

ORIGINAL ARTICLE

Antibiotic susceptibility of *ica*-positive and *ica*-negative MRSA in different phases of biofilm growth

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a clinically relevant pathogen because of its resistance to antibiotics and its ability to form potent biofilm. Both *ica*-negative as well as *ica*-positive MRSA strains are known to produce biofilm. In the present study, these strains were grown in biofilm mode and susceptibility of these to antibiotics was assessed. Our study suggests that antibiotic susceptibility of MRSA biofilm depends on the biochemical composition of its matrix. The biofilm matrix of *ica*-positive MRSA was mainly composed of poly-intercellular adhesion (PIA), whereas eDNA was a major constituent of *ica*-negative MRSA. The results showed that MRSA in planktonic growth was susceptible to clindamycin, vancomycin and minocycline. However, the MIC and MBC of vancomycin for the mature biofilm of *ica*-negative MRSA was 16 and 32 $\mu\text{g ml}^{-1}$, respectively. On the contrary, the MIC and MBC of vancomycin for *ica*-positive MRSA was $> 1024 \mu\text{g ml}^{-1}$. The effect of vancomycin and minocycline on young and old biofilms was also determined. Vancomycin was quite effective in eradicating the young biofilm formed by *ica*-negative MRSA; however, it was completely ineffective on the biofilm of *ica*-positive MRSA. Minocycline at its highest clinical achievable concentration was found to be quite effective in eradicating the young biofilm formed by both the strains. The enzyme-linked immunosorbent assay (ELISA) results and dot blot assay suggest that the presence of *ica* locus influenced PIA production, which probably contributed towards the failure of vancomycin in eradicating the biofilm formed by *ica*-positive strain. However, none of the antibiotics used in this study was effective in eradicating the mature biofilms.

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INTRODUCTION

Methicillin-resistant *S. aureus* (MRSA) strains pose a major threat to patients because of their resistance towards β -lactam antibiotics such as oxacillin. MRSA has spread worldwide both in health-care and community settings.¹ Presently, the prevalence of methicillin resistance among *S. aureus* isolates in intensive care units in the United States is 60%,² and more than 90 000 invasive infections due to MRSA occur in the United States.³ Moreover in recent years, the prevalence of MRSA in India has increased from 12% in 1992 to 80.83% in 2002.⁴

Over the years, vancomycin has been considered as a gold standard treatment for MRSA infections. It is generally considered as the first-line option for the treatment of bacteremia, endocarditis, osteomyelitis, septic arthritis and meningitis, followed by antibiotics such as clindamycin, doxycycline, minocycline and linezolid.⁵ However, to compound this problem, MRSA is also known to form potent biofilm.¹

Earlier, only poly-intercellular adhesion (PIA) molecules encoded by *ica*-operon were known to be responsible for the biofilm-forming ability of *S. aureus*. However, now it is known that an *ica* ADBC-independent biofilm phenotype, which instead depends upon the

fibronectin-binding proteins (FnBPA and FnBPB) and major autoysin (Atl), also exists.^{6–10} Hence, in addition to resistance towards antibiotics, the biofilm formed on the implanted prosthetic biomaterials is a major risk factor in health-care-associated infections. Moreover, during the course of infection, the implant becomes coated with host-derived extracellular matrix proteins,¹¹ resulting in increased antibiotic resistance of biofilm cells that is up to 1000-fold greater than the free-living planktonic bacterial cells.^{12–14} The existence of microbial cells in stationary phase and permeability barrier imposed by aggregative biopolymers that hold the biofilm together contribute towards resistance to antimicrobials.^{13,14}

Till date, no regimen has been established to treat chronic biofilm infections that differ markedly from planktonic cells grown in the same environment. According to the Clinical and Laboratory Standards Institute (CLSI) methods, the killing dose determined for antibiotics is prepared only for planktonic cells, as a result of which they have no effect on bacteria grown within biofilm.^{15–17} The minimum biofilm eradication concentration, which targets complete bacterial killing, would be more meaningful than the biofilm MIC to prevent chronic phase of biofilm infections, which are characterized by less active dispersal.^{12,15} The minimum concentration required for

biofilm eradication is impractical to achieve when conventional antibiotics are used *in vitro*^{18,19} but the biofilm MBC, which targets 99.9% killing of biofilm-grown bacteria, appears to be achievable.

To the best of our knowledge, no report is yet available in which a comparative study has been conducted to evaluate the significance of *ica*-locus in the context of antibiotic susceptibility of different phases of planktonic as well as biofilm growth. In the present study, the biofilm formation has been characterized and quantified in presence and absence of the *ica*-locus. In addition, antibiotic susceptibility of various phases of biofilm growth of MRSA has been studied (planktonic cells at the mid-log phase, planktonic cells at the stationary phase and mature biofilms) at their serum achievable concentration.

MATERIALS AND METHODS

Bacterial strains

MRSA ATCC 33591 and ATCC 43 300 procured from American type Culture Collection Centre, Manassas, VA, USA were used in the present study. All the strains were preserved in semi-solid agar stabs at 4 °C and in brain heart infusion broth with 15% glycerol at -70 °C until further use. All antimicrobial susceptibility experiments were performed in soyabean casein digest medium. Growth media and antibiotics were purchased from HiMedia Laboratories (Mumbai, India). Nutrient agar was used as the culture medium for MRSA and bacterial dilutions were made in sterile 0.85%, normal saline (NS).

Antibiotics

Vancomycin hydrochloride, clindamycin and minocycline (HiMedia Laboratories) representing members of glycopeptides, lincosamide and broad-range tetracycline were used in the present study.

Biofilm development

MRSA biofilm was grown in 96-well microtiter plate according to the modified method of Bedi *et al.*²⁰ Briefly, wells of microtiter plate were inoculated with 50 µl of soyabean casein digest medium and 50 µl of bacterial culture (OD₆₀₀ = 0.3) equivalent to 10⁸ CFU ml⁻¹ of bacteria and incubated at 37 °C overnight. In each test, control wells containing sterile broth were included as plate sterility control. After incubation, planktonic cells in the fluid were removed and wells were washed thoroughly three times with NS. The biofilm matrix was then scraped with a sterile pipette tip, suspended in NS and vortexed for 3 min. Microbial load of biofilm was enumerated by viable cell counting.

For the establishment of 7-day-old biofilm, spent medium was replaced with fresh sterile soyabean casein digest medium in rest of the wells and plate was reincubated at 37 °C overnight. Media in each well was replaced every 24 h until the seventh day of the experiment. Every day, wells were stained with 0.1% crystal violet for 10 min. Following the staining step, the colorant was discarded and the wells were rinsed three times with NS to remove excess stain. Then, the dye incorporated in the biofilm or present in the control wells was solubilized in absolute ethanol. The OD of the stained biofilm and control wells was read at 595 nm using enzyme-linked immunosorbent assay reader (BIORAD iMark Microplate Reader, Bio-Rad Laboratories Inc, Hercules, CA, USA). Microbial load of the biofilm was also enumerated daily by viable cell count method till 7 days.

Slime production

The slime production was assessed by the method of Freeman *et al.*²¹ Briefly, the plates composed of brain heart infusion agar and Congo red 0.8 g l⁻¹ were inoculated and incubated aerobically for 24 h at 37 °C. A positive result was indicated on the basis of colony morphology. A black colony with a dry crystalline consistency was considered positive. Non-slime producers usually remained pink, although occasional darkening at the center of the colony was observed and this was labeled as bull's eye appearance.

Assessment of biochemical nature of biofilm

The composition of biofilm matrix was assessed by the method of Fischer *et al.*²² Briefly, wells of microtiter plate were inoculated with 50 µl of soyabean casein digest medium and 50 µl of bacterial culture (OD₆₀₀ = 0.3) equivalent to 10⁸ CFU ml⁻¹ of bacteria and incubated at 37 °C overnight. In each test, 0.1 U DNase I and Proteinase K (0.2 mg ml⁻¹ in 100 mM TrisNaCl, pH 7.5) were added and plates were incubated for 24 h at 37 °C without agitation. After incubation, planktonic cells in the fluid were removed and wells were washed thoroughly three times with NS. After drying the biofilm, the test and control wells stained with CV as described above. The OD of the stained biofilm and control wells was read at 595 nm using enzyme-linked immunosorbent assay reader (BIORAD iMark Microplate Reader, Bio-Rad Laboratories Inc). In addition, the amount of eDNA and protein content was assessed by measuring OD₂₆₀ and OD₂₈₀ using UV Nanodrop (ThermoFischer Scientific Ltd., Waltham, MA, USA).

Quantification of eDNA and proteins in biofilm

The amount of eDNA was quantified by following the method of Vilain *et al.*²³ Briefly, biofilm was established as described above. Each day the wells were washed three times with NS. The biofilm cells were scraped and centrifuged at 10 000 r.p.m. for 10 min at 4 °C. The supernatant was filtered by using 0.45-µm syringe filter (Millipore Ireland Ltd., Tullagreen, Carrigtwohill, County Cork) to remove all the bacterial cells. The amount of eDNA present in the supernatant was assessed by measuring O.D₂₆₀ using UV Nanodrop 1000 (ThermoFischer Scientific Ltd.).

PIA quantification using dot blot and ELISA

PIA was extracted from biofilm according to the method of Jefferson and Cerca.²⁴ Briefly, the biofilm was grown on 35-mm dishes, scraped off and suspended in phosphate-buffered saline (PBS). The OD₆₀₀ of the suspension was adjusted to 1.5, centrifuged at 9296 g for 5 min and the pellet was suspended in 50 ml 0.5 M EDTA (pH 8.0). The samples were incubated, twice at 100 °C for 5 min with incubation on ice for 5 min in between and then centrifuged at 9296 g for 5 min. The supernatant was treated with proteinase K (2 mg ml⁻¹) for 1 h at 60 °C and then incubated at 80 °C for 30 min to inactivate proteinase K. The samples were stored at -20 °C till further use.

For dot blot, twofold dilutions of PIA extract were prepared in Tris-buffered saline (TBS) and 5 µl of each suspension was spotted on nitrocellulose membrane. The membrane was air-dried, moistened with TBS and fixed in 50-ml blocking solution comprising 1% (w/v) bovine serum albumin (BSA) in TBST (TBS plus 0.05% Tween 20) for 1 h. Fifty milliliters of WGA-horseshoe peroxidase conjugate (WGA-HRP, 130 ng ml⁻¹) plus 1% BSA in TBST were added and the membrane was incubated for 30 min. The membrane was then washed three times for 5 min each in TBST, incubated in 50 ml substrate (0.5 mg diaminobenzidine per ml plus 1 µl H₂O₂ per ml in TBS) for 15 min and rinsed in distilled water. The experiment was performed in duplicate.

PIA in biofilm was quantitated by enzyme-linked immunosorbent assay (ELISA) according to the method of Cramton *et al.*²⁵ Overnight cultures of both the strains were adjusted to an OD₆₀₀ of 0.3, and 200 µl of this culture was incubated per well in flat-bottomed, 96-well polystyrene microtiter plates for 24 h at 37 °C. The resulting biofilm was washed twice with PBS and fixed with 200 µl of 95% ethanol for 15 min. An aliquot of 100 µl blocking solution (1%, w/v, BSA in PBST (PBS plus 0.05% Tween 20)) was added per well and the microtiter plate incubated for 1 h at 37 °C. The blocking solution was then removed, 100 µl WGA-HRP (75 ng ml⁻¹) in 1% BSA in PBST was added per well and the microtiter plate incubated for 30 min at 37 °C. The wells were washed three times for 5 min each in PBST. Substrate (100 µl 0.5 mg ortho-phenylenediamine per ml plus 5 µl H₂O₂ per ml in citrate buffer (pH 5.0)) was then added, the microtiter plate was incubated for 5 min at 37 °C and the reaction was stopped by adding 50 µl of 12.5% H₂SO₄ per well. The A₄₉₀ was measured. Experiments were performed in duplicate.

Antibiotic susceptibility testing

MIC and MBC of planktonic cells at the mid-log phase. The MIC of the planktonic cells was determined in accordance with the Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines.²⁶ Antibiotics

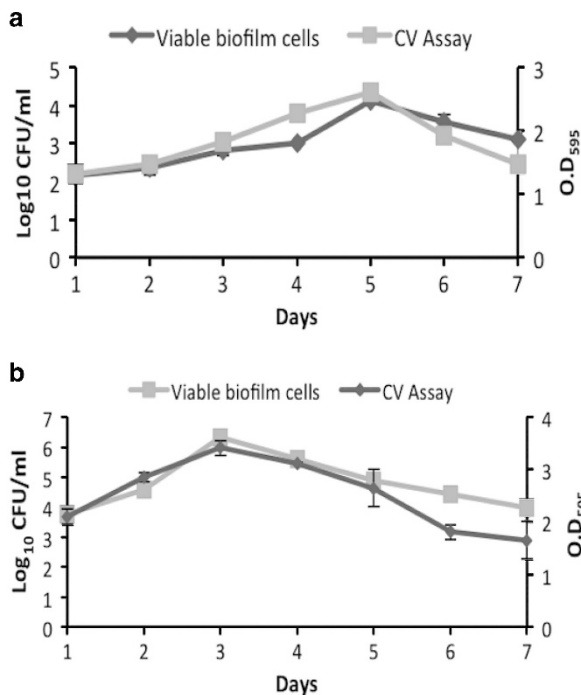


Figure 1 (a, b) Biofilm-forming ability of *ica*-negative and *ica*-positive MRSA over a period of 7 days as determined by crystal violet staining (OD_{595}) and viable biofilm cell quantification. CV, coefficient of variation.

were used at a concentration ranging from 1024 to 0.062 mg^{-1} . The highest dilution of the antibiotic showing visible inhibition of bacterial growth after overnight incubation at 37°C was taken as MIC of the drug.

The MBC of the antibiotics against planktonic bacteria at mid-log phase was determined by plating the contents of the tubes containing MIC and higher concentration of antibiotic on nutrient agar and incubated at 37°C for 24 h. Before plating, the bacteria with or without antibiotic treatment were pelleted with centrifugation at 10 000 r.p.m. for 10 min, washed twice with NS and finally suspended in 1 ml NS. The resulting cell suspension was serially diluted, and viable count was enumerated by drop plating.

MIC and MBC of biofilm cells. The method of Ceri *et al.*¹⁵ was used to determine MIC of biofilm-grown bacteria. Biofilm established in 96-well microplate was exposed to $100 \mu\text{l}$ of twofold serial dilution of antibiotics ranging from 1024 to 0.062 mg ml^{-1} in Tryptose Soy Broth. The microplate was then incubated at 37°C for 24 h. After overnight challenge, the supernatant from each well was carefully transferred to the wells in a new 96-well microplate without disturbing the biofilm, and the turbidity of the contents was visually assessed. The biofilm MIC was defined as the lowest concentration of antibiotic at which no visible growth was observed. When exposed to antibiotics at or above this concentration, the biofilm-shed bacteria showed susceptibility to antibiotic.

MBC of the biofilm bacteria was also assessed on the basis of reduction in viable counts after overnight exposure to antibiotics. The viable bacteria remaining in biofilm in wells containing MIC or higher concentrations of antibiotic were counted by plating on nutrient agar and incubating at 37°C for 24 h. MBC of biofilm was calculated by dividing the predetermined viable count of untreated biofilm by viable count obtained after antibiotic exposure.

MBC of planktonic cells at the stationary phase. MIC of antibiotic at stationary phase could not be tested by standard procedure because of high cell density of bacteria in the overnight culture. The MBC of antibiotics against planktonic bacteria at the stationary phase was determined following the Clinical and Laboratory Standards Institute guidelines,²⁷ except that stationary phase culture replaced log-phase culture. The MBCs for planktonic cells at the

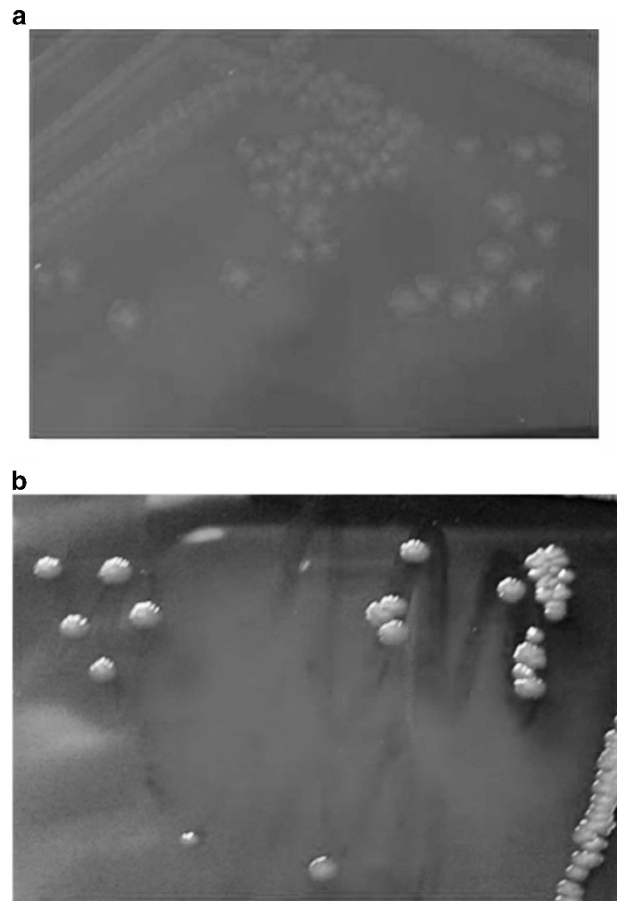


Figure 2 (a, b) Colony morphology of *ica*-negative and *ica*-positive MRSA on Congo red Agar. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

stationary phase and for biofilm was defined as the minimum concentration of antibiotics required to reduce bacterial numbers by at least 3 logs.

Effect of antibiotics on preformed biofilm. As the maximum clinically achievable concentration of vancomycin and minocycline is 60 and $4 \mu\text{g ml}^{-1}$, respectively²⁸ (intravenously), the concentration used in the susceptibility assay was 60 and $150 \mu\text{g ml}^{-1}$ for vancomycin and $4 \mu\text{g ml}^{-1}$ for minocycline. Initially, the control and untreated wells were incubated at 37°C overnight. For each subsequent day antibiotic was added to the well for that day for which it was to be tested. (For example on day 1, antibiotic was added to well untreated for 1 day, on day 2 antibiotic was added to well untreated for 2 days and so on for 7 days.) After the overnight treatment, the viable count was enumerated using drop-plating method.²⁹ Experiments were performed in duplicate.

Statistical analysis

All the experiments were performed in duplicate. The mean and s.d. were calculated from the results. The bacterial count was converted to \log_{10} . The effect of different treatments on biofilm eradication was evaluated by two-way analysis of variance and Student's *t*-test, and $P < 0.01$ was considered significant.

RESULTS

The results of biofilm formed by *ica*-positive and *ica*-negative MRSA are shown in Figures 1a and b. In case of *ica*-positive MRSA, the biofilm formation peaked on the third day of incubation, whereas in *ica*-negative MRSA maximum the biofilm formation was seen on the fifth day of incubation. Following this, a decrease in biofilm

formation was observed as bacterial counts declined with time. The results of semiquantitative estimation were also confirmed by quantification of viable biofilm cells.

Comparison of slime production and biochemical composition

The slime production by both strains under study was assessed by culturing them on CRA plates. As shown in Figures 2a and b, *ica*-negative MRSA gave pink to red colonies, whereas *ica*-positive MRSA showed black colonies with dry crystalline consistency. Further incubation up to 72 h to check delayed slime production by *ica*-negative MRSA showed no significant change in color or darkening of the center. On treating the *ica*-negative MRSA biofilm with DNase and ProteinaseK and subsequently staining it with CV, it was observed that biofilm-forming ability of *ica*-negative MRSA was reduced in comparison with control. As shown in Figure 3a, the biofilm-forming ability of *ica*-negative MRSA plunged on days 1, 2 and 3. The biofilm formation was negligible in comparison with control on days 5 (peak day), 6 and 7. However, in case of *ica*-positive MRSA (Figure 3b), treatment with DNase and Proteinase K led to a decrease in OD₅₉₅ from 3.41 to 1.36 on day 3 (peak day). Similarly, OD₅₉₅ decreased from 1.81 and 1.65 to 1.05 and 1.05 on days 6 and 7, respectively.

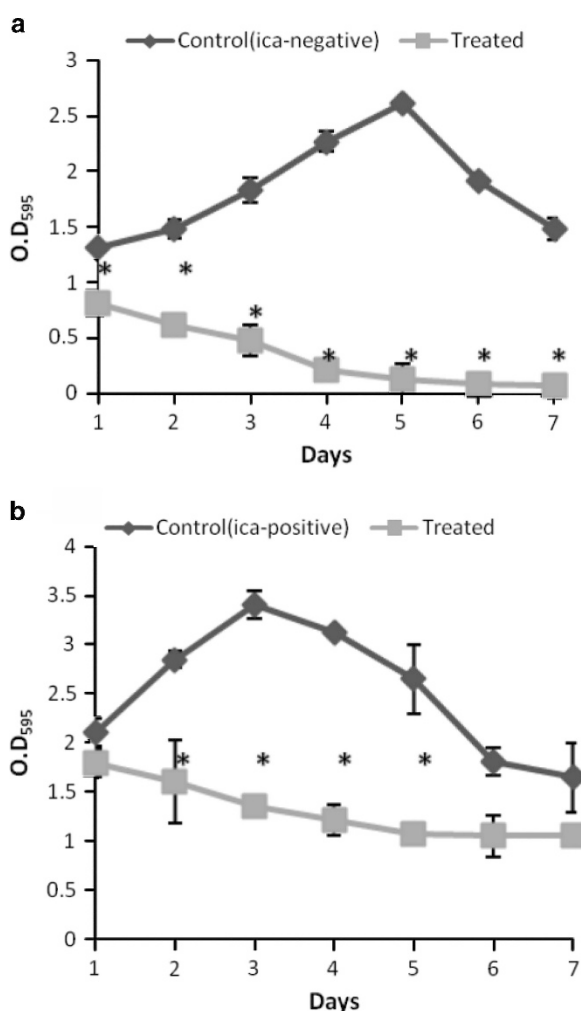


Figure 3 (a, b) Susceptibility pattern of *ica*-negative and *ica*-positive MRSA biofilms to DNase and ProteinaseK. Experiment was performed in duplicate. Error bars indicate s.d. * $P < 0.01$.

Quantification of eDNA in biofilm

The quantity of eDNA released by *ica*-negative MRSA biofilm was assessed over the period of 7 days. An increase in the amount of eDNA release was seen over time and the observed increase was $92.2 \pm 0.08 \text{ ng } \mu\text{l}^{-1}$ on day 1 to $144.1 \pm 0.05 \text{ ng } \mu\text{l}^{-1}$ on day 5 (peak day). The amount of eDNA was 132.72 ± 0.3 and $130.41 \pm 0.11 \text{ ng } \mu\text{l}^{-1}$, respectively, on days 6 and 7, showing no further change in eDNA release.

Estimation of PIA using dot blot and ELISA

The PIA production by *ica*-positive MRSA was assessed both using dot blot and ELISA. In dot blot assay, the amount of PIA was quantified on days 1, 3, 5 and 7. As shown in Figure 4, the PIA signal was detected upto 1:64 on day 7. In addition, PIA was quantified using ELISA on days 1, 3, 5 and 7. PIA production was found to increase with time (day 1: 1.83 ± 0.04 ; $A_{490} \pm \text{s.d.}$) and the maximum amount (2.33 ± 0.09) was seen on the third day (that is, peak day), after which no significant ($P < 0.01$) decrease (day 5: 2.29 ± 0.08) was observed in PIA amount (day 7: 2.28 ± 0.03).

Antibiotic susceptibility of MRSA biofilm at different stages of formation

Differences in MIC and MBC of planktonic cultures and biofilm cells of ica-negative MRSA. The MIC and MBC of clindamycin for biofilm cells was significantly higher than log planktonic cells (that is, $> 1024 \mu\text{g ml}^{-1}$), which was more than the upper detection limit as shown in Table 1. On the contrary, no significant difference in the MIC and MBC of vancomycin and minocycline for biofilm cells was observed when we compared them with log planktonic cells. The MBC of clindamycin for biofilm cells and stationary planktonic cells was more than the upper detection limit. In addition, no significant decrease in MIC and MBC of biofilm cells for vancomycin and minocycline was observed in comparison with stationary planktonic cells.

Differences in MIC and MBC of planktonic cultures and biofilm cells of ica-positive MRSA. The MBC of clindamycin and vancomycin for biofilm cells was more than the highest concentration tested in comparison with log planktonic cells. On the contrary, the MIC of

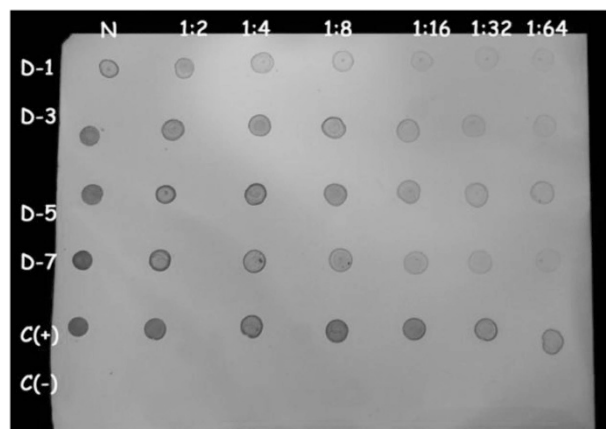


Figure 4 Semi-quantitative estimation of PIA in the biofilm formed by *ica*-positive MRSA on day 1, 3, 5 and 7 by dot blot. PIA was detected using WGA-HRP. *S. aureus* ATCC 12598 and *S. epidermidis* ATCC 12228 were used as positive (+) and negative (-) controls, respectively. N, Neat (undiluted) extract. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 Antibiotic susceptibility of three antibiotics against MRSA biofilms in different phases of biofilm formation

Mode of growth	Initial bacterial density (CFU ml ⁻¹)	Clindamycin (µg ml ⁻¹)		Vancomycin (µg ml ⁻¹)		Minocycline (µg ml ⁻¹)	
		MIC	MBC	MIC	MBC	MIC	MBC
<i>ica</i> -negative MRSA							
Log planktonic	1.2 × 10 ⁵	64	128	8	16	0.25	1
Stationary planktonic	1.1 × 10 ⁷	—	>1024	—	16	—	4
Mature biofilm	1.3 × 10 ⁷	>1024	>1024	16	32	0.5	2
<i>ica</i> -positive MRSA							
Log planktonic	3.4 × 10 ⁵	128	256	8	64	0.5	2
Stationary planktonic	3.5 × 10 ⁸	—	512	—	256	—	8
Mature biofilm	3.2 × 10 ⁸	>1024	>1024	512	>1024	8	16

Abbreviation: MRSA, Methicillin-resistant *Staphylococcus aureus*.

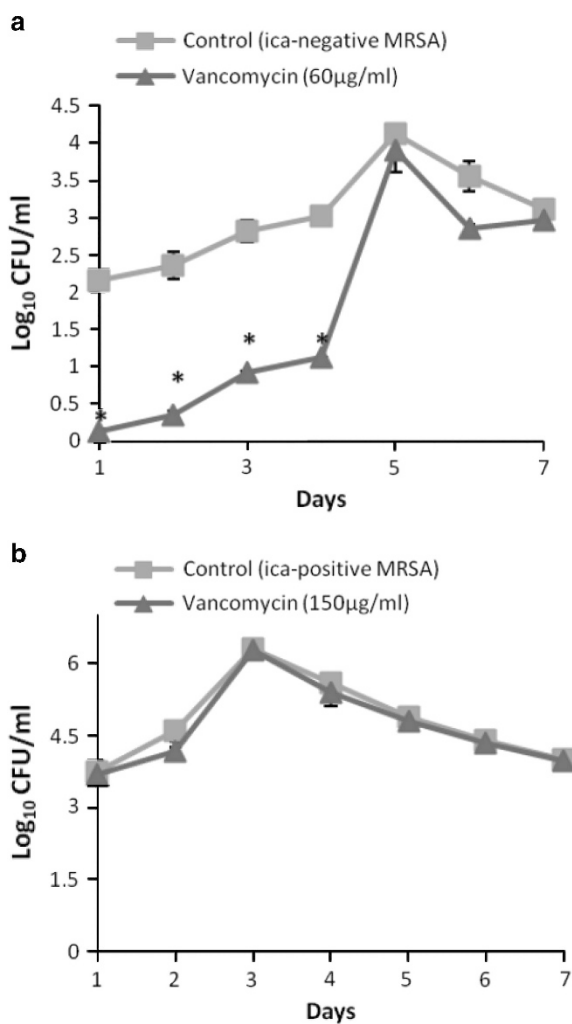


Figure 5 (a, b) Susceptibility pattern of *ica*-negative and *ica*-positive MRSA to vancomycin on different days of incubation. Experiment was performed in duplicates. Error bars indicate s.d. **P* < 0.01.

vancomycin and minocycline for biofilm cells was 64 and 16 times higher, respectively, when compared with log planktonic cells (Table 1). However, no significant decrease in the MBC of vancomycin and minocycline for biofilm cells and stationary planktonic cells was observed.

Differences in MIC and MBC of both types of biofilms. As shown in Table 1, the MIC of vancomycin and minocycline for *ica*-positive MRSA biofilm was 32 and 16 times, respectively, higher than *ica*-negative MRSA biofilm. However, clindamycin was ineffective in eradicating both types of biofilms as the MIC and MBC were found to be higher than the upper detection limit. In addition, the MBC of vancomycin was > 1024 µg ml⁻¹ for *ica*-positive MRSA biofilm.

Effect of antibiotics on preformed biofilm

Susceptibility of preformed MRSA biofilms to vancomycin. The effect of vancomycin was studied on both the *ica*-positive as well as *ica*-negative preformed biofilms. The results showed that a concentration of 60 µg ml⁻¹, which is also its highest clinical achievable concentration, was found to be quite effective in eradicating the biofilm formed by *ica*-negative MRSA (Figure 5a). A significant decrease of 2.02 logs and 2.00 logs (*P* < 0.01) was observed on day 1 and day 2, respectively. A uniform decrease was found on next consecutive days. However, a nonsignificant decrease of 0.71 logs and 0.15 logs (*P* > 0.01) was observed in the bacterial count of older biofilms (that is, peak day onwards). On the contrary, as shown in Figure 5b, vancomycin was completely ineffective in eradicating the biofilm produced by *ica*-positive MRSA biofilm even at a concentration higher than its serum achievable concentration. No significant reduction in biofilm bacterial count (*P* > 0.01) was observed from day 1 to day 7.

Susceptibility of MRSA biofilm to minocycline. The results of susceptibility of MRSA biofilm to minocycline are shown in Figures 6a and b. Minocycline was found to be quite effective on young biofilm formed by *ica*-negative MRSA at a concentration of 4 µg ml⁻¹, which is also its highest clinical achievable concentration. A significant gradual decrease in log values was detected from day 1 to day 4, and corresponding values on each day were found to be as 2.3, 2.26, 2.10 and 2.00 logs, respectively. A significant decrease of 1.90 logs was also observed on the peak day. However, no significant reduction in bacterial count was observed in older biofilm. Similarly, minocycline was able to eradicate the biofilm formed by *ica*-positive MRSA. On day 1, a significant decrease of 2.00 logs was observed. A reduction in bacterial count upto 2.03 logs was also observed on day 3 (that is, the peak day). However, minocycline was ineffective when used on older biofilms of both the MRSA strains.

DISCUSSION

Majority of infections are attributed to biofilm mode of growth.³⁰ However, antibiotics fail to eradicate these cells as concentration of

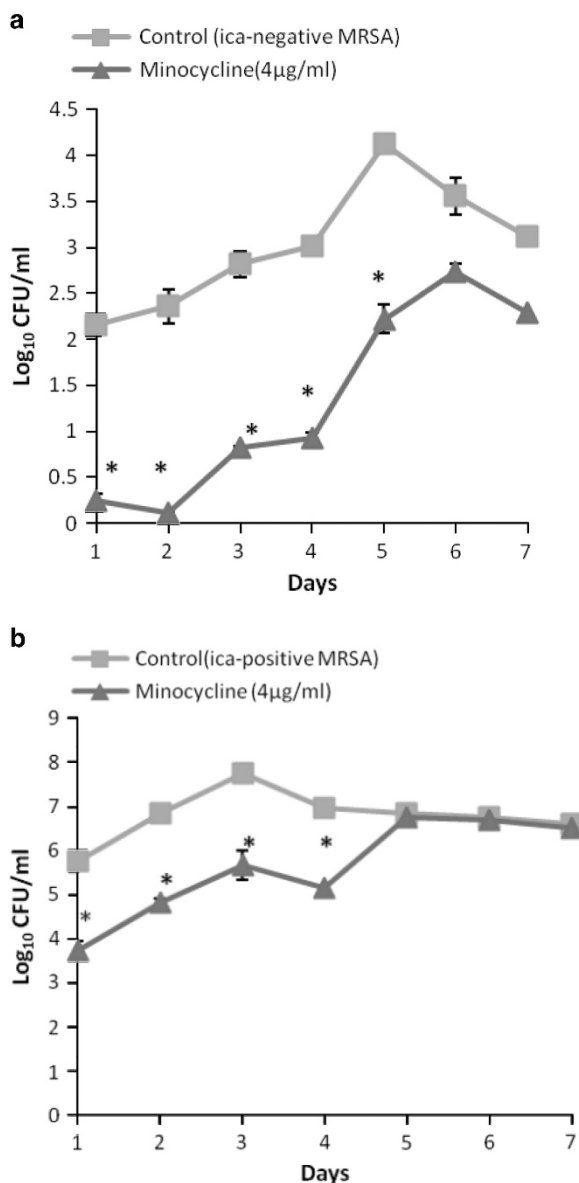


Figure 6 (a, b) Antibiotic susceptibility pattern of *ica*-negative MRSA and *ica*-positive MRSA biofilm to minocycline. Experiment was performed in duplicate. Error bars indicate s.d. * $P < 0.01$.

antibiotic required to eradicate bacteria in biofilm is much higher than MIC of planktonic cells. In this study, an attempt was made to describe a system that may help in elucidating the effectiveness of different classes of antibiotics in the eradication of biofilm produced by MRSA strains with or without *ica*-locus.

Two standard MRSA strains (ATCC MRSA 43300 possessing *ica*-locus³¹ and ATCC MRSA 33591 lacking *ica*-locus³²) were compared on the basis of their biofilm-forming ability, its biochemical/morphological characterization and antibiotic susceptibility. The different phases of growth relevant to biofilm formation were included. MRSA strain lacking *ica*-locus in comparison with *ica*-positive MRSA took longer time in establishing itself in biofilm mode. These results suggest that absence *ica*-locus most probably is a contributing factor in delaying the biofilm formation. Similar results have been proposed previously showing that *ica*-negative *S. epidermidis* was weak biofilm producer and took comparatively

longer time in establishing its biofilm mode than *ica*-positive strains.³³ To further confirm this finding, both the MRSA strains were assessed for their ability to produce slime. Slime not only helps the organism in their attachment to different surfaces but also hinders the penetration of antibiotic by acting as a chemical barrier to them. The difference in colony morphology on CRA agar confirmed the ability of *ica*-positive MRSA to produce comparatively more slime than the *ica*-negative MRSA.

The biochemical composition of biofilm produced by both the strains was significantly altered when treated with proteinase K and DNase, showing that biofilm matrix in both the cases was rich in protein. However, the biofilm-forming ability of *ica*-negative MRSA was significantly less in presence of DNase *vis a vis* *ica*-positive-treated biofilm. Hence, the eDNA content in the biofilm of *ica*-negative MRSA was evaluated. The results showed that eDNA was the major constituent of *ica*-negative MRSA biofilm. In recent study Fisher *et al.*²² have also demonstrated eDNA to be the major component of biofilm matrix in *ica*-independent *S. aureus* biofilm. On the other hand, PIA was found to be the major component of *ica*-dependent *S. aureus* biofilm. The further confirmation was made by estimating the amount of PIA and eDNA. In *ica*-positive MRSA biofilm, an increase in PIA with successive increase in biofilm age was observed. The amount of PIA produced by *ica*-positive MRSA was maximum on the peak day of biofilm formation (that is, third day). Similar increase in the content of eDNA was observed in *ica*-negative MRSA biofilm showing maximum amount of eDNA on the peak day of biofilm formation (that is, fifth day).

The planktonic cultures in the mid-log phase, stationary phase and mature biofilm were assessed for their susceptibility to different classes of antibiotics. The rationale was to compare the MIC and MBC of antibiotics for planktonic and biofilm growth of both the strains and compare the antibiotic susceptibility of two types of biofilm. Our study showed that both the strains in their log planktonic phase were susceptible to clindamycin, vancomycin and minocycline. However, no significant difference was observed in the MIC and MBC of stationary planktonic and mature biofilm. This is consistent with the findings of earlier workers (Spoering and Lewis,³⁴ Qu *et al.*³⁵ and Singla *et al.*³⁶) who also showed that stationary-phase culture possessed showed similar or higher resistance than the biofilm-grown bacteria.

On comparing the MIC and MBC of two types of biofilms, some interesting results were seen. Significant difference was observed in the MIC and MBC of vancomycin for stationary planktonic and mature biofilm of *ica*-positive and *ica*-negative MRSA. The reason for this difference probably lies in the composition of the biofilm matrix of both the biofilms. As mentioned earlier, the biofilm matrix of *ica*-positive MRSA had a dense structure, mainly composed of PIA and vancomycin being a density-dependent antibiotic was not able to penetrate into it effectively. As observed in this study, as well as reported earlier, the *ica*-negative *S. epidermidis* forms weak biofilm in the absence of *ica*-locus. Thus, lack of PIA results in less thicker biofilm, which in turn allows more effective penetration of antibiotic.³⁷ On the other hand, antibiotic belonging to the tetracycline group was found to be quite effective in eradicating both *ica*-positive as well as *ica*-negative MRSA biofilms. In the earlier study, Cerca *et al.*³⁸ also showed tetracyclines to be more effective than cell wall synthesis inhibiting antibiotics because antibiotics that target RNA and protein syntheses have similar activities on planktonic and biofilm cells. Hence, a significant decrease in the young biofilm of *ica*-positive and *ica*-negative MRSA was observed in presence of minocycline. In addition, in *ica*-negative MRSA biofilms Atl (major

autolysin) and FnBPA and FnBPB proteins have also been found to be major factors.^{6–10} According to previous findings of Yamada *et al.*³⁹ and Ledala *et al.*,⁴⁰ tetracyclines suppress the localization of Atl and this in turn lead to impaired autolysin activity. This major autolysin (Atl) not only helps in initial attachment of biofilm to various surfaces but also helps in the lysis of bacteria to release eDNA in the biofilm matrix.⁴¹

Biofilm age is also a decisive factor in determining the outcome of a therapeutic regimen.^{36,42} Older biofilms are known to require higher antibiotic concentrations for their eradication than planktonic cells as well as younger biofilm.⁴³ Our study showed that vancomycin at its highest serum achievable concentration was quite effective in eradicating the young biofilm formed by *ica*-negative MRSA. On the contrary, the antibiotic failed to eradicate the biofilm formed by *ica*-positive MRSA. Even when used at a higher concentration (that is, 150 µg ml⁻¹), it failed to give significant decrease in the number of biofilm cells. On the other hand, using minocycline at its serum achievable concentration both the biofilms were effectively eradicated. Minocycline significantly decreased the biofilm cell count in young biofilm of *ica*-positive MRSA (that is, upto 2 days) and *ica*-negative (that is, upto 4 days) MRSA. Both the antibiotics, however, were ineffective in eradicating the older biofilm. This might be because of the inaccessibility of antibiotic to bacteria as an increase in extracellular matrix was observed in older biofilm.^{44,45}

Our study confirms that biofilm kinetics, morphological characteristics and antibiotic susceptibility of MRSA biofilm are dependent upon the biochemical composition of the biofilm. The production of PIA in significant amount altered the antibiotic susceptibility of the isolates confirming that it was dependent upon the presence of *ica*-locus. The cell wall-inhibiting antibiotic such as vancomycin was not effective against *ica*-positive biofilm cells but the same could eradicate the biofilm produced by *ica*-negative MRSA. On the other hand, tetracyclines (minocycline) appeared to be most effective to treat young biofilm of both the MRSA strains. The results of this study also suggest that early initiation of treatment of bacteria with antibiotics in the biofilm mode is essential because as the biofilm grows older, there is increased production of extracellular polysaccharide and metabolically less active cells. These changes make the older biofilm resistant to antibiotics. However, the data generated in this study are based on the observations made on the standard strains of MRSA. It is suggested that future studies should concentrate on wild-type isolates from clinical sources so as to have a more meaningful data.

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