (Sigma) solution was added to each well, plate were re-incubated for an additional 2 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 **(Martin A, Palomino JC and Portaels F 2005).**

Results

Characteristics of mycobacterial biofilm-: The biofilms produced by fast grower *M. smegmatis* and *M.fortuitum* appeared as surface pellicles (i.e., they appear at the air-liquid interface). The surface pellicle of maturing biofilm of *M.smegmatis* MC ^{2} 155 and clinical isolates of *M.smegmatis* , *M. fortuitum* at day 5 after inoculation had a characteristic ridge-like appearance **(Figure 1&2).** At day 7, the complex network of ridges appeared to be more distinct. The differences between the day 5-and day 7- biofilms are difficult to ascertain. The air-liquid interface, where these biofilms form, can be seen in each tissue culture flask in the adjacent wall. The biofilm appeared to be pulling away from the sides of the PVC plastic wall of the flask revealing the bulk fluid sequestered underneath the surface pellicle.

Drug Susceptibility Testing against NTM, 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 MIC of *M. fortuitum* was 8 µg/ml. The MIC of Isoniazid for *M. smegmatis, M. fortuitum* was 4 µg/ml and 2 µg/ml. respectively.

The inhibitory effect of Rifampicin was 4 µg/ml for *M. fortuitum* and the concentration 8 µg/ml, was for *M. smegmatis*. Ethambutol also exhibits the inhibitory effect for *M. smegmatis* at 6µg/ml and for *M. fortuitum* were 3µg/ml. The inhibitory effect of Pyrazinamide was 50µg/ml for *M. fortuitum*, and 100µg/ml for *M. smegmatis.*

***Drug Efficacy in biofilm cells of M. smegmatis, M. fortuitum*-:**

The cultures of *M. smegmatis* and *M. fortuitum* were grown in Sauton's media. Biofilm cultures were grown in the 200-µl dishes of96-well, U-bottom, polyvinyl chloride plates (**Axygen Cat.No.2797)** with lids (**Axygen-Cat. No-1179.GEN-MTP-L-S)** as describedby (**Teng R 2003)** with two modifications.(i) To produce a biofilm growth curve from start tostationary phase within 1 day (rather than several days)incubation was at 37^{0}C (ii) To increase reproducibility of the growth curve 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 10^7cfu) 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. **(Table. 2).** 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.

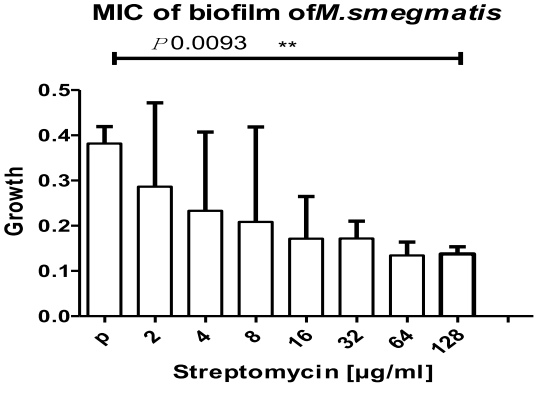


Fig. 5 (i)

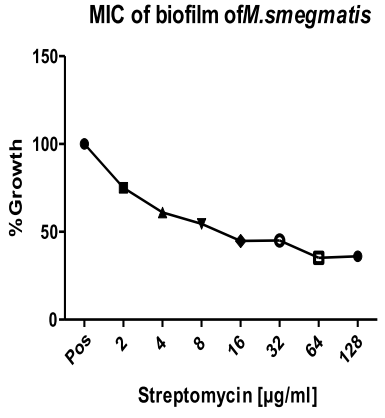


Fig. 5 (ii)

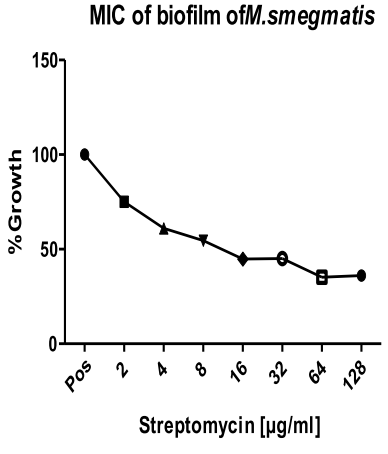


Fig. 6 (i)

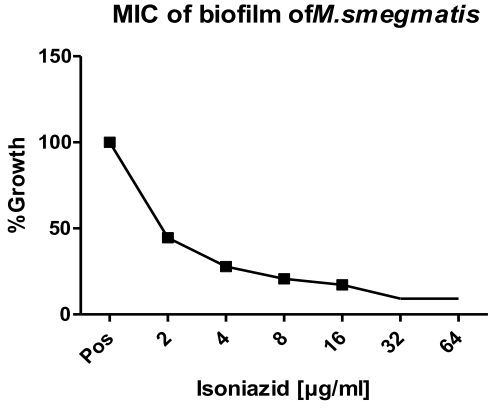


Fig. 6 (il)

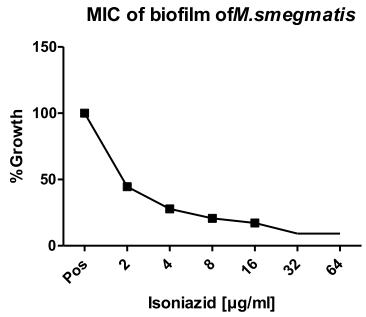
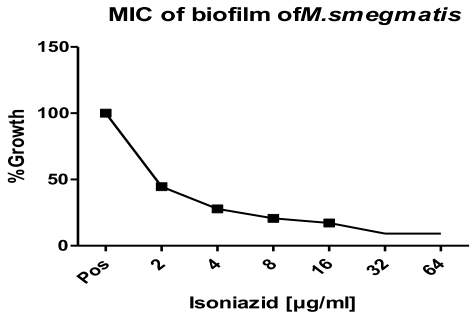


Fig. 7 (i)



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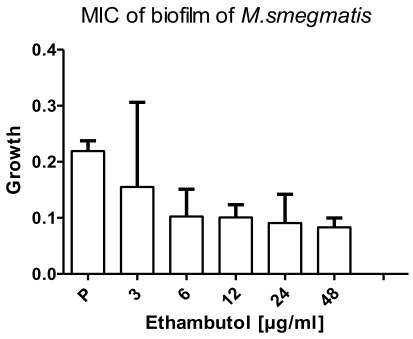

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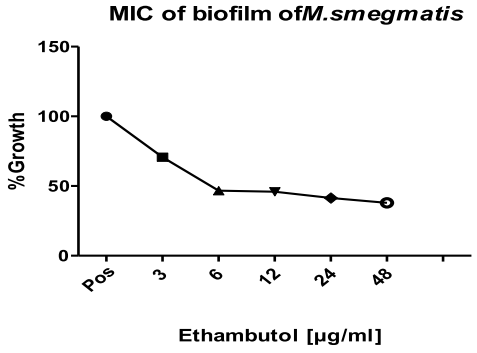
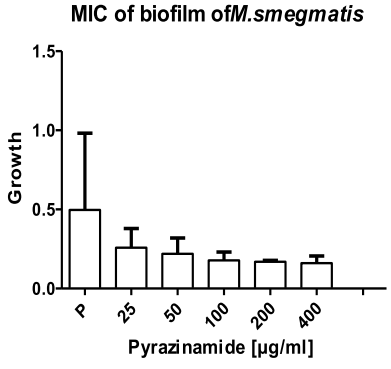


Fig. 8 (ii)



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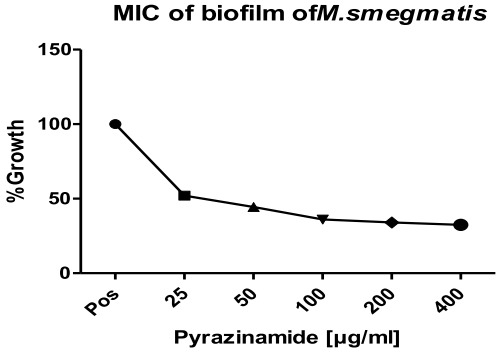

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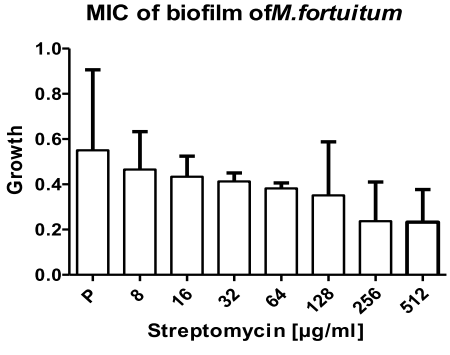


Fig. 10 (i)

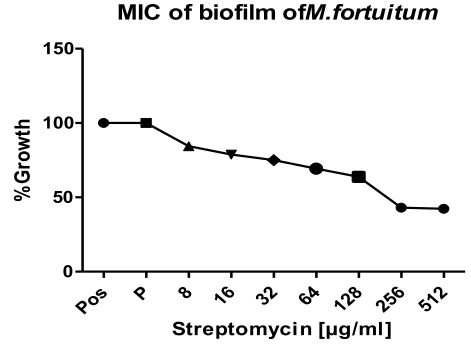
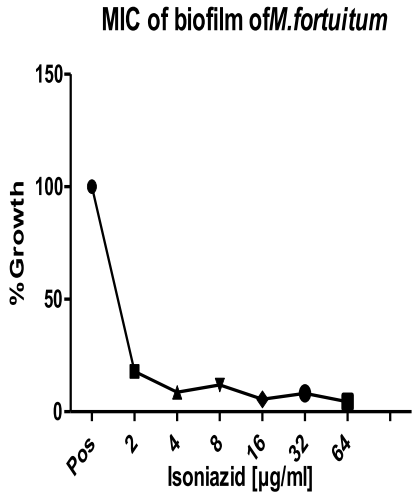


Fig. 10 (ii)



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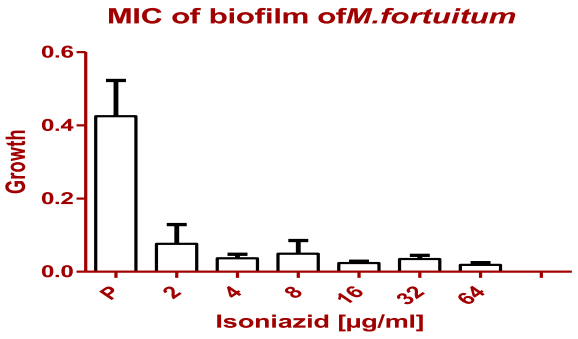
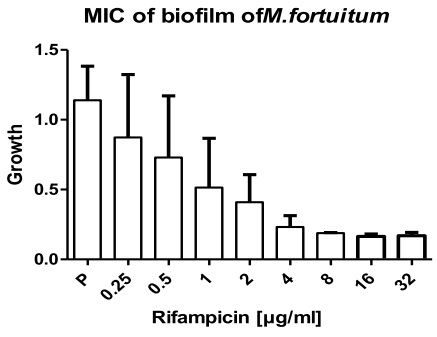


Fig. 11 (ii)



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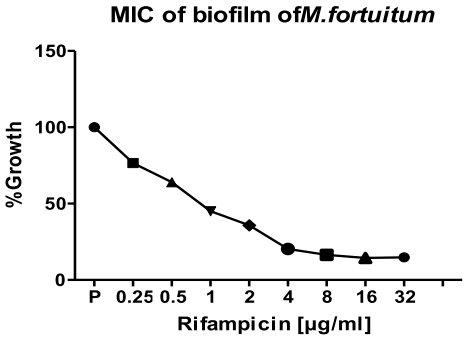

Fig. 12 (ii)

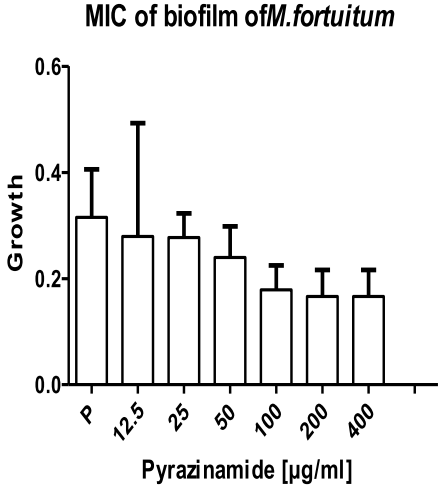

Fig. 13 (i)

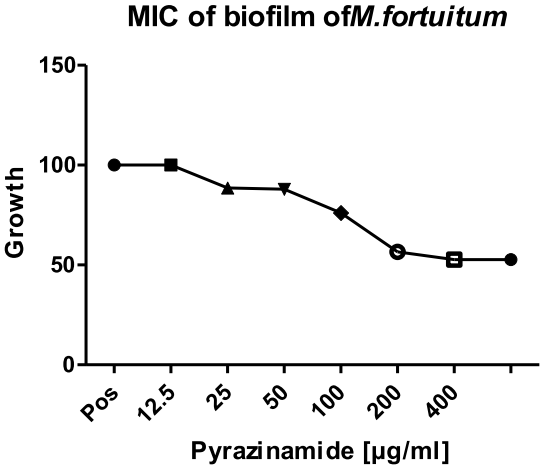

Fig. 13 (ii)

Drug efficacy for biofilm of M.smegmatis:

The drug response curves for *M.smegmatis* biofilm cultures shown in **Fig.1,** demonstrate the strong inhibitory effect of Isoniazid on suspension cultures, independent of the age of the exponentially growing culture. 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 (**Table-2**). The MIC for Streptomycin was 4 µg/ ml for planktonic cells

and for biofilm cells the MIC was 64 µg/ ml. Rifampicin also shows strong inhibitory effect for planktonic cells at concentration 8 µg/ ml and for biofilm cells the inhibitory effect was 64 µg/ ml. Ethambutol and Pyrazinamide showed inhibitory effect for biofilm cells at concentration 48 µg/ ml and 400 µg/ ml but for planktonic cells the effect was at concentration 6 µg/ ml and 100 µg/ ml. Statistically significant differences were found for Streptomycin at concentration 128 µg/ ml **Fig No,5,6,7,8,9. (i&ii).**

Drug efficacy for biofilm of M. fortuitum:

M. fortuitum developed strong biofilm as compared to other bacteria. The inhibitory effect of Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide was 8 µg/ ml,2 µg/ ml, 4 µg/ ml, 3 µg/ ml, 50 µg/ ml for planktonic cells and for biofilm cells the MIC was 256 µg/ ml, 32 µg/ ml, 16 µg/ ml, 24 µg/ ml, 200 µg/ ml for the same drugs. Statistically no significant differences were found between the isolates. **Fig No-10, 11, 12, 13, (i&ii).**

Discussion

Bacteria that adhere to implanted medical devices or damaged tissue can become the cause of persistent infections. **(Costerton JW. et al., 1999; Costerton JW. et al., 2000).** These bacteria encase themselves in a hydrated matrix of polysaccharide and protein, forming a slimy layer known as a biofilm. Biofilm formation is important because this mode of growth is associated with the chronic nature of the subsequent infections, and with their inherent resistance to antibiotic chemotherapy. Periodontitis and chronic lung infection in cystic fibrosis patients are examples of diseases that are generally acknowledged to be associated with biofilms. **(Darveau RP. et al., 2000; Singh PK. et al., 2000)**.Various nosocomial infections such as those related to the use of central venous catheters,**(Passerini L. et al., 1992)** urinary catheters, (**Morris NS. et al., 1999)**prosthetic heart valves**,(Hyde JA. et al., 1998)** and orthopaedic devices (**Gristina AG. et al., 1994)** are clearly associated with biofilms that adhere to the biomaterial surface. These infections share common characteristics even though the microbial causes and host sites vary greatly. The most important of these characteristics is that bacteria in biofilms evade host defences and withstand antimicrobial chemotherapy. Even in individuals with competent innate and adaptive immune responses, biofilm-based infections are rarely resolved. In fact, tissues adjacent to the biofilm might undergo collateral damage by immune complexes and invading neutrophils **(Høiby N. et al., 1995**). Susceptibility tests with in-vitro biofilm models have shown the survival of bacterial biofilm after treatment with antibiotics at concentrations hundreds or even a thousand times the minimum inhibitory concentration of the bacteria measured in a suspension culture. In vivo, antibiotics might suppress symptoms of infection by killing free-floating bacteria shed from the attached population, but fail to eradicate those bacterial cells still embedded in the biofilm. When antimicrobial chemotherapy stops, the biofilm can act as a nidus for recurrence of infection. Biofilm infections usually persist until the colonised surface is surgically removed from the body. As an example of sequelae of biofilms, let us consider the case of a patient with pacemaker endocarditis. A man aged 56 years was admitted with a 4-day history of nausea, vomiting, and shaking chills. Physical examination showed a temperature of 39·2°C and tenderness in his upper right quadrant. *Staphylococcusaureus* grew from blood cultures. He was treated intravenously with 12 g cloxacillin daily for 4 weeks. 1 week after discharge he developed nausea, vomiting, fever, and sweating. Again, *S aureus* grew from blood cultures for 6 weeks he was treated with 12 g intravenous cloxacillin daily and with 600 mg oral rifampicin daily. There were no signs of endocarditis. He promptly responded to antibiotic therapy, but was readmitted a third time 9 days after discharge with the same symptoms. Once again, *S aureus* grew from blood cultures. The entire pacing system was removed and intravenous cloxacillin was continued for 4 weeks. He remained well thereafter. Swabbing of the infected pacemaker lead recovered *S aureus*, and examination by electron microscopy showed localised accretions of coccoid bacteria. Bacteria in biofilms persist in the body by a strategy that might be characterised as tenacious survival as opposed to aggressive virulence. Biofilm infections can linger for months, years, or even a lifetime. Although they compromise quality of life, these

infections are rarely fatal and are often traced to species of bacteria, suchas *Pseudomonas aeruginosa* or *S epidermidis*, that are ubiquitous in water, air, soil, or skin. These are opportunistic pathogens that persist because they are adopt at biofilm forming, in which they are protected.

The bacteria enclosed within the biofilm are extremely resistant to antibiotic treatments. Such resistance can be explained by hypotheses, not necessarily limited to the following ones (Figure 8 a). First, the EPS secreted by biofilm bacteria, acts as a physical/chemical barrier, thus preventing penetration by antibodies or many antibiotics **(Lewis et al., 2001; Lewis et al., 2001; Costerton 1995)**. Moreover, EPS is negatively charged and functions as an ion-exchange resin which is capable of binding a large number of the antibiotic molecules that are attempting to reach the embedded biofilm cells. Second, embedded biofilm bacteria are generally not actively engaged in cell division, are smaller in size and less permeable to antibiotics. Virtually all antimicrobials are more effective in killing rapidly-growing cells. Further, transition from exponential to slow/no growth are generally accompanied by expression of antibiotic-resistant factors (**Lewis et al., 2001, Brown et al., 1988; Wentland et al., 1996**). Slow growth activates the RelA-dependent synthesis of ppGpp, which inhibits anabolic processes in bacterial cells56. Interestingly, ppGpp suppressed the activity of a major *E. coli* autolysin, SLT57, which would make the cells more resistant to autolysis and could explain the mechanism of tolerance to antibiotics in slowly growing. ppGpp inhibits peptidoglycan synthesis, which would explain the decreased levels of activity of cell-wall synthesis inhibitors under starvation conditions.

Third, antibiotic degrading enzymes such as *b*-lactamase may also be immobilized in the EPS matrix, so that the incoming antibiotic molecules can be inactivated effectively. It is interesting to note that biofilm cells of the *P. aeruginosa* have been shown to produce 32-fold more *b*-lactamase than cells of the same strain grown planktonically**(Potera C. et al., 1999; Tuomanen E. et a., l986).** Fourth, up to 40% of the cell-wall protein composition of bacteria in biofilms is altered from that of its planktonic brethren **(Potera C. et al., 1999;O'Toole G. A., 2000).** The membranes of biofilm bacteria might be better equipped to pump out antibiotics before they can cause damage, or even antibiotics targets may disappear. Fifth, the antimicrobial agent is deactivated in the outer layers of the biofilm, faster than it diffuses. This is true for reactive oxidants such as hypochlorite and H2O2 (**Lewis et al., 2001; Costerton et al., 1995; De Beer et al., 1994;Xuet al., 1996**). These antimicrobial oxidants are products of the oxidative burst of phagocytic cells and poor penetration of these may partially account for the inability of phagocytic cells to destroy biofilm microorganisms. Biofilms also provide an ideal niche for the exchange of extrachromosomal DNA responsible for antibiotic resistance, virulence factors and environmental survival capabilitiesat accelerated rates, making it a prefect milieu for emergence of drug resistance pathogens. Plasmidcarrying strains have also been shown to transfer plasmids.

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. The biofilm literature offers essentially three possible explanations for biofilm-associated drug resistance: (i) Slower biofilm growth, (ii) Decreased drug penetration into the bioflm due to architecture and/or extracellular matrix of the biofilm, (iii) Resistance mechanisms expressed specifically in biofilm bacilli but not in planktonic cells. The generation times of biofilm and planktonic *M. smegmatis,* and *M.fortuitum* cultures were found to be similar. Isoniazid shows specific activity of *M.smegmatis* and *M.fortuitum* biofilm cell growths. As we have previously reported the component of mycobacterial 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 **Suzuki et al 1995.** Hence it is showed the specific activity. The another important possibility is slower growth of the biofilm culture as a mechanism for the increase in MIC appears not to play a role. This leaves

drug penetration problems and expression of biofilm-specific resistance mechanisms as possible causes for the observed resistance against isoniazid. We used the genetically tractable and fast-growing *M. smegmatis, M.fortuitum* for this study. Hence, our work provides the basis for a genetic dissection of the biofilm-associated drug resistance phenomenon uncovered in this work. Isolation of mutant strains that have lost the ability to form a bio- film at elevated isoniazid levels is currently under way. The work reported here shows that the bacterium is furthermore capable of differentiating into a multicellular, surface-attached form that is resistant to growth inhibition by antimycobacterials. A more comprehensive understanding of processes connected with biofilm development in different stress conditions is expected to lead to new knowledge that would help in developing novel and effective control strategies for prevention of biofilms in clinically relevant situations in mycobacterial diseases and hence would stimulate new thinking that would be of help in improvement in patient management.

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