Indian J Med Res 140, November 2014, pp 665-671

Time dependent enhanced resistance against antibiotics & metal salts by planktonic & biofilm form of *Acinetobacter haemolyticus* MMC 8 clinical isolate

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Received May 7, 2013

***Background & objectives*: Available literature shows paucity of reports describing antibiotic and metal resistance profile of biofilm forming clinical isolates of *Acinetobacter haemolyticus*. The present study was undertaken to evaluate the antibiotic and metal resistance profile of Indian clinical isolate of *A. haemolyticus* MMC 8 isolated from human pus sample in planktonic and biofilm form.**

***Methods*: Antibiotic susceptibility and minimum inhibitory concentration were determined employing broth and agar dilution techniques. Biofilm formation was evaluated quantitatively by microtiter plate method and variation in complex architecture was determined by scanning electron microscopy. Minimum biofilm inhibiting concentration was checked by Calgary biofilm device.**

***Results*: Planktonic *A. haemolyticus* MMC 8 was sensitive to 14 antibiotics, $AgNO_3$ and $HgC1_2$ resistant to streptomycin and intermediately resistant to netilmycin and kanamycin. MMC 8 exhibited temporal variation in amount and structure of biofilm. There was 32 – 4000 and 4 – 256 fold increase in antibiotic and metal salt concentration, respectively to inhibit biofilm over a period of 72 h as against susceptible planktonic counterparts. Total viable count in the range of 10^5 -10^6cfu / ml was observed on plating minimum biofilm inhibiting concentration on Muller-Hinton Agar plate without antimicrobial agents. Biofilm forming cells were several folds more resistant to antibiotics and metal salts in comparison to planktonic cells. Presence of unaffected residual cell population indicated presence of persister cells.**

***Interpretation & conclusions*: The results indicate that biofilm formation causes enhanced resistance against antibiotics and metal salts in otherwise susceptible planktonic *A. haemolyticus* MMC 8.**

Key words *Acinetobacter haemolyticus* - antibiotic resistance - Calgary biofilm device - MBIC - MIC - persister cells - SEM

Members of genus *Acinetobacter* are considered as one of the most important nosocomial pathogens[1] which exhibit multiple antibiotic and metal resistance and ability to form biofilm[2]. One of the members of the genus, *A. haemolyticus* is predominantly found on skin of tribal[3] and urban[4] Indian population. It has been reported to exhibit multidrug resistance against commonly used drugs for treatment[5-8] and production of haemolytic enzymes[9].

Biofilm is an important mode of bacterial life fostering elevated resistance towards antibiotics and metal salts used in medicine[10]. Earlier, biofilm of

Staphylococcus spp.[11] and *Escherichia coli*[12] have been reported to exhibit elevated resistance against antibiotics. Studies on *Pseudomonas aeruginosa* have also indicated planktonic and biofilm cells to be resistant to antimicrobial agents[13]. Mechanisms of resistance in biofilm vary significantly from that of planktonic forms[14]. Biofilm resistance mechanisms include *(i)* production of exopolymeric substances (EPS), *(ii)* slow growth rate, *(iii)* spatial and physiological heterogeneity, *(iv)* persister cells, and *(v)* small colony variants[15,16]. However, none of these mechanisms have been reported to be involved in biofilm mediated resistance of genus *Acinetobacter*.

Information on biofilm formation and comparison of antibiotic and metal resistance profile in planktonic and biofilm form of *A. haemolyticus* is not available. Thus, the present study was aimed at studying sensitivity pattern of *A. haemolyticus* MMC 8 against 20 antibiotics belonging to five classes and metal salts commonly used in treatment. The study was further extended to understand temporal variation in biofilm formation and its effect on resistance in biofilm form of *A. haemolyticus* MMC 8 in comparison to planktonic counterparts.

Material & Methods

Bacterial cultures: Sixteen *Acinetobacter* strains isolated from clinical samples in the departments of Microbiology at three different hospitals namely Madurai Medical College (MMC), Madurai, Sri Ramchandra Medical College (SRMC), Chennai. All India Institute of Medical Sciences (AIIMS), New Delhi, India, were procured and identified by 16S rRNA gene sequencing and were used for biofilm screening at Institute of Bioinformatics and Biotechnology, University of Pune, Pune (data not shown). On the basis of biofilm formation ability *A. haemolyticus* MMC 8 (GenBank EU779836) was used for further studies. For antibiotic and metal susceptibility, *Escherichia coli* NCIM 2931 (ATCC 25922) was used as per recommendations of CLSI Document M07-A9[17]. Cultures were maintained in 50 per cent (v/v) glycerol at - 80°C. Planktonic and biofilm cultures were grown at 37°C with 150 rpm and static conditions, respectively.

Susceptibility testing against antibiotics and metal salts: Susceptibility of *A. haemolyticus* MMC 8 was checked against 20 antibiotics, by Kirbey Bauer disc diffusion assay[4]. The antibiotics include amikacin, gentamicin, tobramycin, kanamycin, netilmycin, streptomycin, ampicillin, amoxycillin, amoxicillin/clavulanic acid combination, penicillin-G, ceftazidime, ceftriaxone, cefepime, meropenem, imipenem, lomefloxacin, chloramphenicol, polymyxin B, rifampicin and tetracycline. Overnight broth culture (100 µl, 10^5 cfu/ml) was spread on Muller-Hinton agar (MHA) plates and antibiotic discs (Hi-Media Ltd, Mumbai) were placed aseptically. After 24 h of incubation, zone diameters were measured. Metal susceptibility was determined by agar dilution technique as described previously[18]. MHA plates containing concentrations of 0.01, 0.1, 1, 10 mM of $AgNO_3$ and $HgCl_2$ were prepared, spot inoculated (5 µl) with 10^5 cfu / ml and incubated. After 24 h, plates were observed for presence or absence of growth.

Determination of minimum inhibitory concentration (MIC): MIC of antibiotics was determined by broth dilution technique as described by CLSI[17]. Briefly, gentamycin, tobramycin, amoxycillin/clavualnic acid, rifampicin and polymyxin B in range of 2 - 2048 µg/ml were prepared in Muller-Hinton broth (MHB). Each tube was inoculated to maintain final density at 10^5 cfu/ml and incubated. For metal salts, MHA plates containing metal concentrations in the range of 0.1 to 1 mM ($AgNO_3$) and 0.01 - 0.1 mM ($HgCl_2$) were prepared. Culture was spotted (5 µl) and the plates were incubated. After 24 h lowest concentration of antibiotic and metal salt which inhibited growth was recorded as MIC.

Biofilm formation: Biofilm formation was scored in microtiter plate as described earlier[19] with a few modifications. Briefly, culture (5 µl) was inoculated in 195 µl Luria Bertani (LB) broth maintaining final cell density of 10^5 cfu/ml. After every 24 h broth was aspirated and wells were washed with PBS (200 µl). To the dried plate, aqueous 0.1 per cent (w/v) gentian violet (200 µl) was added and allowed to stand for 15 min. After careful aspiration bound stain was solubilized in alcohol (200 µl) and absorbance was measured at 570 nm on multiplate reader (Molecular devices, USA). Sterile uninoculated L-B broth was used as negative control.

Scanning electron microscopic (SEM) analysis of biofilm: SEM analysis of challenged and unchallenged pegs was performed as described earlier[19].

Determination of minimum biofilm inhibiting concentration (MBIC): MBIC was determined employing Calgary Biofilm Device (CBD) (Innovotech, Canada). The plate trough was filled with 24.375 ml of MHB and inoculated with 0.625 µl culture (final cell

density = 10^5 cfu/ml). These plates were incubated for a period of 24, 48 and 72 h in static conditions. After the respective period of incubation CBD lid was washed with PBS and transferred to microtiter plate containing gentamycin, tobramycin, amoxycillin/clavualnic acid, rifampicin, polymyxin B, $AgNO_3$ and $HgCl_2$ dilutions. After incubation for 24 h, pegs were washed with PBS and transferred to a fresh plate containing 200 µl MHB in each well. This pegs, were sonicated in Ultrasonic cleaner, (Equitron, India) at 40 per cent power for 10 min and incubated for 24 h. The well which did not allow growth was recorded as MBIC and spotted on MHA plate. After 24 h of incubation, the spots which did not grow were recorded as minimum biofilm eradicating concentration (MBEC).

Results

Planktonic A. haemolyticus MMC 8 is sensitive to antibiotics and metal salts: *A. haemolyticus* MMC 8 was sensitive to 14 antibiotics (amikacin, gentamicin, tobramycin, ceftazidime, ceftriaxone, cefepime, lomefloxacin, ampicilin, amoxicillin, amoxicillin, clavulanic acid combination, penicillin chloramphenicol, rifampicin, tetracycline). However, it was resistant to streptomycin and intermediately resistant to kanamycin, netilmycin. In case of metal salts it was sensitive to both $AgNO_3$ and $HgCl_2$. MIC of antibiotics which are commonly used for treatment suggested *A. haemolyticus* MMC 8 to be resistant to amoxicillin/clavulanic acid with highest MIC of 2048 µg/ml. For other antibiotics like gentamicin, tobramycin and rifampicin, MIC was 8, 64 and 2 µg/ml, respectively while for polmyxin B it was 39.06 U/ml. MIC values of $AgNO_3$ and $HgCl_2$ were 0.4 and 0.03 mM, respectively (Fig. 1 a, b, c, e). Moreover, the MIC values for tobramycin and polymyxin B increased with culture age (Fig.1c, d), while for $AgNO_3$ and $HgCl_2$ these decreased to 0.1 and 0.02 mM, respectively (Fig.1f, g).

Biofilm formation of A. haemolyticus MMC 8 increases with incubation period: Biofilm formation by microtiter plate method demonstrated that *A. haemolyticus* MMC 8 established its biofilm over a period of time. The O.D. value of 24 h old biofilm was 0.192±0.0003, while for 48 and 72 h old biofilm these were 0.302 ± 0.006 and 0.85 ± 0.01, respectively. This indicated that the amount of biofilm formed by *A. haemolyticus* MMC 8 increased with increase in period of incubation (Fig.2a). Further, SEM analysis showed structural variation in compactness and architecture of biofilm. One day old biofilm (Fig.2b) comprised cellular aggregates while two days old (Fig.2c) biofilm was organized forming cellular heaps embedded within exopolymeric matrix. The three days old (Fig.2d) biofilm appeared like an intrinsically entangled aggregates of cells.

Biofilm formation enhances resistance in A. haemolyticus MMC 8 against antibiotics and metals salts: *A. haemolyticus* MMC 8 sensitive to four antibiotics (gentamycin, tobramycin, polymyxin B, rifamficin) and two metal salts ($AgNO_3$, $HgCl_2$) became resistant to all after forming biofilm. This resistance increased with age of biofilm. The minimum antibiotic concentration required for inhibiting biofilm growth was about 1000 fold higher for 24 and 48 h biofilm and 4000 fold for 72 h old biofilm (Table, Fig.1b-h). This was observed for all antibiotics. Further, on spot inoculating the well containing MBIC a few viable cells in the range of 10^5 - 10^6 cfu/ml survived the antibiotic and metal treatment. Presence of this unaffected cell population was confirmed by SEM analysis (Fig. 2 d, e). This indicated presence of metabolically inactive cells known as persister cells thriving even at high concentration of antibiotic and metals salts.

Discussion

In the present study, it was observed that *A. haemolyticus* MMC 8 produced and stabilized its biofilm over a period of 72 h. Concurrent with our results, Nancharaiah *et al*[20] have reported increasing biofilm formation of *P. putida* over a period of time.

It is important to note that one day old biofilm formed by *A. haemolyticus* MMC 8 was resistant to almost all the antibiotics and metal salts. However, three days old biofilm required relatively much higher concentrations of antibiotics and metal salts for inhibition. Earlier reports on *P. aeruginosa* have demonstrated five days old biofilm susceptible, while seven days old biofilm resistant towards tobramycin and pipercillin[21]. This indicates the severity of the infection caused by biofilm of *A. haemolyticus* and the necessity to treat at the earliest.

It was we observed that 32 - 4000 and 4 - 256 fold increase in antibiotic and metal salt concentration, respectively was required to inhibit biofilm even after 24 h of exposure as compared to planktonic counterparts. Earlier studies on *S. aureus*, *E.coli* and *P. aeruginosa* have shown it to be 2–64 times more resistant in biofilm form than in logarithmically growing planktonic cells against antibiotics and metal ions after 24 h of exposure[22]. Our results are concurrent with those of Teitzel and Parsek[23] who reported *P.*

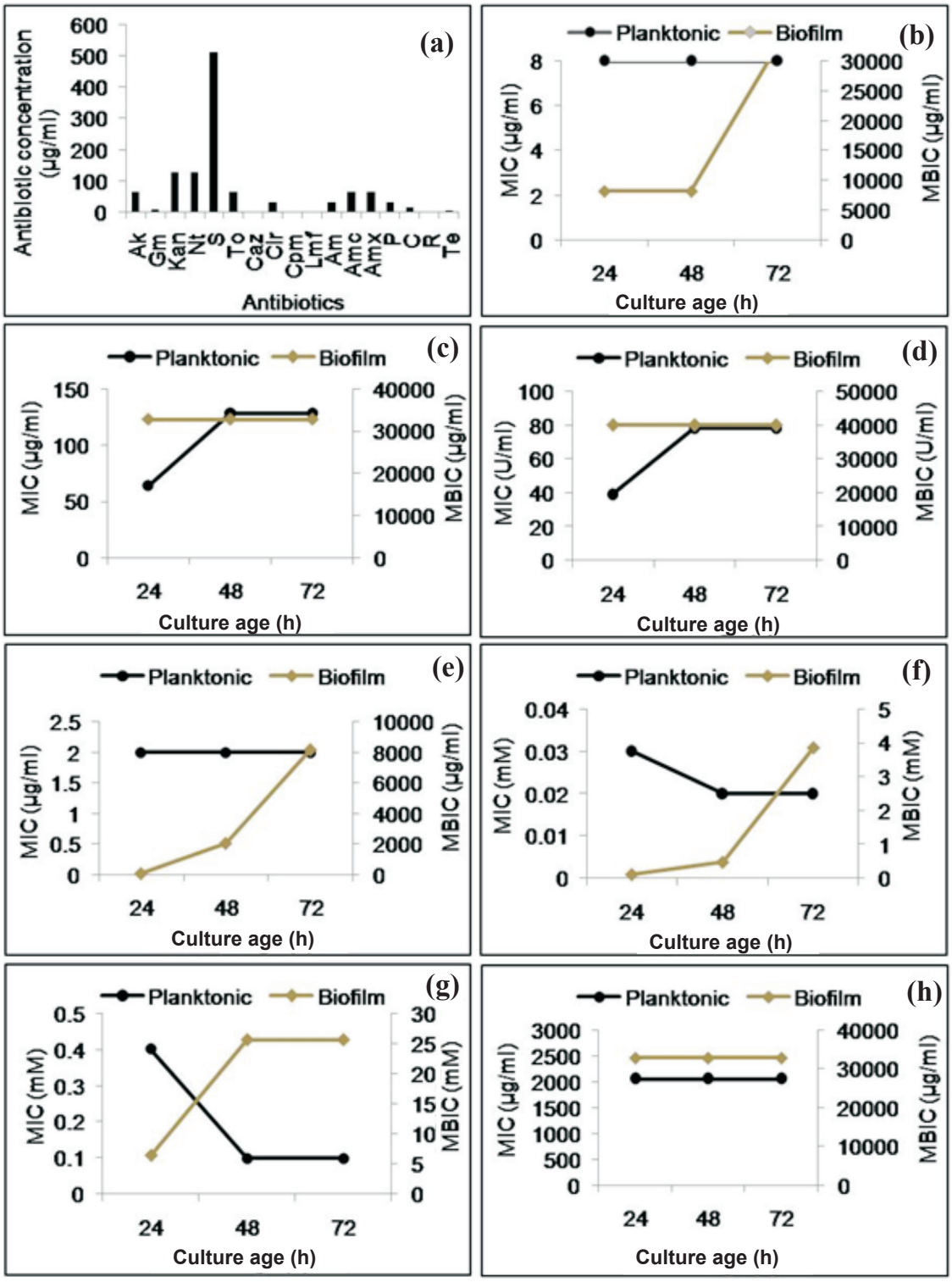

Fig. 1. MIC and MBIC of antibiotics and metal salts against *A. haemolyticus* MMC 8 **(a)** MIC of antibiotics (Ak, amikacin; Gm, gentamicin; Kan, kanamycin; Nt, netilmycin; S, streptomycin; To, tobramycin; Caz, ceftazidime; Cir, ceftriaxone; Cpm, cefepime; Lmf, lomfloxacin; Am, ampicillin; Amc, amoxycillin/clavuanic acid combination; Amx, amoxycillin; P, penicillin G; C, chloramphenicol; R, rifampicin; Te, tetracycline), **(b-h)** Comparison of MIC and MBIC of antibiotics and metal salts against *A. haemolyticus* MMC 8 over a period of 72 h; **(b)** gentamicin, **(c)** tobramycin, **(d)** polymyxin B, **(e)** rifampicin, **(f)** $HgCl_2$, **(g)** $AgNO_3$, **(h)** amoxicillin/clavulanic acid. Each experiment was repeated twice and average value is plotted.

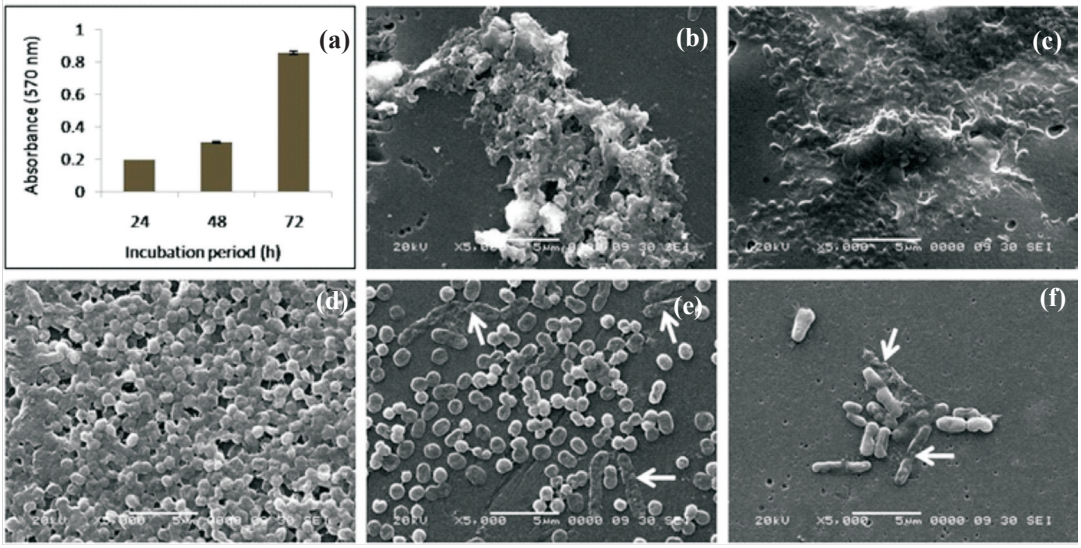

Fig. 2. Biofilm formation by *A. haemolyticus* MMC 8 **(a)** Time course by microtiter plate method, Bars and error bars indicate mean and standard deviation of six readings obtained in two sets Scanning electron micrographs **(b)** 24 h, **(c)** 48 h, **(d)** 72 h of unchallenged biofilm, **(e)** biofilm challenged with gentamicin, **(f)** $AgNO_3$. White arrows indicate affected cells due to gentamycin (8192 µg/ml) and silver nitrate (6.4 mM).

aeruginosa biofilm to be more resistant than planktonic free floating cells.

Biofilm formed by *A. haemolyticus* MMC 8 was found resistant to amoxicillin/calvulanic acid and positive for β-lactamse production. Prior studies on β-lactamse positive *Klebsiella pneumoniae* have demonstrated increased rate of antibiotic degradation than penetration in biofilm[24]. Similar mechanism may be exhibited by *A. haemolyticus* MMC 8. In our earlier studies on *A. haemolyticus* MMC 8 was found to produce exopolymeric substances (EPS) which increased with incubation period (unpublished data). EPS overproducing mutant of *P. aeruginosa* was found to produce thick biofilm which posed major problem

Table. MIC and MBIC of *A. haemolyticus* MMC 8 towards antibiotics and metal salts

Antibiotics	MIC Incubation period (h)			MBIC Incubation period (h)			Fold increase Incubation period (h)		
	24	48	72	24	48	72	24	48	72
Aminoglycosides									
Gentamycin*,#	8	8	8	8192	8192	32768	1024	1024	4096
Tobramycin*,#	64	128	128	> 32768	> 32768	> 32768	2048	256	256
Penicillins									
Amoxycillin/ Clavulanic acid*,#	2048	>2048	>2048	> 32768	> 32768	> 32768	16	16	16
Others									
Rifampicin**,#	< 2	< 2	< 2	64	2048	> 8192	32	1024	2048
Polymyxin B*,##	39.06	78.12	78.12	> 40000	> 40000	> 40000	1024	512	512
Metal salts									
Silver nitrate*,$	0.4	0.1	0.1	6.4	25.6	25.6	16	256	256
Mercuric chloride*,!,$	0.03	0.02	0.02	0.12	0.48	3.84	4	24	192

*Autoclaved distilled water was used as solvent, **DMSO (Hi-Media, Ltd, India) was used as solvent, #concentration in µg/ml, ##concentration in Units/ml, $concentration in mM, ! 0.1 M HCl was used as solvent. Fresh stocks were prepared every time before use. Values represent mean of two sets consisting of two replicates. No significant deviation was observed in two sets

in antibiotic diffusion leading to elevated resistance[25]. Moreover, EPS is known to be composed of charged polymers such as polypeptides[26], nucleic acids[27] and polysaccharides[28]. These charged molecules make EPS act as ionic resin, thereby reducing the number of antibiotic molecules and metal ions entering in the interiors of biofilm[23]. This may be the reason for elevated resistance against aminoglycosides and metals.

In the course of biofilm formation, physiological gradients are produced within the biofilm decreasing growth rate of bacteria in small areas of biofilm[29] leading to development of subpopulation which remains unaffected by antibiotic and metal salts and known as persister cells[30]. Persister cells exhibit a typical phenotype of not growing in presence of high concentrations of antimicrobial agents or rather do not die and hence contribute to resistance[31]. In the present study, a small population (10^5-10^6 cfu/ml) of cells was seen growing on MHA plates after removing antibiotic and metal salt stress. This indicated development of persister cells within biofilm formed by *A. haemolyticus* MMC 8 leading to an increase in resistance. Our results are concurrent with those of Singh *et al*[32] who have reported role of persister cells in antibiotic resistance in biofilm of *S. aureus*. Presence of metal ion resistant persister cell population has also been reported in *E.coli*[33]. Previously, higher concentrations of antibiotics have also been reported to be ineffective in eradicating biofilm of *P. aeruginosa*[34].

In conclusion, the study describes antibiotic and metal resistant profile of planktonic and biofilm of *A. haemolyticus* MMC 8. Increasing complexity of biofilm, production of exopolymeric substances and presence of persister cells may be the reason leading to elevated resistance in biofilm formed over a period of 72 h.

Acknowledgment

Authors acknowledge financial assistance to UPE Phase I and II awarded to University of Pune. The study was partly supported by a major research project awarded by the University Grants Commission, New Delhi, India, to the last author (BAC). Authors acknowledge the technical assistance provided by the scanning electron microscopy facility of Department of Physics, University of Pune.

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