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ORIGINAL ARTICLE

Artificial *Klebsiella pneumoniae* biofilm model mimicking *in vivo* system: altered morphological characteristics and antibiotic resistance

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The purpose of this study was to develop a biofilm model of *Klebsiella pneumoniae* B5055, mimicking *in vivo* biofilm system so as to determine susceptibility of different phases of biofilm to antibiotics by three-dimensional analysis. Artificial mature biofilm of *K. pneumoniae* was made on black, polycarbonate membranes. Biofilm structure was visualized by scanning electron microscope (SEM) and confocal laser scanning microscopy (CLSM). Viable count method, CLSM and SEM analysis confirmed that mature, uniform and viable biofilms can be formed on the polycarbonate membranes by this method. The three-dimensional heterogeneity of biofilm was confirmed on the basis of results of CLSM, which is an important characteristics of *in vivo* biofilm system. Staining with the LIVE/DEAD *Bac*Light viability kit and acridine orange suggested that the center of biofilm had more inactive cells compared with actively dividing cells on the periphery. Amikacin at a concentration of 40 μg ml^{-1} was effective against younger biofilm whereas ineffective against older biofilm that showed sparsely populated dead cells using the *Bac*Light viability staining kit. Role of altered morphological characteristics toward increased antibiotic susceptibility was also studied for different phases of *K. pneumoniae* biofilm by CLSM and light microscopy. Thickness of biofilm increased from 0.093 to 0.231 mm with time. So, both heterogeneity and thickness of the biofilm are likely to influence the ineffectiveness of amikacin in older biofilm. The present model holds considerable clinical relevance and may be useful for evaluating the efficacy of antimicrobial agent on bacterial biofilms *in vitro*.
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Keywords: antibiotic resistance; biofilm model; confocal laser scanning microscopy (CLSM); thickness

INTRODUCTION

Biofilms are aggregates of microorganisms that are formed on abiotic and biotic surfaces. The microorganisms present in the biofilm state exhibit different rates of growth and metabolic activity. This is due to heterogeneity in terms of oxygen and nutrient concentrations in microbial biofilms. Even pure cultures of microorganisms growing in biofilms experience these gradients and thus represent a range of metabolic states. The variety of growth states that can be represented in a biofilm, surely contribute to the antimicrobial tolerance manifested by biofilms. The infections caused by bacteria in biofilm mode are difficult to treat as they are highly resistant to antibiotics and hence often show adverse therapeutic outcome.[1,2]

To examine potential of antimicrobial strategies for treating biofilm-related infections, animal models have been used in the past. However, there is a growing need to establish pure or mixed artificial mature biofilms *in vitro* that can be used to inoculate animals for establishing biofilm-related infections. So an artificial biofilm may not only be useful for understanding biofilm formation and its biological activities, but such an approach will also help in establishing the infection in an *in vivo* system. In the past, various methods like CDC (Centers for Disease Control) biofilm reactor, modified Robbin's device, Calgary Biofilm Device and modified CDC biofilm reactor have been reported to produce mature biofilms.

However, the development of simple and sensitive methods for biofilm monitoring are essential as most of the approaches used earlier required either destructive sampling of biofilms, or did not provide information about their architecture, spatial structure and physiological heterogeneity.[3] The development of non-invasive and non-destructive techniques such as confocal laser scanning microscopy (CLSM) in recent years has helped in better understanding of microbial biofilms. There have been few investigations in which growth pattern of different phases of biofilms has been visualized using this technique.

In this study, we report a simple method to produce mature biofilm of *Klebsiella pneumoniae* B5055 compared with other methods, which are not only tedious but also involve the use of sophisticated instruments. The combination of CLSM with viable cell count method provided an additional advantage in assessing the effect of antibiotic on the biofilm cells, especially of different phases of *K. pneumoniae* biofilm.

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MATERIALS AND METHODS

Bacterial strain, media and antibiotic

Klebsiella pneumoniae B5055 obtained from Dr M Trautman, Department of Medical Microbiology and Hygiene UIm, Germany was used in the present study. Nutrient broth was used routinely for bacterial culture. Nutrient agar was used as the culture medium for biofilm production of *K. pneumoniae*. Normal saline (0.85% sodium chloride in water w/v) was prepared and used for culture dilutions.

Biofilm development

Colony biofilms of *K. pneumoniae* were grown on black, polycarbonate disc (EMD Millipore Corporation, Billerica, MA, USA) according to the method described by Anderl *et al.*[4] with modifications. Briefly, culture of *K. pneumoniae* was grown overnight in nutrient broth at 37 °C and diluted to an OD (600 nm, 1 cm path length) of 0.300 in normal saline. In all, 10 µl of diluted planktonic culture was used to inoculate individual sterile, black, polycarbonate membrane filters (diameter, 47 mm; pore size, 0.2 µm) resting on agar culture medium. The membranes were sterilized by UV exposure (15 min per side) before inoculation. The plates were inverted and incubated at 37 °C till 7 days, with the membrane-supported biofilms transferred to fresh culture medium every 24 h.

Viable bacteria enumeration

The biofilms were sampled every 24 h. The biofilms were then washed with normal saline by agitation to remove non-adherent cells. For the determination of number of CFU, each membrane-supported biofilm was placed in centrifuge tube containing 9.0 ml of normal saline, the mixture was vortexed at high speed for 3 min with a Vortex mixer and then serially diluted in normal saline. Viable bacteria were enumerated by plating serially diluted samples onto nutrient agar by the drop plating method[5] and the plates were incubated at 37 °C for 24 h. The experiments were performed in duplicate.

Scanning electron microscopy of biofilm

Untreated biofilm was examined by scanning electron microscope (SEM) according to the method of Yasuda *et al.*[6] with modifications. Briefly, the colony biofilms were fixed with 2.5% glutaraldehyde (pH 7.2) at room temperature for 1 h, washed thrice in the rinsing buffer (Sorensen's phosphate buffer (0.1 mol l^{-1}, pH 7.2) + 7% sucrose) at 4 °C for 15 min each, and fixed with 1% osmium tetraoxide at 4 °C for 30 min. This was followed by three washings in rinsing buffer at 4 °C for 15 min each and dehydration of the biofilms in a series of ethanol solutions (50%, 10 min; 70%, 10 min; 80%, 15 min; 90%, 15 min; and 100%, 20 min). The specimens were then critical point dried, coated with gold, and observed under scanning electron microscope (Jeol JSM-6100, JEOL Ltd., Tokyo, Japan). The experiments were performed in quadruplicate.

Biofilm susceptibility assay

The MIC of amikacin (Hi Media Laboratories Pvt. Ltd., Mumbai, India) for planktonic cells of *K. pneumoniae* B5055 was determined according to the CLSI guidelines.[7] The MIC was defined as the lowest concentration of antibiotic that prevented visible growth after overnight incubation at 37 °C. To study the effect of antibiotic treatment on biofilms, the biofilm laden discs were treated with antibiotic at 37 °C by placing them on agar plates containing amikacin at a concentration of 40 µg ml^{-1} and bacterial load of the treated biofilm was estimated. The biofilms were sampled after every 24 h up to 7 days.

Thickness of biofilm

The biofilm thickness was determined by light microscopy using an Olympus CH20i light microscope (Olympus America Inc., Center Valley, PA, USA) according to the method of Bakke and Olsson[8] with modifications.

Scanning confocal laser microscopy of biofilms

Antibiotic treated and untreated biofilms were examined by scanning confocal laser microscopy (SCLM) (Nikon Ti eclipse, Nikon Corporation, Tokyo, Japan). For SCLM, biofilm-coated polycarbonate membrane filters were stained with the *Bac*Light Live/Dead fluorescent stain (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Untreated biofilms were also stained with acridine orange (Hi Media Laboratories Pvt. Ltd.) for 5 min to study spatial patterns of growth rate. A stock solution of 2 mg ml^{-1} acridine orange in phosphate buffer (pH 7.2) was prepared. From the stock solution, a fresh solution of 4 mg l^{-1} acridine orange in phosphate buffer was prepared for colony staining. Filters were then placed on glass slides, covered with a coverslip and examined with inverted SCLM. The Live/Dead stain differentiates live from dead bacteria by staining living bacteria fluorescent green and the dead ones fluorescent red, while the background remains virtually non-fluorescent.

Statistical analysis

All experiments were performed in duplicate and repeated at least three times on different days. The bacterial count was expressed as $\log_{10}$.[4] On different days of biofilm formation, all the data from a particular treatment and time point were taken and log reduction in comparison with untreated biofilm at the respective time point was calculated. The effect of different treatments on biofilm eradication was evaluated by the Student's *t*-test and $P<0.01$ was considered as significant. Data were analyzed using the Excel software.

RESULTS

Bacterial Quantification

When the biofilm of *K. pneumoniae* was allowed to grow on polycarbonate discs, 2-day biofilm showed a constant bacterial count of ~8.81 logs CFU per membrane. However, estimation of bacterial numbers in untreated biofilms on disc showed an increase, with a peak on fifth day of incubation, after which the biofilm bacterial counts decreased progressively (Figure 1).

SEM and CLSM analysis of mature biofilm

The biofilms developed on polycarbonate discs were observed using SEM for direct evidence of mature biofilm formation on discs. SEM images of 2-day biofilm on polycarbonate discs after sample fixation and dehydration indicated biofilm formation on the membrane surfaces (Figure 2a). Upon SEM analysis, all the three indicators of a mature biofilm were detected; that is, significant exopolysaccharide production (Figures 2b and c), possible water channel development (Figure 2d) and three-dimensional structures of the biofilm extending vertically from the surface of the polycarbonate disc (Figures 2b and c). Moreover, staining of biofilm using Live/Dead fluorescent stain indicated the presence of live cells within the biofilm as majority of the cells stained green rather than red (Figure 2e).

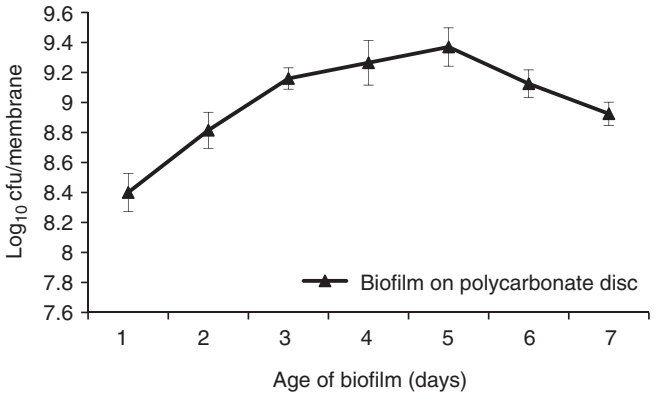

Figure 1 Kinetics of biofilm growth on polycarbonate membrane when biofilms were grown for 7 days. The experiment was repeated at least three times and error bars = ± s.d.

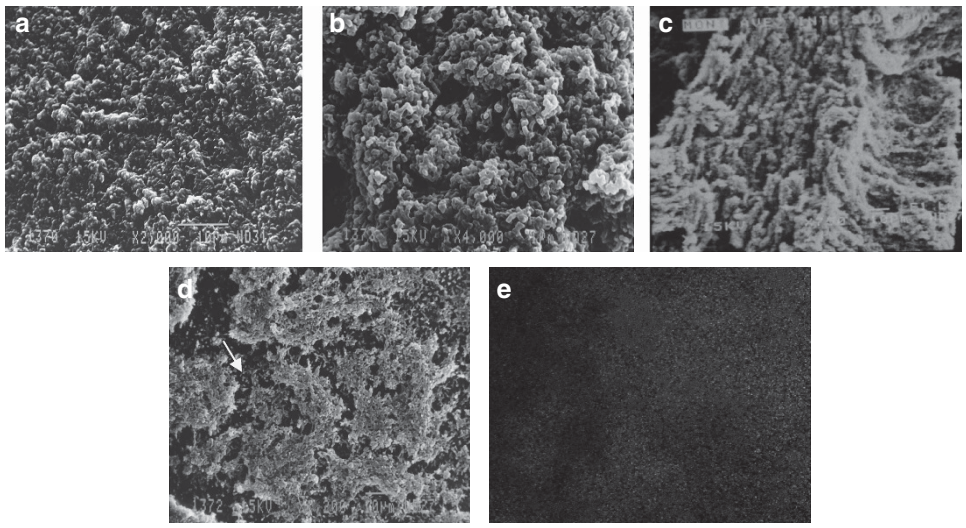

Figure 2 (**a**) Representative SEM image of the *Klebsiella pneumoniae* biofilm. (**b**, **c**) Representative image showing a three-dimensional structure of the biofilm extending vertically from the surface of the membrane. (**d**) Representative image showing possible water channels within the biofilm structure (white arrow). (**e**) Image showing a Z projection slice of the *K. pneumoniae* biofilm collected using CLSM. Original magnification was ×60. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Morphological characteristics of biofilm

Biofilm was developed in both XY and XZ direction during a 7-day period. With the increase in the biofilm age, the microorganisms multiplied horizontally (XY direction) and the area of biofilm increased from 19.62 to 153.86 mm^2 over a period of 7 days (Figure 3). At the same time, the organisms also multiplied vertically (XZ direction), forming thick bacterial masses. Thickness of biofilm increased from 0.093 to 0.231 mm up to day 5 and it remained constant afterwards (Figure 3).

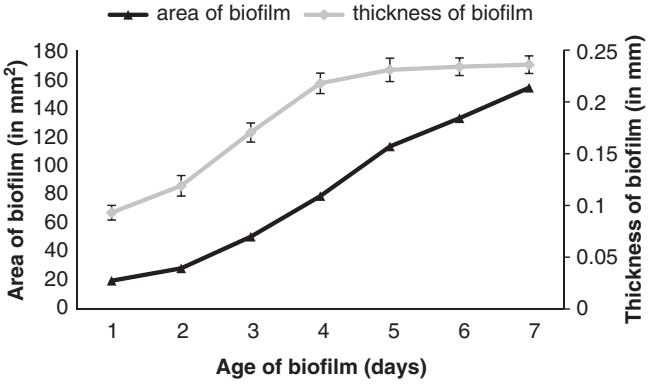

Figure 3 Area and thickness of *K. pneumoniae* biofilm developed on polycarbonate membrane.

Antibiotic susceptibility of biofilm

On exposure of planktonic culture of *K. pneumoniae* to different concentrations of amikacin, 16 μg ml^{-1} was found to be the MIC of the antibiotic. Treatment of biofilm with amikacin (40 μg ml^{-1}) led to a significant reduction of 2.1 logs ($P<0.01$) in the bacterial count up to day 4 of biofilm formation, indicating that amikacin was effective against younger biofilm at 40 μg ml^{-1}. However, it was ineffective against older biofilm (that is, fifth day onwards) as insignificant reduction in the log bacterial count; that is, 0.62 ($P>0.01$) was observed (Figure 4).

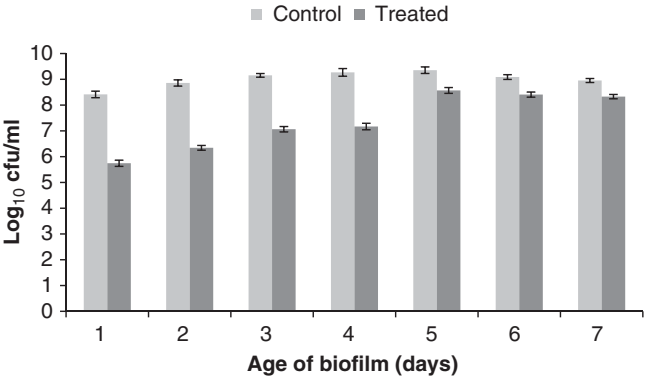

Figure 4 Susceptibility of *K. pneumoniae* biofilm to amikacin. Experiment was performed in duplicate. Error bars indicate standard deviation.

CLSM analysis of untreated *K. pneumoniae* biofilms

To establish the live/dead pattern within *K. pneumoniae* biofilm, young and older biofilms grown on black, polycarbonate discs were stained with the Live/Dead *Bac*Light reagent. The Live/Dead stain uses two fluorescent nucleic acid stains, Syto9 and propidium iodide. Syto9 is used to quantify 'live' cells because it can permeate cells under all conditions; propidium iodide is used to quantify 'dead' cells because, as a highly charged molecule, it is unable to permeate into cells with a strong electrochemical gradient across the membrane (that is, the chemiosmotic potential). At young biofilm stage, almost all of the biofilm cells stained 'live' (Figure 5a) whereas almost all the cells in the middle of the biofilm structure stained 'red' and peripheral cells as live in older biofilms (Figure 5b). Less information is available how the membrane properties of cells in biofilms change with time. Given this uncertainty that cells in the center of 7-day old biofilm stained as dead cells, acridine orange was employed for staining to identify regions of biofilms that contain rapidly or slow growing cells based on their relative RNA–DNA content. Staining of 7-day-old biofilms showed orange fluorescence close to the air and agar interfaces and green at the center of the biofilm (Figure 6).

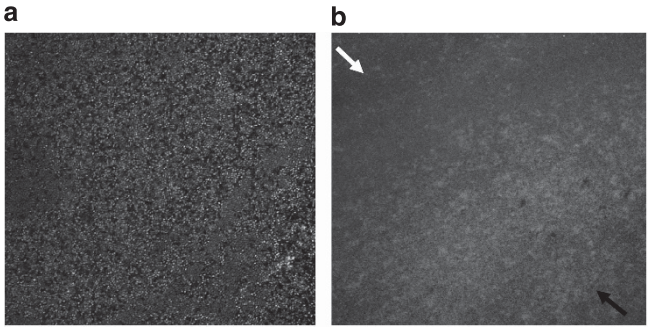

Figure 5 Live/dead staining of (**a**) young and (**b**) old *K. pneumoniae* biofilms. Green indicates the 'live' cells (Syto9) and red indicates the 'dead' cells (propidium iodide). White and black arrows indicate the regions of the periphery and the center of the biofilm, respectively. Periphery and center in this context refer to the region representing the inner and outer regions, respectively, of the biofilm surface when viewed by confocal microscope. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

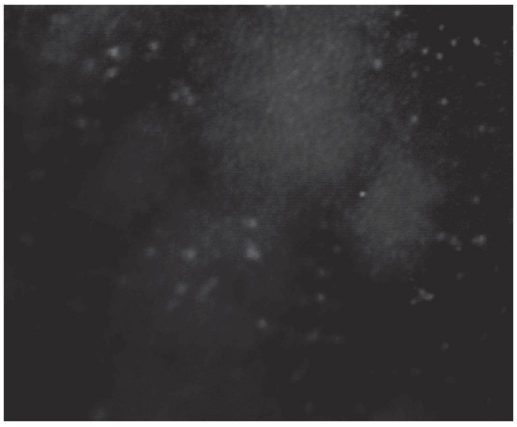

Figure 6 Physiological heterogeneity in biofilms. The spatial pattern of growth rate within a *Klebsiella pneumoniae* biofilm, as judged by acridine orange staining. In this figure, areas of orange staining correspond to a high relative RNA content and thus rapid growth whereas cells stained green have low relative RNA content and show a slower growth rate. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Green color was most evident in the thickest parts of the colony. These results indicated that there are clearly distinct regions of faster and slower growth throughout the biofilm.

LIVE/DEAD staining of antibiotic treated *K. pneumoniae* biofilms

The young and old biofilms (3 days and 7 days old) were treated with antibiotic, stained with the live/dead *Bac*Light kit and visualized by confocal microscope. The results showed predominance of red fluorescence indicating damaged biofilm cells following antibiotic treatment in comparison with the control showing green color dominance in young biofilm (Figures 5a and 7a). However, antibiotic treatment of 7-day old biofilm showed relatively less number of red fluorescing cells as compared with 3-day treated biofilm, which indicated its relatively lower potential to eradicate older biofilms (Figure 7b).

DISCUSSION

An increased understanding of the important role played by biofilms in device-related and chronic infections has been recognized. In the present study, an attempt was made to develop an *in vitro* model of artificial mature biofilm that closely mimicked *in vivo* biofilm. The results showed that uniform mature and viable biofilms of *Klebsiella pneumoniae* could be easily established on the polycarbonate membranes. The viable bacterial cells could be obtained in high number after 40 h of incubation. The staining of a 3-day-old biofilm with the commercial *Bac*Light viability kit further confirmed that majority of bacteria in this biofilm were viable. SEM analysis of biofilm revealed complex structure as enhanced exopolysaccharide production, water channel formation and three-dimensional structures were observed. This method of biofilm formation was found to be simple and cost effective as compared with a recently reported method of Williams *et al*.[9] who demonstrated formation of biofilm of *S. aureus* on the surface of polyetheretherketone membranes by using a modified CDC biofilm reactor. In addition the method reported in this study showed a heterogeneous three-dimensional structure that mimicked *in vivo* biofilm formation. On the contrary microtiter plate method and Calgary Biofilm Device method, however, lack this feature as homogeneous biofilms are formed differing greatly from *in vivo* biofilms.

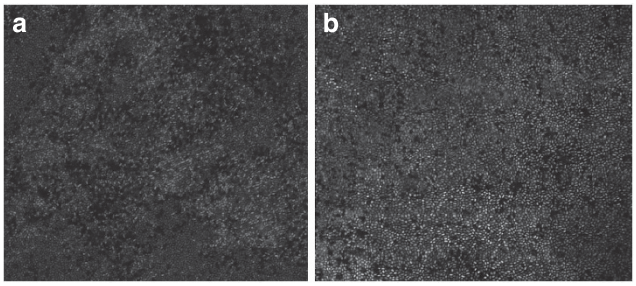

Figure 7 (**a**) Young biofilm (**b**) old biofilm stained with the Live/Dead *Bac*Light Bacterial viability kit following treatment with antibiotic. The green fluorescence represents viable cells while red fluorescence represents dead cells. All the discs were visualized using $\times 60$. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Three-dimensional information is important because biofilms are known for their structural and physiological heterogeneity.[10–12] Biofilm model described in the study can also be used to study spatial heterogeneity (three-dimensional arrangement of cells) in bacterial biofilms. In the present study, CLSM analysis using the LIVE/DEAD *Bac*Light bacterial viability kit showed existence of spatial heterogeneity in older biofilm as majority of the cells in the middle of the biofilm were dead cells. On the contrary, bacterial cells in young biofilms were viable as all of them stained green. In biofilms where microorganisms are present in high cell densities, it is likely that a significant fraction of the population is not actively growing.[13,14] The dead cells identified in the middle of the older biofilm are therefore metabolically inactive forms. To provide further support to this observation, older biofilm of *K. pneumoniae* was stained with acridine orange. Spatiometabolic heterogeneity was observed in older biofilm as orange areas marked in the biofilm–nutrient interface represented cells with fast growth rates and yellow/green areas marked in the center of the biofilm represented slow growing cells. Wentland *et al*.[15] have also reported spatial heterogeneity in older biofilm of *Shewanella oneidensis* using CLSM study.

No attempt has been made earlier to determine the efficacy of antibiotic during different phases of *K. pneumoniae* biofilm while maintaining its structural heterogeneity as observed in natural biofilms. In the present study, the efficacy of amikacin ($40\,\mu g\,ml^{-1}$) in eradicating the biofilm of *K. pneumoniae* B5055 was studied. Viable

count method showed that antibiotic treatment resulted in a significant reduction ($P<0.05$) in the growth of younger biofilms (that is, up to 4-day-old biofilm) while it remained ineffective against older biofilm (that is, fifth day onwards). Treated biofilm of *K. pneumoniae* B5055 when visualized under confocal microscope after staining with the Live/Dead *Bac*Light bacterial viability staining kit showed comparable results with that obtained by viable count method. Qualitative analysis of viability of antibiotic treated biofilm showed predominance of dead cells in 2-day-old biofilm compared with older biofilm.

Researchers have reported that many antibiotics, such as aminoglycosides, are less effective against the same organism under anaerobic conditions than under aerobic conditions.[16] This may be one of the reasons for the observed ineffectiveness of amikacin against older biofilm. Stewart *et al.*[17] reported that not only biofilm age but also its thickness produces a barrier-like effect, limiting the transport of antimicrobial agents. So, light microscopy was used to determine the thickness of *K. pneumoniae* biofilm over a period of 7 days. Thickness of biofilm was found to increase from 0.093 to 0.231 mm up to day 5 and remained constant afterwards. This in turn might be another factor responsible for increased resistance of older biofilm of *K. pneumoniae* to amikacin, as observed in this study. On the basis of the results, it was found that transport through thick biofilm matrix resulted in resistance to antimicrobials compounds.[17]

The system reported in this study will help to decide the dose of antibiotics accurately for eliminating biofilm-related infections because the concentration of antibiotic measured by using planktonic cells or homogeneous biofilms has been found to be inadequate for eradicating *in vivo* biofilms.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **284,** 1318–1322 (1999).
2 Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2,** 95–108 (2004).
3 Rozak, D. B. & Colwell, R. R. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* **51,** 365–379 (1987).
4 Anderl, J. N., Franklin, M. J. & Stewart, P. S. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* **44,** 1818–1824 (2000).
5 Miles, A. A., Misra, S. S. & Irwin, J. O. The estimation of the bactericidal power of the blood. *J. Hyg. (Lond)* **38,** 732–749 (1938).
6 Yasuda, H., Ajiki, Y., Koga, T. & Yokota, T. Interaction between clarithromycin and biofilms formed by *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* **38,** 138–141 (1994).
7 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically-Seventh Edition: Approved Standard M7-A7* (CLSI, Wayne, PA, USA, 2006).
8 Bakke, R. & Olsson, P. Q. Biofilm thickness measurements by light microscopy. *J. Microbiol. Methods* **5,** 93–98 (1986).
9 Williams, D. L., Woodbury, K. L., Haymond, B. S., Parker, A. E. & Bloebaum, R. D. A modified CDC biofilm reactor to produce mature biofilms on the surface of PEEK membranes for an *in vivo* animal model application. *Curr. Microbiol.* **62,** 1657–1663 (2011).
10 Rani, S. A. *et al.* Spatial patterns of DNA replication, protein synthesis and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J. Bacteriol* **189,** 4223–4233 (2007).
11 Stewart, P. S. Diffusion in biofilms. *J. Bacteriol.* **185,** 1485–1491 (2003).
12 Xu, K. D., Stewart, P. S., Xia, F., Huang, C.-T. & McFeters, G. A. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl. Environ. Microbiol.* **64,** 4035–4039 (1998).
13 Davey, M. E. & O'Toole, G. A. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64,** 847–867 (2000).
14 Tolker-Nielsen, T. & Molin, S. Spatial organization of microbial biofilm communities. *Microb. Ecol.* **40,** 75–84 (2000).
15 Wentland, E. J., Stewart, P. S., Huang, C. T. & McFeters, G. A. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnol. Prog.* **12,** 316–321 (1996).
16 Tack, K. J. & Sabath, L. D. Increased minimum inhibitory concentrations with anaerobiasis for tobramycin, gentamicin, and amikacin, compared to latamoxef, piperacillin, chloramphenicol, and clindamycin. *Chemotherapy* **31,** 204–210 (1985).
17 Stewart, P. S., Grab, L. & Diemer, J. A. Analysis of biocide transport limitation in an artificial biofilm system. *J. Appl. Microbiol.* **85,** 495–500 (1998).