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Effect of subinhibitory concentrations of fluoroquinolones on biofilm production by clinical isolates of *Streptococcus pyogenes*

Kannan Balaji, Ramalingam Thenmozhi & Shunmugiah Karutha Pandian

Department of Biotechnology, Alagappa University, Karaikudi, India

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***Background & objectives*: Subinhibitory concentrations (sub-MICs) of antibiotics, although not able to kill bacteria, but influence bacterial virulence significantly. Fluoroquinolones (FQs) which are used against other bacterial pathogens creates resistance in non-targeted *Streptococcus pyogenes.* This study was undertaken to characterize the effect of sub-MICs of FQs on *S. pyogenes* biofilm formation.**

***Methods*: Biofilm forming six M serotypes M56, st38, M89, M65, M100 and M74 of *S. pyogenes* clinical isolates were challenged against four FQs namely, ciprofloxacin, ofloxacin, levofloxacin and norfloxacin. The antibiofilm potential of these FQs was analysed at their subinhibitory concentrations (1/2 to 1/64 MIC) using biofilm assay, XTT reduction assay, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).**

***Results*: Among the four FQs tested, ofloxacin and levofloxacin at 1/2 MIC showed the maximum inhibition (92%) of biofilm formation against M56 and M74 serotypes. FQs effectively interfered in the microcolony formation of *S. pyogenes* isolates at 1/2 to 1/8 sub-MICs. Inhibition of biofilm formation was greatly reduced beyond 1/16 MICs and allowed biofilm formation. XTT reduction assay revealed the increase in metabolic activity of *S. pyogenes* biofilm against the decrease in FQs concentration. SEM and CLSM validated the potential of sub-MICs of FQs against the six *S. pyogenes*.**

***Interpretation & conclusions*: Our results showed that the inhibitory effect all four FQs on *S. pyogenes* biofilm formation was concentration dependent. FQs at proper dosage can be effective against *S. pyogenes* and lower concentrations may allow the bacteria to form barriers against the antibiotic in the form of biofilm.**

Key words Biofilms - confocal laser scanning microscopy - fluoroquinolones - *Streptococcus pyogenes* - subinhibitory concentration

Streptococcus pyogenes is an important human pathogen responsible for a wide array of infections such as pharyngitis, scarlet fever, cellulitis, bacteremia, impetigo, acute rheumatic fever, glomerulonephritis, necrotizing fascitis and streptococcal toxic shock syndrome. *S. pyogenes* possess the inherent capacity to form biofilms that are associated with specific M serotypes[1,2]. Biofilms play an important role in more than 50 per cent of the human bacterial infections[3]. The biofilms of *S. pyogenes* have been observed in skin and root canal infections[4,5].

The presence of exopolysaccharide matrix in biofilm leads to increased resistance to antimicrobial treatments and host defenses which favour the growth of microorganisms in hostile or suboptimal environments[6]. Generally higher concentration of the antibiotics is required to kill bacteria in the biofilm phase than their planktonic counterparts[7]. Significant limitations to biofilm penetration have been reported for beta-lactams and aminoglycosides class of antibiotics[8]. Penicillin remains the drug of choice for the treatment of *S. pyogenes* infections because it remains susceptible to this antibiotic despite its intensive use. The increasing use of fluoroquinolones (FQs) due to their excellent activities against some other bacterial pathogens, has led to the emergence of FQs resistant *S. pyogenes* strains[9]. Several studies showed the emergence of FQs resistance in *S. pyogenes* isolates though these were not used against *S. pyogenes* infections[10-12]. It has been implied that subinhibitory concentrations (sub-MICs) of certain antibiotics can suppress the formation of biofilms by disrupting the adhering capacity[13] and higher concentrations of FQs may result in the induction of resistance[14]. Hence concentrations of antibiotics may influence the bacterial virulence parameters such as adherence[15], motility, biofilm formation[16] and sensitivity to oxidative stress[17].

Therefore, studying the effect of sub-MICs of antibiotics on microorganisms is of continuing interest to microbiologists[18,19]. In this study, we investigated the effect of subinhibitory concentrations of FQs namely, ciprofloxacin (CIP), levofloxacin (LEV), norfloxacin (NOR) and ofloxacin (OFL) on biofilm production by *S. pyogenes*.

Material & Methods

Six clinical isolates of *S. pyogenes* with M serotypes of M56, st38, M89, M65, M100 and M74 already identified as effective biofilm formers in our previous study[2] were used. These isolates were obtained from pharyngitis patients, attending Government Rajaji Hospital, Madurai, India using 5 per cent sheep blood agar plates and routinely maintained in Tryptose agar plates (Hi media Laboratories, India).

Susceptibility testing: Minimal inhibitory concentrations (MICs) of ciprofloxacin (CIP) (Himedia Laboratories, India), levofloxacin (LEV), norfloxacin (NOR) and ofloxacin (OFL) (Sigma, USA) were determined using modified form of broth microdilution method outlined by the Clinical and Laboratory Standards Institute[20]. The broth microdilution method involves exposing bacteria to decreasing concentrations of FQs in liquid media. The bacterial suspension (10^6 CFU/ml) was added to Todd Hewitt Broth (THB)[21] supplemented with 5 per cent lysed sheep blood and the antibiotics were serially diluted two folds to give final concentrations ranging from 0.5 to 128 µg/ml and incubated at 37°C for 18 h. The lowest concentration of FQs at which there was no visible growth was taken as the MIC for that isolate.

Biofilm assay: The effects of four FQs were tested against biofilm forming M serotypes of *S. pyogenes* isolates in 24 well microtiter plates as described earlier[20]. Briefly, the FQs at sub-MICs (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) were added to THB containing the bacteria of 10^6 cfu/ml. Culture without adding any antibiotics was used as control and the wells containing THB alone were used as blanks. The percentage of biofilm inhibition was calculated by the formula:

Percentage of inhibition = ([Control $OD_{570\ nm}$ - Test $OD_{570\ nm}$] / Control $OD_{570\ nm}$) x 100

XTT reduction assay: A semiquantitative measurement of metabolic activity of *S. pyogenes* biofilms were obtained from the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium-hydroxide (XTT) reduction assay[20]. Biofilm formed wells were washed twice with PBS to remove planktonic as well as adhered cells. Then, 50 µl of XTT salt solution (1mg/ml in PBS) and 4 µl of menodione solution (1mM in acetone; Sigma, USA) were added to each well. Microtiter plates were incubated at 37ºC in the dark for 90 min. Bacterial dehydrogenase activity reduces XTT tetrazolium salt to XTT formazan, resulting in colorimetric change (turns to orange) which was correlated with cell viability. The colorimetric changes were measured spectrophotometrically at 492 nm[22].

Light microscopy: For determining the biofilm formation of *S. pyogenes*, small sterile glass slides (1x1cm) were placed into the wells of 24 well polystyrene plates. The four FQs at sub-MICs (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) along with THB containing the bacteria of 10^6 cfu/ml were added into the 24 well plates and incubated at 37ºC for 24 h. After incubation the small glass slides were removed and gently washed twice to remove planktonic cells. Crystal violet staining was performed and the presence of biofilms was inspected by light microscopy (Euromex GE 3045, Holland) at magnifications of 40x.

Scanning electron microscopy (SEM): Sample preparation for SEM analysis was performed as

described by Lembke *et al*[1]. Biofilms on the glass pieces were fixed for 2 h in solution containing 2.5 per cent glutaraldehyde. Further, the glass pieces were washed in 0.1 M sodium acetate buffer (*p*H 7.3). Samples were dehydrated through a graded series of ethanol, critical point dried, gold sputtered and examined under scanning electron microscope (Hitachi S-3000H, Japan).

Confocal laser scanning microscopy (CLSM): CLSM was used to determine the three dimensional architecture, thickness and morphology of biofilms formed by *S. pyogenes* isolates. Staining of biofilms and CLSM analysis were performed as described previously[21]. The biofilms formed on cover slips were stained with 0.2 per cent acridine orange (Hi-Media Laboratories, Mumbai) for 2 min. The stained slides were subjected to visualization under confocal laser scanning microscope (Zeiss LSM710 meta, Germany). Images were captured and processed by using Zeiss LSM Image Examiner Version 4.2.0.121.

Statistical analysis: All experiments were performed in triplicates. Comparative results for different isolates were statistically analyzed using SPSS 15.0 statistical package (SPSS Inc., Chicago, USA). All pair-wise comparisons were performed using Dunnett's test. $P<0.05$ was considered significant.

Table. Per cent inhibition of biofilm formation by *S. pyogenes* isolates on six concentrations of four fluoroquinolones

		% inhibition of *S. pyogenes* biofilm against four FQs					
Isolates	MIC (µg/ml)	1/2MIC	1/4MIC	1/8MIC	1/16MIC	1/32MIC	1/64MIC
M56	0.3	77.4 ± 0.65	70.2 ± 2.5	63.4 ± 0.6	59.2 ± 0.7	53.2 ± 1.1	29.3 ± 3.1
st38	0.3	80.7 ± 1.7	74.8 ± 2.4	69 ± 0.7	62 ± 0.3	58.8 ± 0.7	54.6 ± 1.4
M89	0.3	78.7 ± 1.5	65.7 ± 2.7	57.9 ± 1.9	52.6 ± 2	45.6 ± 1.1	36.6 ± 0.3
M65	0.3	57 ± 1.9	48.8 ± 0.9	39.8 ± 0.4	33.8 ± 0.7	28.2 ± 4.7	18 ± 0.6
M100	0.2	80.6 ± 1.1	76 ± 0.9	72.4 ± 0.6	69.1 ± 0.6	62.8 ± 0.5	56.8 ± 0.6
M74	0.4	83.3 ± 1	79.3 ± 0.9	70.6 ± 0.5	66.6 ± 1.1	58.7 ± 1	52.2 ± 0.7
OFL							
M56	0.9	92.3 ± 2.3	88.7 ± 2	84.5 ± 3	82.3 ± 2.5	75 ± 2.1	68.1 ± 2.5
st38	1	81.7 ± 2.4	81.3 ± 1.7	74.3 ± 1.4	71.1 ± 2.5	66.3 ± 2.7	55.9 ± 2.8
M89	1	78.9 ± 0.3	70.2 ± 0.2	63.7 ± 0.3	55.2 ± 0.2	49.9 ± 0.1	42.5 ± 0.5
M65	1	66.3 ± 1	56 ± 1.1	46.8 ± 0.7	40.9 ± 0.8	31.3 ± 1.2	20.7 ± 0.5
M100	0.7	78.7 ± 0.4	74.2 ± 0.3	67.2 ± 0.2	62 ± 0.05	58.7 ± 0.4	56.1 ± 0.1
M74	1	81 ± 1	72.4 ± 1.8	65.9 ± 1.2	57.2 ± 1	52.2 ± 0.9	46.6 ± 1.7
LEV							
M56	0.5	75.1 ± 0.7	69.2 ± 1	66.7 ± 10	54.1 ± 0.9	49.5 ± 0.6	44.4 ± 1.3
st38	0.5	80.4 ± 0.7	75.9 ± 0.4	71.2 ± 0.2	65.7 ± 0.2	60.1 ± 0.17	53 ± 0.2
M89	0.3	72.2 ± 0.05	62.4 ± 0.8	58.5 ± 0.5	50.1 ± 0.2	45.1 ± 0.5	39.3 ± 0.2
M65	0.4	72.3 ± 1	66.7 ± 5.6	56.5 ± 2.4	48 ± 2.6	42.1 ± 3.1	30.3 ± 2.9
M100	0.2	79 ± 0.1	75.8 ± 0.2	73 ± 0.6	67.1 ± 0.7	65.5 ± 0.3	61 ± 0.9
M74	0.4	92.7 ± 0.5	91.6 ± 0.1	89.6 ± 0.5	88.7 ± 0.1	88.4 ± 0.4	87.6 ± 0.2
NOR							
M56	5	74.5 ± 3.6	65.7 ± 0.6	59.2 ± 0.6	55.1 ± 0.6	49.1 ± 0.5	37.2 ± 1.8
st38	8	82 ± 0.05	77.8 ± 0.6	70.6 ± 0.7	61.6 ± 0.5	56.8 ± 0.3	50.7 ± 0.7
M89	3	64.1 ± 0.8	60 ± 0.05	50.4 ± 0.4	46.1 ± 0.1	36.8 ± 1	33.5 ± 0.5
M65	7	74.1 ± 0.8	60.7 ± 1.1	47.8 ± 0.5	42.3 ± 0.9	39.6 ± 0.6	34.6 ± 4.7
M100	4	78.9 ± 0.2	76.6 ± 0.3	68.8 ± 0.2	62.6 ± 1	59 ± 0.4	57.5 ± 0.4
M74	8	78.9 ± 0.6	73.8 ± 0.5	70.3 ± 1	61.2 ± 0.7	51.2 ± 0.4	43.9 ± 0.6

CIP, ciprofloxacin; OFL, ofloxacin; LEV, levofloxacin; NOR, norfloxacin
Values are mean ± SD of three observations

Results

MIC values of all six isolates against four FQs are shown in the Table. NOR demonstrated the highest MIC value of 8 µg/ml for st38 and M78 isolates. M100 showed the lowest MIC value of 0.2 µg/ml against CIP and LEV. All the four FQs at their sub-MICs did not show any antibacterial activity.

Effect of subinhibitory concentrations of FQs on S. pyogenes biofilms: FQs showed substantial inhibition in the biofilm formation of *S. pyogenes* isolates. It was evident that OFL and LEV showed a promising antibiofilm activity with a maximum inhibition of 92 per cent against the potent biofilm former M56 and M74 serotypes at 1/2 MIC, followed by CIP and NOR with 83 and 82 per cent inhibition against M74 and st38 serotypes respectively (Table). On an average, the FQs showed 70 and 50 per cent inhibition ($P<0.05$) against all the M serotypes tested at 1/4 and 1/8 MIC, respectively when compared to the control. The percentage inhibition of biofilm observed in the M65 serotype was comparatively low against all the four FQs tested, while the least was observed in M89 treated with NOR. With an increase in dilution of sub-MICs of the antibiotics, the percentage inhibition of the biofilm formation was also reduced considerably. This result demonstrates that the formation of biofilms was influenced in a concentration dependent manner.

Assessment of metabolic activity on preformed biofilms: XTT assay revealed the metabolic activity of *S. pyogenes* biofilms against the FQs after 24 h of incubation at 37ºC. Biofilms at 1/2 and 1/4 MICs showed less metabolic activity. It was evident that the metabolic activity of *S. pyogenes* against FQs was concentration dependent and metabolically active biofilm were more in lower sub-MICs (Fig. 1).

Surface topography and architecture of biofilms: The structural morphology of the biofilm formation viewed under light microscopy and was further confirmed by SEM analysis. SEM analysis revealed the potential effect of FQs against biofilm forming M serotypes of *S. pyogenes*. LEV at sub-MICs (1/2-1/8 MIC) restricted the attachment of bacterial cells greatly when compared to the control. Figure 2 (representative SEM images) depicts the antibiofilm activity of the FQs against the serotype M56. At sub-MICs (1/16-1/64 MIC), incompetence of FQs on biofilm formation was observed and at these lower concentrations FQs lost their antibiofilm activity and allowed the bacteria to form biofilm. Increase in biofilm formation upon exposure to lower concentrations of FQs (1/8-1/64 MIC) was observed from SEM analysis (Fig. 2 c-f). Magnified SEM image showed a visible slimy matrix around *S. pyogenes* cell (Fig. 3) comparably more slimy outer cover than that of the control.

CLSM was used to analyze the surface topography and three dimensional architecture of the biofilm formed by *S. pyogenes* isolates. The thickness of *S. pyogenes* biofilm ranged from 60-120 µm. All FQs acted well on *S. pyogenes* biofilms up to 1/8 MIC whereas at 1/16-1/64 MIC there was a proportional increase in the thickness of biofilms. LEV reduced the formation of biofilms at 1/2 to 1/8 MIC in M56 serotype (Fig. 4a-d as a representative for all FQs tested) whereas antibiotics beyond 1/16 MIC showed a gradual increase in the establishment of biofilms (Fig. 4e-g).

Discussion

In bacteria, the formation of biofilms is the major barrier that protects the pathogen from many environmental stresses like antibiotics and human immune system. The biofilm formed by *S. pyogenes* restricts the diffusion of antimicrobials agents and thereby develops tolerance to these substances, which is of major clinical importance[23]. The effectiveness of various antibiotics at their sub-inhibitory concentrations against the adherence property and virulence factors production by the pathogens has been studied[13,24]. The effect of sub-MICs of various antibiotics and FQs has been studied on *P. aeruginosa*[13], *S. typhimurium*[19], *Staphylococcus* spp.[25] and *Streptococcus* spp.[26] and FQs showed a strong bactericidal activity against the biofilm forming cells than other antibiotics[27]. Tanaka *et al*[27] reported that the levels of some exoproteins were increased in *S. pyogenes* after treatment with macrolides[28], whereas gatifloxacin showed no effect on exoprotein production. The LEV has been shown to have a substantial therapeutic efficacy against biofilm producing *P. aeruginosa* [7]. Odenholt-Tornqvist *et al*[29] reported that only a small amount of FQs is necessary to prevent normal cell growth once the bacteria have been damaged by a suprainhibitory concentration of the antibiotic. Extensive use of one particular antibiotic can develop resistance against that antibiotic in non-targeted bacteria.

In this study, the four FQs at the sub-MICs (1/2, 1/4 and 1/8) reduced the biofilm formation by 40-90 per cent in *S. pyogenes*. These FQs at the sub-MICs may reduce the development of biofilms by interfering with

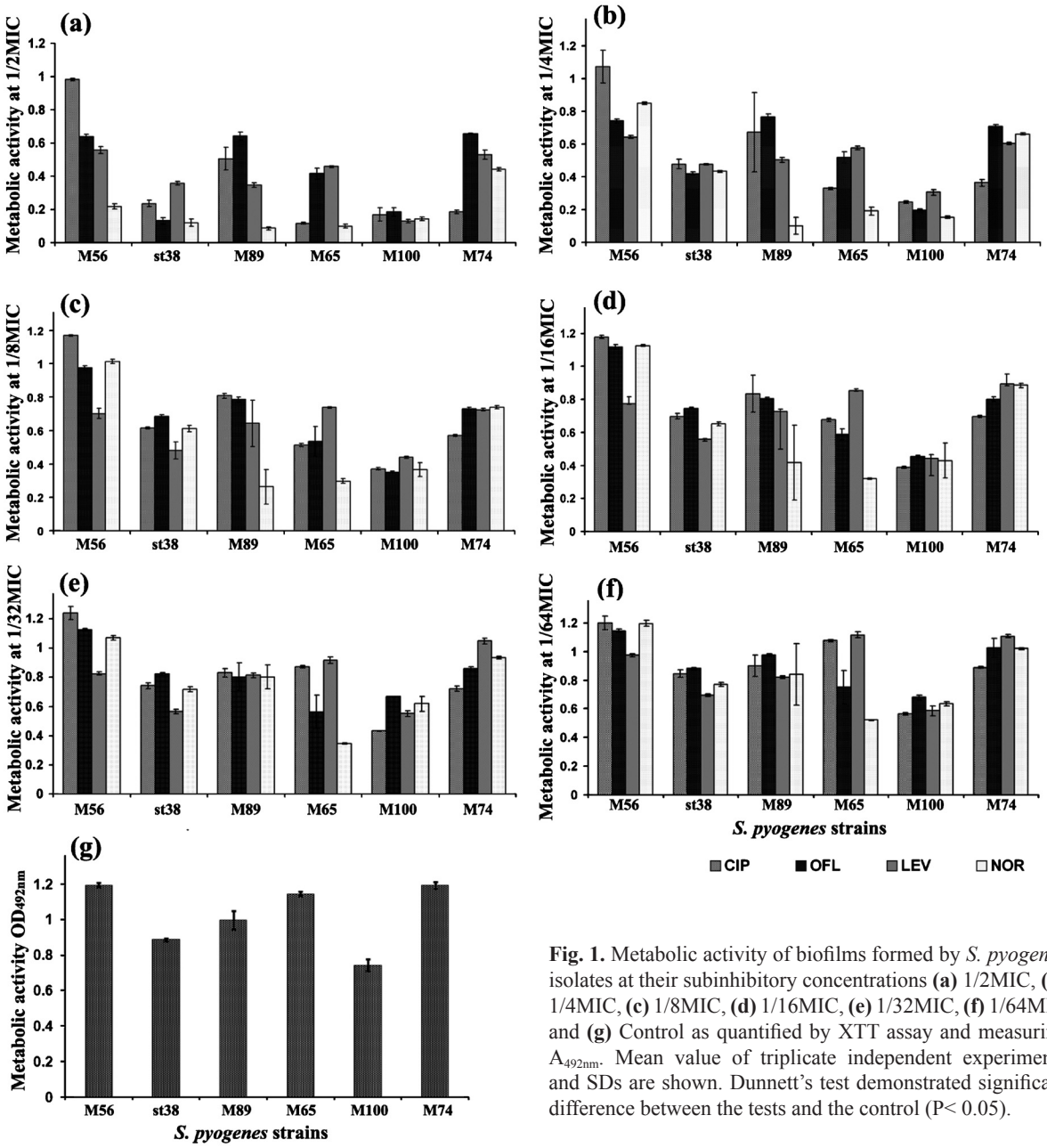

Fig. 1. Metabolic activity of biofilms formed by *S. pyogenes* isolates at their subinhibitory concentrations **(a)** 1/2MIC, **(b)** 1/4MIC, **(c)** 1/8MIC, **(d)** 1/16MIC, **(e)** 1/32MIC, **(f)** 1/64MIC and **(g)** Control as quantified by XTT assay and measuring A_{492nm}. Mean value of triplicate independent experiments and SDs are shown. Dunnett's test demonstrated significant difference between the tests and the control ($P< 0.05$).

the adherence property of the bacterium[30]. Of the four FQs used, OFL worked efficiently in disintegrating the microcolony formation of *S. pyogenes* biofilms. In the present study, all four FQs showed antibiofilm activity up to 1/8 MICs whereas an earlier study[27] reported *P. aeroginosa* showing different sub-MICs with respect to different FQs for the complete eradication of their biofilms. FQs rapidly diffuse deep into the biofilms of Gram-negative bacteria, in the similar way it might have gained entry and disrupted the biofilms of *S. pyogenes*, a Gram-positive bacterium[31]. According to Schmitz *et al*[9] at lower concentrations, FQs act in a bacteriostatic way since these block the DNA replication process, while at higher concentrations these are bactericidal[9]. Similarly, the cell density of the *S. pyogenes* isolates at the MICs was bactericidal whereas at sub-MICs the cell

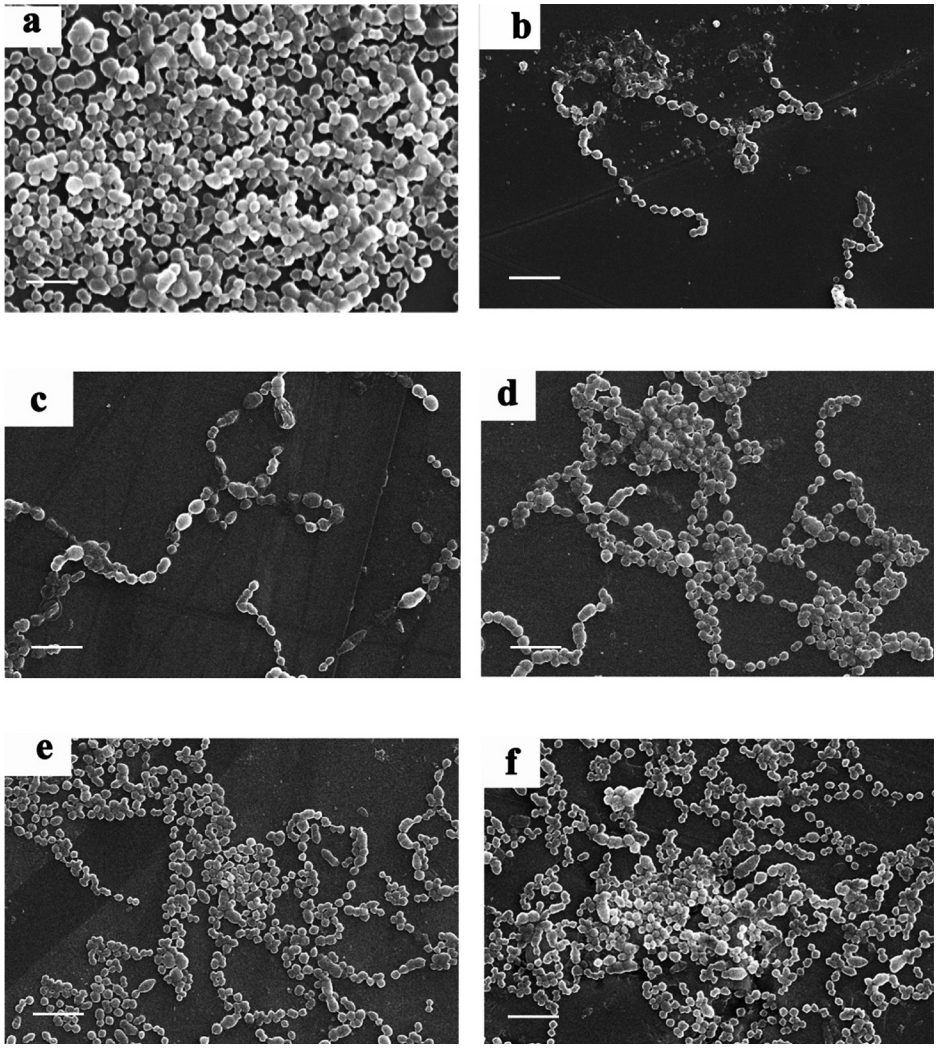

Fig. 2. Scanning electron micrographs of *S. pyogenes* serotype M56 biofilms and their treatment with LEV at sub-MICs **(a)** Control, **(b)** 1/4MIC, **(c)** 1/8MIC, **(d)** 1/16MIC, **(e)** 1/32MIC and **(f)** 1/64MIC (Scale bar = 10 µm).

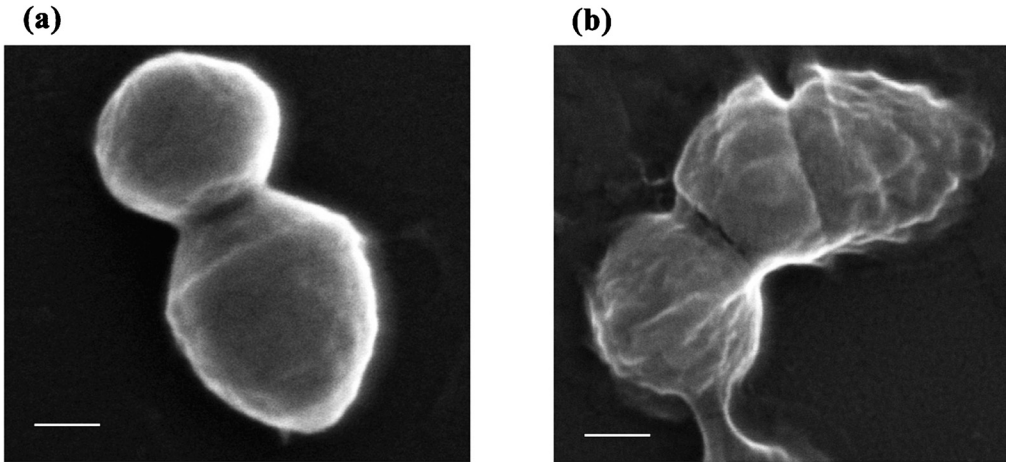

Fig. 3. Scanning electron micrographing image of **(a)** Control and **(b)** st38 cell at 1/32MIC of LEV treatment after 24h of incubation (Scale bar = 1 µm).

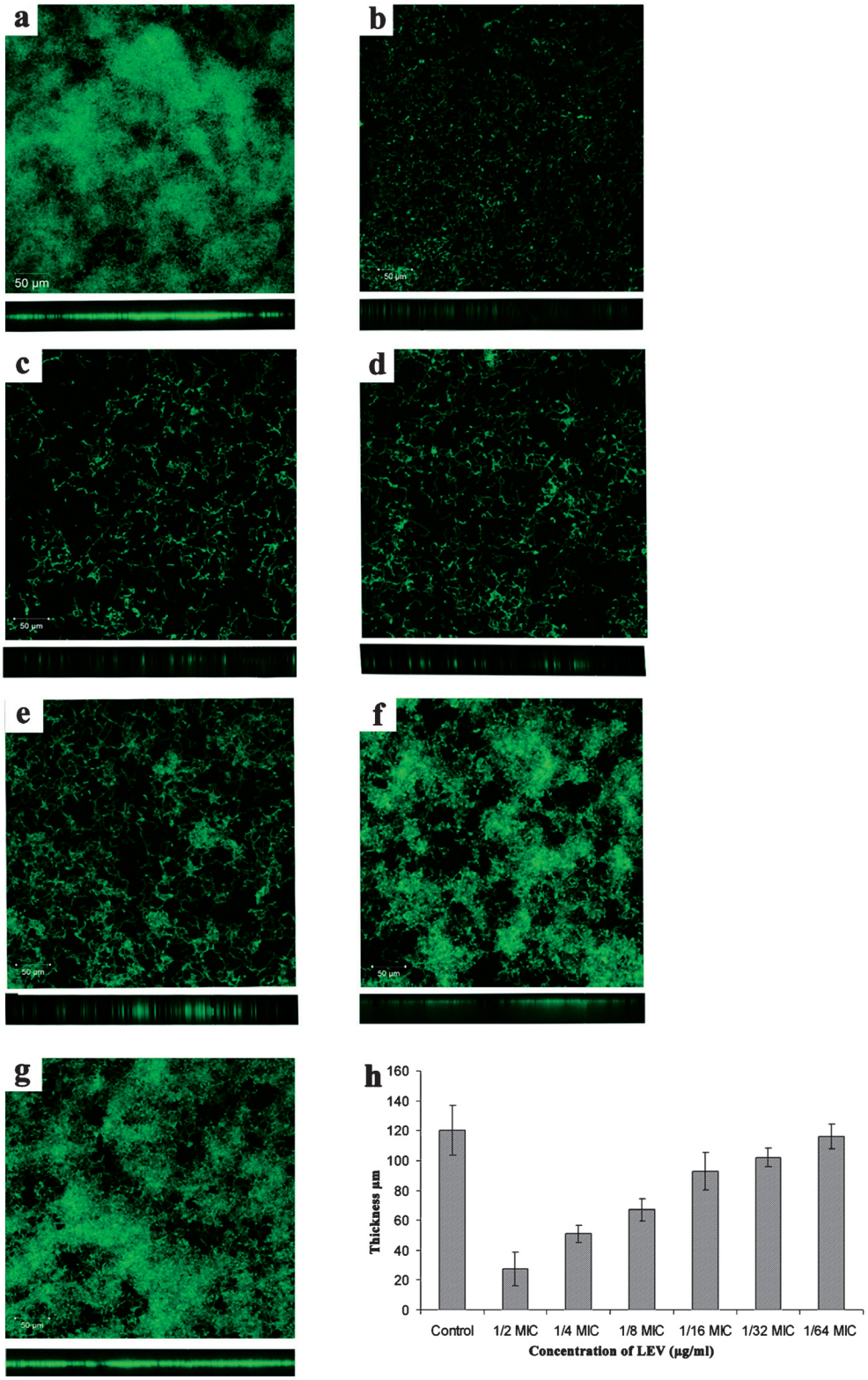

Fig. 4. Confocal Laser Scanning Microscopic image showing gradual increase in biofilm formation by *S. pyogenes* serotype M56 **(a)** Control, **(b)** 1/2MIC, **(c)** 1/4MIC, **(d)** 1/8MIC, **(e)** 1/16MIC, **(f)** 1/32MIC, **(g)** 1/64MIC and **(h)** Thicknesses of biofilms were determined from merging all the z-stack images using CLSM-assosiated software. Magnification: 20 x and Scale bar = 50 µm.

density values were similar to the control, eventhough the FQs were bacteriostatic and the metabolic activity of *S. pyogenes* biofilms determined by XTT assay was associated with the cell density values (data not shown).

In the present study, FQs at lower sub-MICs (1/16-1/64 MIC) lost their antibiofilm effect and allowed biofilm formation in the isolates. There are several reports regarding the induction of biofilms while treatment with antibiotics. For example, Linares *et al*[32] also reported the induction of biofilms at sub-MICs of ciprofloxacin, tobramycin and tetracycline. Another important fact is that microbes exhibit inherent antibiotic resistant mechanisms to overcome the hostile environments[8,23] likewise they may induce biofilm formation as a protective mechanism. *In situ,* FQs used for the treatment of other bacterial pathogens may enhance biofilm formation in non-targeted *S. pyogenes*. Several factors may also be involved in the induction of *S. pyogenes* biofilm at the lower concentrations of FQs. Reduced concentrations of the antibiotics lead to an adverse condition, which in turn may activate the quorum of signaling molecules in *S. pyogenes* by inducing the virulence trait such as biofilm formation[18].

In conclusion, our results document that all four FQs used in this study efficiently inhibited the biofilm formation at their sub-MICs (1/2-1/8 MIC) and at lower sub-MICs (1/16 and 1/64 MIC). These lower concentrations of FQs may provide a chance to protect the pathogen by forming biofilm. SEM and CLSM analyses portrayed the surface topography and architecture of biofilms formed by the six M serotypes of *S. pyogenes* strains. Considerable reduction in thickness of the biofilms at the (1/2-1/8 MIC) and increasing thickness at lower sub-MICs by CLSM analysis demonstrated FQs ability to interference with *S. pyogenes* biofilms. Further expression analysis of *S. pyogenes* isolates challenged with FQs at subinhibitory concentration may unravel the exact mechanism involved in the concentration dependent biofilm inhibition. Hence, the outcome of this study suggests that appropriate FQs should be used at proper dosage for other bacterial infections else their effects over non-targeted pathogens like *S. pyogenes* could either worsen or attenuate the disease. FQs usage based on optimal concentrations of the antibiotics and target specificity is crucial to protect the mankind from life threatening infections.

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Reprint requests: Prof. S. Karutha Pandian, Department of Biotechnology, Alagappa University,
Karaikudi 630 003, Tamil Nadu, India
e-mail: sk_pandian@rediffmail.com