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Streptomycin mediated biofilm inhibition and suppression of virulence properties in *Pseudomonas aeruginosa* PAO1

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Abstract

Pseudomonas aeruginosa is known as an opportunistic pathogen whose one of the antibiotic resistance mechanisms includes biofilm formation and virulence factor production. The present study showed that the sub-minimum inhibitory concentration (sub-MIC) of streptomycin inhibited the formation of biofilm and eradicated the established mature biofilm. Streptomycin at sub-MIC was also capable of inhibiting biofilm formation on the urinary catheters. In addition, the sub-MIC of streptomycin attenuated the bacterial virulence properties as confirmed by both phenotypic and gene expression studies. The optimal conditions for streptomycin to perform anti-biofilm and anti-virulence activities were proposed as alkaline TSB media (pH 7.9) at 35 °C. However, sub-MIC of streptomycin also exhibited a comparative anti-biofilm efficacy in LB media at similar pH level and temperature. Furthermore, this condition also improved the biofilm inhibition and eradication properties of streptomycin, tobramycin and tetracycline towards the biofilm formed by a clinical isolate of *P. aeruginosa*. Findings from the present study provide an important insight for further studies on the mechanisms of biofilm inhibition and dispersion of pre-existing biofilm by streptomycin as well as tobramycin and tetracycline under a specific culture environment.

Keywords Anti-biofilm · Biofilm · Eradication · Inhibitory concentrations · Motility · Streptomycin

Introduction

Pseudomonas aeruginosa is known as one of the common causes of nosocomial bloodstream and urinary tract infections (Miyoshi-Akiyama et al. 2017; Talwalkar and Murray 2016). The bacterial pathogenesis which results in chronic infections is governed by biofilm formation and virulence factor production (Ben Haj Khalifa et al. 2011; Jamal et al. 2018; Kannan et al. 2018). Biofilm is a community of sessile cells enclosed within extracellular polymeric substances (EPS) which is composed of exopolysaccharides, lipid, proteins, and extracellular DNA (e-DNA) (Billings et al. 2015; Flemming et al. 2016). *P. aeruginosa* is able to form biofilm in the living host and on several clinical devices such as ventilators and catheters (Olejnickova et al. 2014; Ramirez-Estrada et al. 2016). As compared to the planktonic counterparts, the biofilm lifestyle provides protection to the bacterial cells from the host immune system, tolerance to extreme environmental conditions, and resistance to a majority of conventional antibiotics (Flemming and Wingender 2010; Mah and O'Toole 2001). In most cases, antibiotics resistance derived from biofilm formation is proposed to attribute to (1) the biofilm architecture and thickness, which minimize drug penetration and diffusion; (2) the slow metabolic rate, which lowers susceptibility to drug; and (3) the components such as negatively charged e-DNA, exopolysaccharides, and antibiotic-modifying enzymes, which hinder the penetration of antibiotics (Hall and Mah 2017; Mulcahy et al. 2008; Penesyan et al. 2015; Shaikh et al. 2015; Singh et al. 2016).

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Combining with the heterogenicity and rapid gene transfer capability of the bacterial community lying within the biofilm, there is no doubt that most of the traditional antibiotic therapies were unable to combat the spread of biofilm formation as well as other antibiotic resistance mechanisms.

Due to the complications in biofilm nature, one of the recent anti-biofilm approaches is proposed to target several virulence phenotypes (Allen et al. 2014; Pattnaik et al. 2018; Qiu et al. 2019). Instead of "killing" the bacteria, this anti-virulence approach attempts to attenuate a wide range of other virulence factors, e.g., pyocyanin, rhamnolipid, iron-acquiring siderophores, proteases, and hemolysins, which are vastly produced along with biofilm formation and also regulated by quorum sensing (QS) signaling system (Khan et al. 2019a), thereby weakening the bacterial pathogenicity, reducing the infection severity and combatting the correlated capability of forming biofilm (Fleitas Martinez et al. 2019; García-Contreras et al. 2013). In fact, this approach has been reported as a highly potential replacement for traditional antibiotic treatments as to some extent, it could reduce the selective pressure caused by antibiotics, which in turn limits the possibility of developing resistance by gene mutation (Cegelski et al. 2008; Parrino et al. 2018). Therefore, attenuating virulence properties could be considered as a potential anti-biofilm strategy; hence, extensive research for a novel effective anti-virulence agent has been on demand.

Anti-virulence agents and their efficacy are extremely varied in sources and active concentrations. Among the new sources of these agents, common antibiotics at their sub-minimum inhibitory concentrations (sub-MICs) were also recognized for their inhibitory activity towards numerous Gram-negative and Gram-positive bacteria (Bala et al. 2011; El-Mowafy et al. 2017; Gupta et al. 2016; Haddadin et al. 2010; Imperi et al. 2014; Vidya et al. 2005). Aminoglycosides with polycationic nature are one of the traditional antibiotics that are currently re-examined for their virulence inhibition potentials. A majority of aminoglycoside applications are now being used in combination therapy in which the antibiotic is conjugated with other chemical compounds or immobilized onto a carrier, which was previously proposed to help minimize the side effects which resulted from high concentration and frequent use of the antibiotic (Alipour et al. 2010; Chanda et al. 2017; Furiga et al. 2015). To our updated knowledge, although gentamycin, tobramycin, and fluoroquinolone either alone or in combination have been reported as effective anti-biofilm agents against *P. aeruginosa*, the prevalence of resistance emergence urges the search for alternative approaches such as combination, anti-virulence, and anti-quorum sensing strategies (Antoniu 2015; Borges et al. 2017; Chanda et al. 2017; Das et al. 2016; Parai et al. 2018). Currently, despite being known as a commonly used member of aminoglycoside family, the action on *P. aeruginosa* biofilm at sub-MIC of streptomycin has been limitedly known. Therefore, the present study has selected streptomycin to evaluate its performance at sub-MIC under different environmental conditions (pH, culture media, and temperature) towards *P. aeruginosa* biofilm formation and production of several virulence properties, thus suggesting the promising role of this antibiotic as an anti-virulence agent for treating *P. aeruginosa* infections.

Materials and methods

Bacterial strains, chemicals, and growth conditions

Pseudomonas aeruginosa PAO1 KCTC1637 was obtained from Korean Collection for Type Cultures (KCTC, Daejeon, Korea). *P. aeruginosa* GNUH-NCCP 6039 (isolated from pleural fluid) was purchased from Gyeongsang National University-Hospital Branch of the National Culture Collection for Pathogens (GNUH-NCCP, Gyeongsang, Korea). Cultivation of *P. aeruginosa* strains was performed in tryptic soy broth (TSB; Difco Laboratory Inc., Detroit, MI, USA), Mueller-Hinton broth (MHB), Luria-Bertani (LB) broth, and TSB-agar. Streptomycin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The pH of each media was adjusted to 5.9, 7.2, and 7.9 using HCl and NaOH. The stock concentration of streptomycin was maintained acidic (pH 5.9) by preparing in 10^{-5} N HCl. The temperature used for the growth of *P. aeruginosa* was 25 °C, 30 °C, and 35 °C in aerobic condition. The reagents and chemicals used in the whole experiment at the present were of analytical grade.

Determination of minimum inhibitory concentration and sub-MIC levels of streptomycin

For determination of minimum inhibitory concentration (MIC) of streptomycin, the *P. aeruginosa* cell culture grown overnight in TSB was diluted (1:100) in fresh and sterile TSB media (pH 7.9). The diluted culture (250 μl) was transferred to a 96-well microtiter plate in triplicates. Streptomycin at the concentrations ranged from 0.0625 to 16 μg ml^{-1} was then added to the microtiter plate. Subsequently, the microtiter plate was incubated at 35 °C under shaking condition (567 cycle per minute (cpm)) in microplate reader for 28 h. At the end of incubation, the optical density (OD) of the grown cell culture was measured at 600 nm of wavelength using the microplate reader (BioTek, Winooski, VT, USA).

Similarly, the sub-MIC of streptomycin was also determined by incubating the overnight grown *P. aeruginosa* cell culture (1:100 dilution) in a 96-well microtiter plate. The concentration of streptomycin was taken in the range from 0.0625 to 8 μg ml^{-1}. The 96-well microtiter plate was incubated under shaking condition in microplate reader at 35 °C for 28 h

and the OD was measured at 600 nm wavelength at every 2-h time interval.

Biofilm inhibition assays

The formation of biofilm by *P. aeruginosa* was quantitatively estimated by carrying out the crystal violet staining as discussed previously (Lee et al. 2011). The *P. aeruginosa* biofilm formation was carried out in TSB media (pH 7.9) by placing the diluted overnight grown cell culture (1:100 dilution) in a 96-well microtiter plate. The cells were treated with sub-MIC of streptomycin (ranging from 0.0625 to 4 μg ml^{-1}) and incubated at 35 °C for 24 h. The biofilm inhibition assay by sub-MIC of streptomycin to *P. aeruginosa* was also tested in MHB and LB growth media at different pH (5.9, 7.2, and 7.9) and temperature (25, 30, and 35 °C). After incubation for 24 h, the growth of both planktonic and attached bacterial cells was measured at OD at 600 nm. The procedure of biofilm inhibition assay included three-time washing of the attached cells with distilled water after planktonic cells being discarded and staining with 0.1% crystal violet dye. The residual dye was removed by three-time washing with distilled water and the attached cells were re-suspended in 95% ethanol. The total biofilm-forming cells were measured by OD measurement at 570 nm. The experiment was performed in triplicates.

Biofilm assays on the surfaces of urinary catheter

Urinary catheter which is a medical device was also used to examine the effect of sub-MIC of streptomycin on *P. aeruginosa* biofilm formation as described earlier with a slight modification (Al-Mathkhury et al. 2011; Kart et al. 2017). Polyvinyl chloride urinary catheter was purchased from Hyupsung Medical Co., Ltd., Korea, and was aseptically cut into coupons (15 mm × 15 mm). The catheter coupons were placed in a 6-well plate containing overnight grown cell culture (with an initial OD_{600} of 0.05) in TSB (pH 7.9) and sub-MICs of streptomycin. The titer plate was incubated at 35 °C for 24 h. Two methods were adopted to quantify the biofilm cells: 1-crystal violet staining and 2-bacterial viable cells count. In crystal violet staining, the planktonic cells were removed and the cells attached on the coupons were washed three times with distilled water and stained with 0.1% aqueous crystal violet. After 20 min of staining, the residual crystal violet was discarded and the catheter coupons were washed thrice with distilled water. The stained cells present on the catheter coupons were dissolved into 95% ethyl alcohol in test tubes and the tubes were sonicated (10 min) and vortexed at high speed in order to detach the residual attached cells into solution. The OD of cell suspension was measured at 570 nm. In viable cell count method, the colony-forming unit (CFU) of biofilm cells was determined. Briefly, catheter coupons along with overnight grown *P. aeruginosa* cell culture and sub-MIC of streptomycin in 6-well plate were incubated at 35 °C for 24 h. After 24 h of incubation, the free-floating planktonic cells were discarded and the catheter coupons were washed three times with fresh TSB. Each catheter coupon was then placed in a sterile test tube containing 1 ml fresh TSB and was vortexed at high speed for 10 min. A serial dilution of cell suspension was carried out up to 10^{-7} dilutions in the fresh and sterile TSB. The diluted cell suspension (100 μl) was spread onto TSA agar plate, followed by incubation at 35 °C for 24. The colonies formed on the TSA agar plate were counted. The experiment was performed in triplicates.

Eradication of established mature biofilm

The dispersion of old established biofilm formed by *P. aeruginosa* was performed by inoculating the diluted (100-fold dilution) overnight grown cell culture in a 96-well microtiter plate. The experiment was performed in TSB media (pH 7.9) in three separated 96-well microtiter plates for the evaluation of dispersion at different time periods. The inoculated cells were allowed to establish biofilm without streptomycin during incubation at 35 °C up to 24 h under static condition (Li et al. 2017). After 6, 12, and 24 h of incubation, the planktonic cells were removed while the adhered cells were washed twice with TSB media. Each well was loaded with fresh TSB along with various concentrations of streptomycin ranging from 0.5 to 128 μg ml^{-1}. After further incubation at 35 °C for 24 h, dispersion of the adhered cells was determined by staining with 0.1% aqueous crystal violet dye followed by OD measurement at 570 nm. The experiment was carried in triplicates.

Microscopic observation of biofilm cells

Scanning electron microscope

The biofilm inhibition property of streptomycin in *P. aeruginosa* was visualized by scanning electron microscope (SEM) following the previous protocol (Lee et al. 2011). Briefly, the overnight grown culture of *P. aeruginosa* cells at 100-fold dilution was allowed to grow over the nylon membrane surface (0.5 × 0.5 cm) which were positioned in a 24-well plate containing sub-MIC (2 μg ml^{-1}) streptomycin. After incubation at 35 °C for 24 h, the cells were subjected to fixation with formaldehyde and glutaraldehyde for overnight at 4 °C. The planktonic cells were gently discarded and the attached cells were washed with phosphate buffer saline (PBS) (pH 7.4) for three times. These cells were further dehydrated with different concentrations of ethanol (50, 70, 80, 90, 95, and 100%), each for 20 min. The membranes with adhered cells were freeze-dried using a freeze dryer (model no. FD8518, ilShinBiobase Co. Ltd., Korea). The dried membranes were affixed to SEM stubs, followed by coating with

white gold for 120 s using an ion sputter (E-1010, Hitachi, Tokyo, Japan). The biofilms formed on the nylon membrane surface were examined using the JSM-6490LV microscope (JEOL, Tokyo, Japan) at magnification of × 5000 and a voltage of 15 kV.

Fluorescence microscope

Similarly, the biofilm inhibition effect of streptomycin was also examined using fluorescence microscope. Visualization of cells was performed by using the acridine orange dye (Sigma-Aldrich St. Louis, MO, USA). The cells (1:100 dilution) were allowed to form biofilm over the surface of glass pieces (1 × 1 cm) kept in a 12-well plate along with sub-MIC (2 μg ml^{-1}) streptomycin at 35 °C. The cells attached on the glass pieces were washed thrice with PBS and stained using acridine orange (10 μg ml^{-1}). The residual dye was discarded by washing three times with PBS. The cells adhered to the glass pieces were examined by the Leica DMI300B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with × 40 magnification.

Assays of *P. aeruginosa* virulence factors

Hemolysis assay

The effect of streptomycin on *P. aeruginosa* hemolytic property was determined by following the methodology discussed previously (Lee et al. 2012). Firstly, the *P. aeruginosa* cell culture (1:100 dilutions with TSB) grown overnight was added in the 96-well microtiter plate along with sub-MICs of streptomycin. The microtiter plate was incubated overnight at 35 °C under shaking at 567 cpm. To perform the hemolysis assay, the grown cell culture (50 μl) was added into 950 μl of diluted sheep red blood cells (RBCs) (obtained from MBcell Ltd., Seoul, Korea) in centrifuge tubes (1.5 ml). The tubes were incubated at 35 °C with shaking (250 rpm). After incubation for 1 h, the hemolysis of the RBCs was quantitatively determined by measuring the OD of supernatant at 543 nm.

Assays for the virulence factor productions

The impacts of streptomycin on the production of numerous virulence factors such as rhamnolipid, pyocyanin, and siderophore, e.g., pyoverdine and LasA protease, synthesized by *P. aeruginosa* were also studied. To determine the production of rhamnolipid and pyocyanin, the overnight grown cell culture at the dilution of 1:100 in TSB was treated with sub-MIC streptomycin and incubated for 12 h at 35 °C under shaking (250 rpm). The orcinol colorimetric assay was performed for the quantification of rhamnolipid production at 421-nm wavelength following the previous protocol (Wilhelm et al. 2007). Determination of pyocyanin was carried out using the supernatant after extraction with chloroform according to the previous methodology (Essar et al. 1990). The extracted green-blue color turned into pink color as a result of acidification with 0.2 N HCl and the OD of the pink color was measured at 520 nm. For the quantification of pyoverdine production, iron-limited minimal salt media and 2% sodium succinate were used for the cultivation of *P. aeruginosa* in the presence and absence of streptomycin at sub-MIC. The OD of the culture supernatant was then measured at 405 nm (Stintzi et al. 1998). Production of each virulence factor from *P. aeruginosa* was estimated in triplicates.

Protease activity assay

The secretion and activity of *P. aeruginosa* protease were performed spectrophotometrically as well as on the casein agar plates following the previous protocol (Lee et al. 2012; Luo et al. 2017). The content of LasA protease released from supernatant of the samples treated with sub-MICs of streptomycin was measured by the azocasein assay according to previous studies (Luo et al. 2017). Briefly, the streptomycin-treated samples after 12 h of incubation at 35 °C were centrifuged and filtered. The filtered supernatant (150 μl) was added to the tube containing 250 μl azocasein (2%) (prepared in 50 mM Tris-HCl, pH 7.8) and the mixture was incubated at 4 °C for 4 h. After incubation, the reaction was terminated with 10% trichloroacetic acid, followed by incubating at 4 °C for 15 min. The reaction mixture was centrifuged (10,000 rpm for 10 min) and the obtained supernatant was neutralized with NaOH (1 M). The activity of LasA protease was determined by OD measurement at 440-nm wavelength using microtiter plate reader and the relative value was compared to control. For testing the protease activity on the agar plate, the agar plate was prepared from 2% Bacto agar and 10 g casein powder. The overnight grown *P. aeruginosa* cell culture was diluted at 1:100 dilutions in TSB media (pH 7.9) and grown along with sub-MIC of streptomycin. After 12 h of incubation at 35 °C under shaking condition, the cell culture was centrifuged and filtered using sterile 0.2-μm filter. The cell-free filtered culture supernatant was added in the holes which were made in casein-agar plate and incubated at 35 °C overnight. After incubation, the zone of clearance (which indicated the digestive activity of casein protein) around the hole was assumed as the positive protease activity.

Motility assays

The motility properties such as swimming, swarming, and twitching of *P. aeruginosa* were evaluated in the presence of streptomycin at sub-MIC levels. The methods used for the study of motility were adopted from the previous methods (Lee et al. 2012; Luo et al. 2017). The swarming assay was performed on agar plates prepared from LB broth containing

0.5% casamino acids, 0.5% glucose, and 0.4% Bacto agar according to the procedure developed by Luo et al. (2017). The swimming motility was also performed on agar plate which are prepared from 1% tryptone, 0.25% NaCl, and 0.3% Bacto agar according to the protocol described previously (Lee et al. 2012). To perform the motility assays, the *P. aeruginosa* cell culture grown overnight (5 μl) was positioned in the center of each agar plate. The twitching experiment was performed by placing the overnight grown cell culture in the center of the Petri plate using toothpick following the previous protocol by Luo et al. (2017). The placed cell culture was embedded by pouring LB medium containing 0.2% casamino acids, 30 mM glucose, and 1.5% Bacto agar. The agar plates used for swimming, swarming, and twitching assays were supplemented with sub-MICs (from 0.25 to 2.0 μg ml^{-1}) of streptomycin. After incubation at 35 °C for 24 h, the diameters (cm) of cells traveling on the swarming, swimming, and twitching agar plates were measured.

Real-time polymerase chain reaction

The expression of genes associated with biofilm formation, QS signaling, virulence factor production, and motilities was accessed using real-time polymerase chain reaction (qRT-PCR). The primers used in this study were taken from the earlier studies reported elsewhere such as QS signaling genes (*rhlR-rhlI* and *lasR-lasI* systems) (Furiga et al. 2015), biofilm matrix-forming exopolysaccharide genes (*algA*, *algU*, and *pslM*) (Fu et al. 2017), virulence genes (*phzC*, *phzE*, *pvdA*, *pvcC*, and *lasB*) (Furiga et al. 2015; Lee et al. 2011, 2014), and motility gene (*flgG*) (Lee et al. 2011; Oura et al. 2015). In this study, the pyrroline-5-carboxylate reductase (*proC*) housekeeping gene (Lee et al. 2014) was selected as the control for normalization of expression data of all the genes mentioned above. RNA extraction was carried out by growing the *P. aeruginosa* cell culture overnight (1:100 dilution) in 25 ml TSB (pH 7.9) in the 250-ml Erlenmeyer flask in the presence and absence of sub-MIC (2 μg ml^{-1}) streptomycin under shaking (250 rpm) at 35 °C for 12 h following the previous protocol with slight modifications (Lee et al. 2011). The grown cell culture was firstly chilled in dry ice for 30 s, followed by centrifugation (13,000 rpm; 5 min). From the cell pellets, total RNA was extracted using AccuZol™ reagent kit (Bioneer, South Korea). The concentration at 260 nm and purity of RNA at 260/280 nm were determined by NanoDrop spectrophotometer. From the extracted RNA, the first complementary DNA strand (cDNA) was synthesized by using AccuPower® GreenStar™ qRT-PCR premix kit (Bioneer, South Korea). The cDNA acted as a template and the primers mentioned above were used for the qRT-PCR by using DNA engine Chromo4 real-time Detector (Bio-Rad, Hercules, CA, USA). The PCR program and procedure were adopted from the method described earlier (Luo et al. 2017). After normalizing the gene expression level with the *proC* housekeeping gene, the relative expression level of each gene was measured following the methodology described earlier (Schmittgen and Livak 2008).

Statistical analysis

All the presented graphs were plotted by using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA) and the results were shown as means ± SD. Similarly, analysis of the data in this study was also carried out by performing one-way ANOVA.

Results

Determination of minimum and sub-minimum inhibitory concentration of streptomycin

Prior to the study of biofilm inhibition and suppression of virulence factors by streptomycin, firstly we studied the growth of *P. aeruginosa* for establishing the MIC values of streptomycin. The MIC of streptomycin against *P. aeruginosa* was found as 8 μg ml^{-1} (Fig. 1a). Furthermore, to determine the sub-MIC of streptomycin, *P. aeruginosa* was incubated with streptomycin at different concentrations below the MIC. The bacterial growth at 4 μg ml^{-1} after 28 h of incubation was found to be slightly reduced as compared to the control or below 4 μg ml^{-1} (Fig. 1b). However, the lag phase at 4 μg ml^{-1} concentration was found to prolong up to 16 h. Although at 2 μg ml^{-1} concentration there was also 6 h of lag phase, after 28 h of incubation, the growth was found to be almost equal to 4 μg ml^{-1} of concentration. Above 4 μg ml^{-1} concentration of streptomycin, which is 8 μg ml^{-1}, there was a complete growth inhibition. Based on the above results, the sub-MIC ranging from 0.0625 to 4 μg ml^{-1} was selected for the subsequent studies of biofilm inhibition and virulence factor production of *P. aeruginosa*.

Streptomycin inhibited biofilm formation and eradicated the mature biofilm of *P. aeruginosa*

With the attempts of investigating the *P. aeruginosa* biofilm formation in response to streptomycin activity, three sub-MIC levels of streptomycin (0.5, 1, and 2 μg ml^{-1}) were selected to culture with the bacterial cells along with several different culture conditions, using three different culture media (TSB, MHB, and LB), three pH levels (5.9, 7.2, and 7.9), and three temperature levels (25, 30, and 35 °C). The biofilm inhibition results obtained from the aforementioned conditions are summarized in the Supplementary Fig. S1. In terms of media types, the culture conditions of MHB media along with all tested temperature and pH levels were unable to suppress the

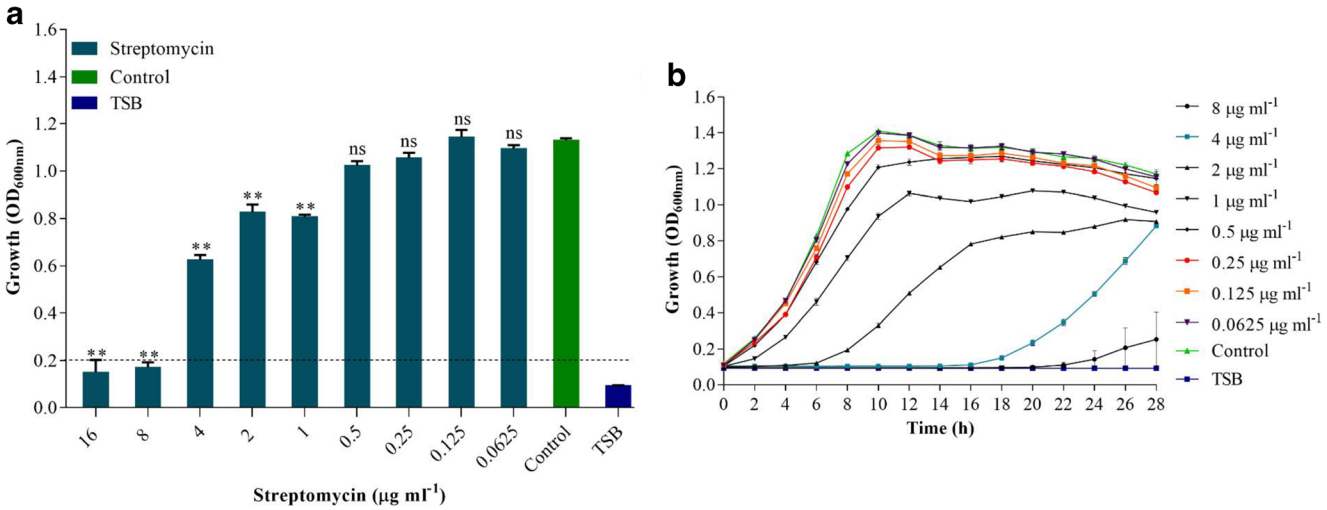


Fig. 1 MIC determination of streptomycin against *P. aeruginosa* PAO1. **a** Bactericidal effect of streptomycin against *P. aeruginosa*. The absorbance of negative control (only TSB) was found to 0.093 ± 0.003; hence, based on this value, the positive growth of the culture was considered when absorbance was found to > 0.2. **b** Growth curve of *P. aeruginosa* in the presence of sub-MIC of streptomycin. $^{**}P < 0.01$ was accepted as statistically significant and ns indicated non-significance

bacterial biofilm formation. Furthermore, it was found that the acidic pH (pH 5.9) at all temperatures from 25 to 35 °C has promoted biofilm formation. In contrast, when environmental pH became alkaline (pH 7.9) and at 35 °C of temperature, a reduction in bacterial biofilm formation was observed in both LB and TSB media. As compared to LB media, the anti-biofilm effect was slightly improved in TSB culture media. Particularly, in TSB media with pH 7.9 at 35 °C, treatment of streptomycin at sub-MIC (2 μg ml^{-1}) and sub-MIC (4 μg ml^{-1}) inhibited 78% and 76% of bacterial biofilm formation, respectively (Fig. 2). Considering all results from pH, temperature, and media types, TSB culture media, pH 7.9, and 35 °C of temperature were selected for further detailed study of biofilm inhibition and several other phenotypic properties.

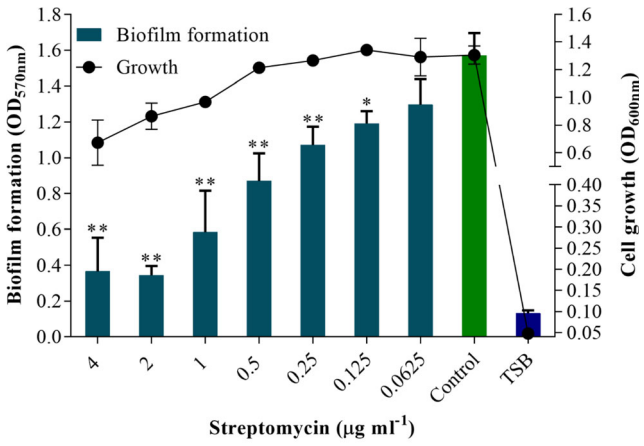


Fig. 2 Effects of sub-MIC of streptomycin on *P. aeruginosa* PAO1 biofilm formation and cell growth of *P. aeruginosa* in the presence of sub-MICs of streptomycin. $^{*}P < 0.05$ and $^{**}P < 0.01$ were accepted as statistically significant

The analysis of the biofilm architecture in the presence of streptomycin at sub-MIC (2 μg ml^{-1}) was evaluated by SEM and fluorescence microscope (Fig. 3a, b). The SEM analysis was carried out to check the impact of streptomycin on the biofilm architecture. As shown in the SEM image, in the presence of sub-MIC of streptomycin, *P. aeruginosa* cells became wrinkled (Fig. 3a) as compared to the control, whereas the non-treated cells clearly showed a dense colonization and highly compact biofilm architecture. The fluorescence microscopy results also showed a reduction in fluorescence intensity, which reflected the inhibitory activity of streptomycin at the sub-MIC against the bacterial biofilm. The streptomycin-treated cells showed a scanty architecture while the non-treated control showed a dense colonization and highly compact architecture of the cells on the glass slide surface (Fig. 3b). The relative fluorescence intensity determination of the cells also showed a clear difference between the treated samples and non-treated sample (control) (Fig. 3c).

In most cases, eradication of pre-existing bacterial biofilm is considered highly challenging as the structure has (1) attached to a surface, (2) produced a wide array of virulence factors and adhesins, and (3) formed a more complicated morphological structure that enhances the level of resistance (Das et al. 2014; Jamal et al. 2018; Sheraton et al. 2018). Previous studies have found several anti-biofilm agents which were highly active in inhibiting biofilm yet were unable to disperse the pre-existing mature one (Otto 2013; Zhao et al. 2017). However, results from the present study showed a significant disruption of 24-h mature biofilm by all tested concentrations of streptomycin (Fig. 4). The effectiveness of streptomycin application reached the highest level during the first 6-h period and gradually decreased at the end of 24 h of treatment.

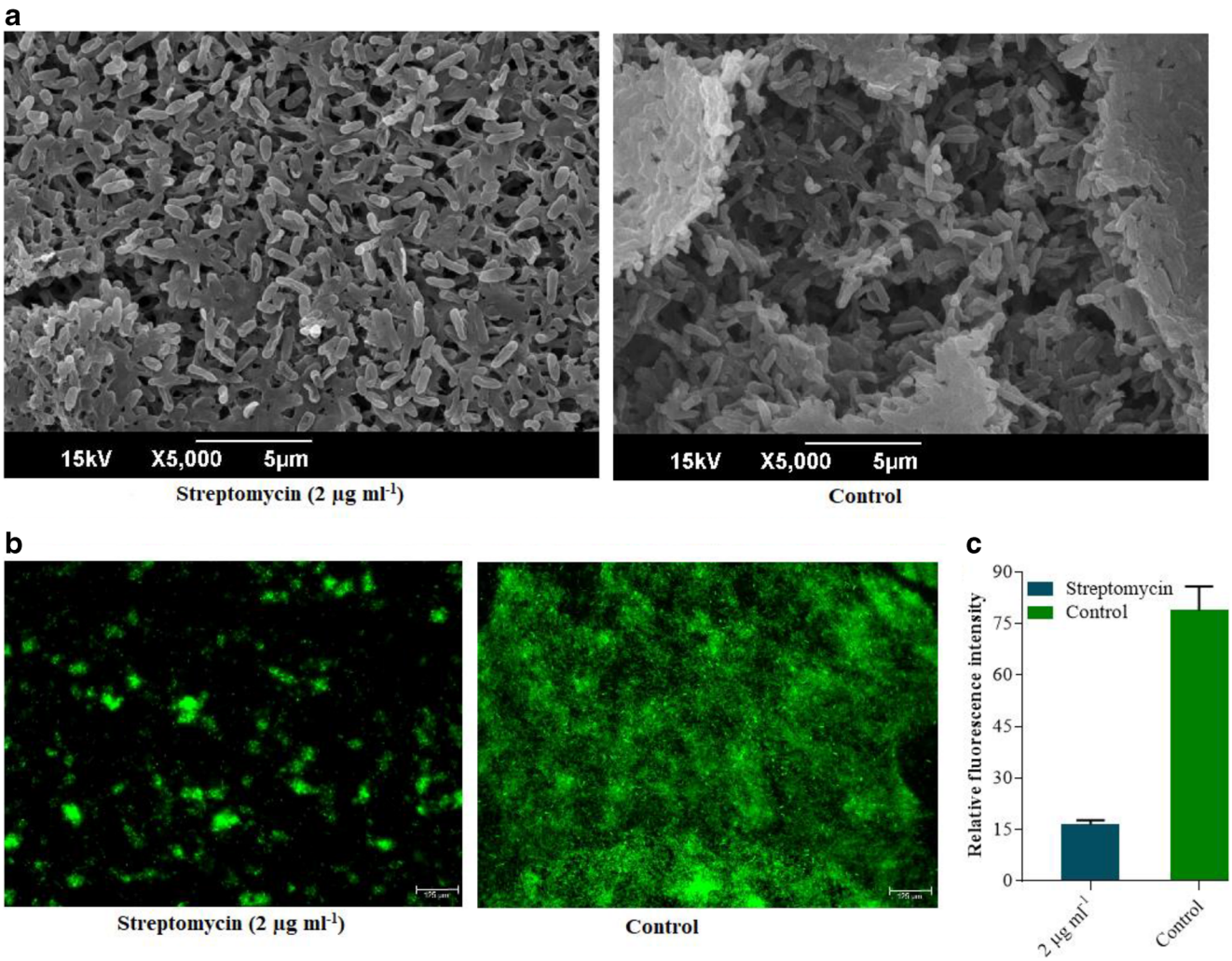


Fig. 3 Visualization of biofilm structure by SEM and fluorescence microscopes in the presence of sub-MIC (2 μg ml^{-1}) of streptomycin. **a** SEM image of the biofilm cells cultured on the nylon membrane. **b** Fluorescence image of the biofilm cells cultured on the glass pieces. **c** Relative fluorescence intensity of biofilm cells

For further confirmation the anti-biofilm activity of streptomycin, we have determined the MIC levels, followed by biofilm inhibition and mature biofilm eradication activities of tetracycline and tobramycin, using the same protocols as streptomycin. The culture conditions were also maintained similar to streptomycin, which were proposed as pH 7.9, 35 °C of temperature, and TSB media. As shown in Fig. 5a, the MIC levels of tetracycline and tobramycin were determined as 8 μg ml^{-1} and 0.5 μg ml^{-1}, respectively. Although at sub-MIC levels tetracycline and tobramycin did not show the concentration-depended biofilm inhibition, both exhibited statistically significant inhibition of biofilm under the similar conditions as specified for the streptomycin (Fig. 5b, c). However, both of them showed a comparative significant concentration-dependent dispersal of preformed mature biofilm of *P. aeruginosa* (Fig. 5d).

Due to the variations of *P. aeruginosa* biofilm and virulence phenotypes, in addition to the standard laboratory strain PAO1, a clinical strain isolated from pleural fluid of a patient in hospital (i.e., GNUH-NCCP 6039) was also employed to evaluate the anti-biofilm effects of streptomycin, tobramycin, and tetracycline. A similar sequence of experiments was performed under the proposed culture conditions (pH 7.9, 35 °C, and TSB) for all three antibiotics, including MIC determination, biofilm inhibition assay, and eradication of preformed mature biofilm. The results are shown in Fig. S2 and S3. Firstly, the MIC values of streptomycin, tetracycline, and tobramycin were found as 16 μg ml^{-1}, 32 μg ml^{-1}, and 0.5 μg ml^{-1}, respectively. Secondly, the anti-biofilm and eradication activities of the three antibiotics were evaluated using crystal violet staining. The biofilm inhibition by streptomycin, tetracycline, and tobramycin of the clinical strain GNUH-NCCP 6039 was significant but not in a concentration-dependence manner as compared to PAO1 (Fig. S2b, c, d). However, streptomycin, tetracycline, and tobramycin

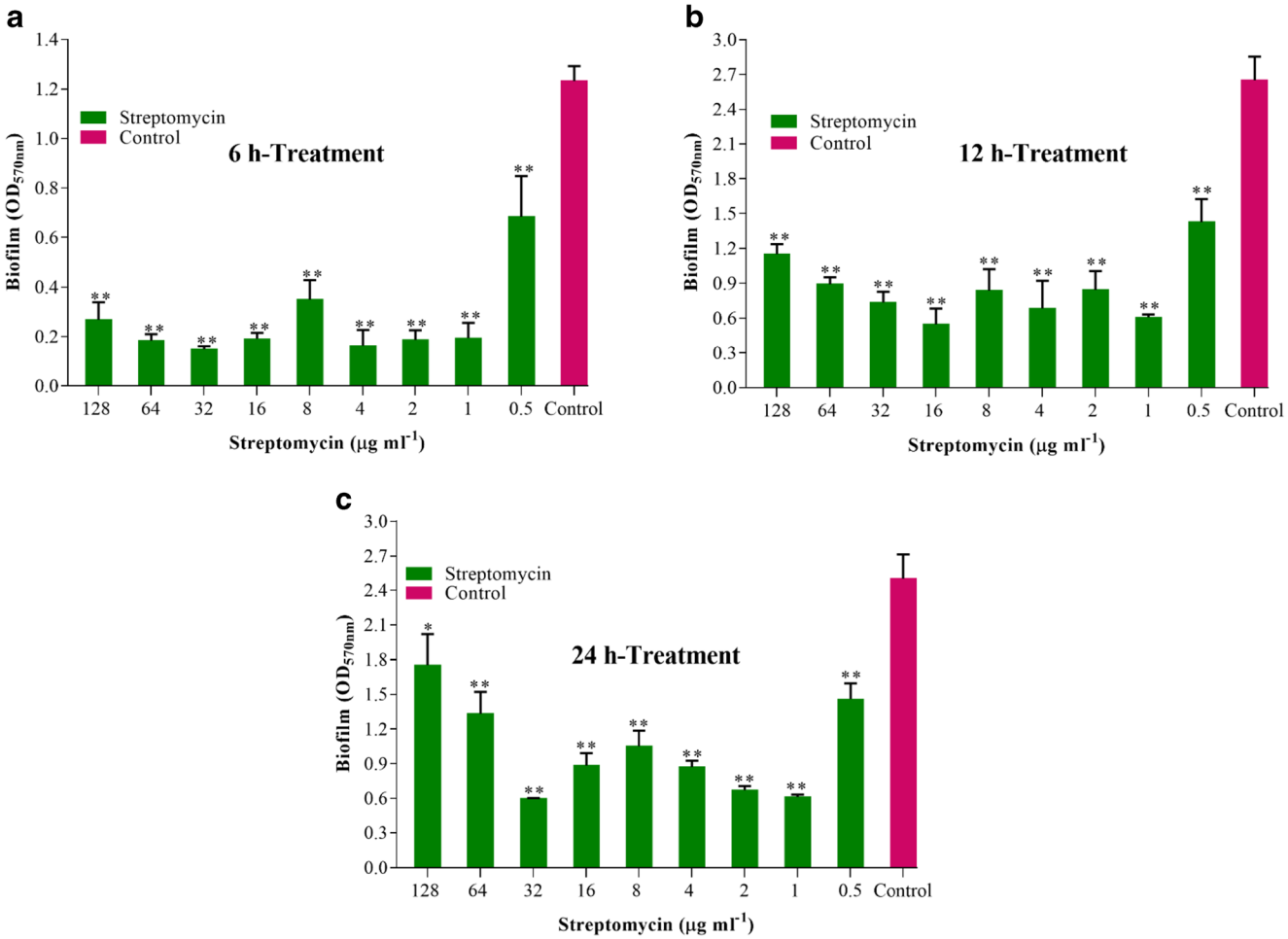


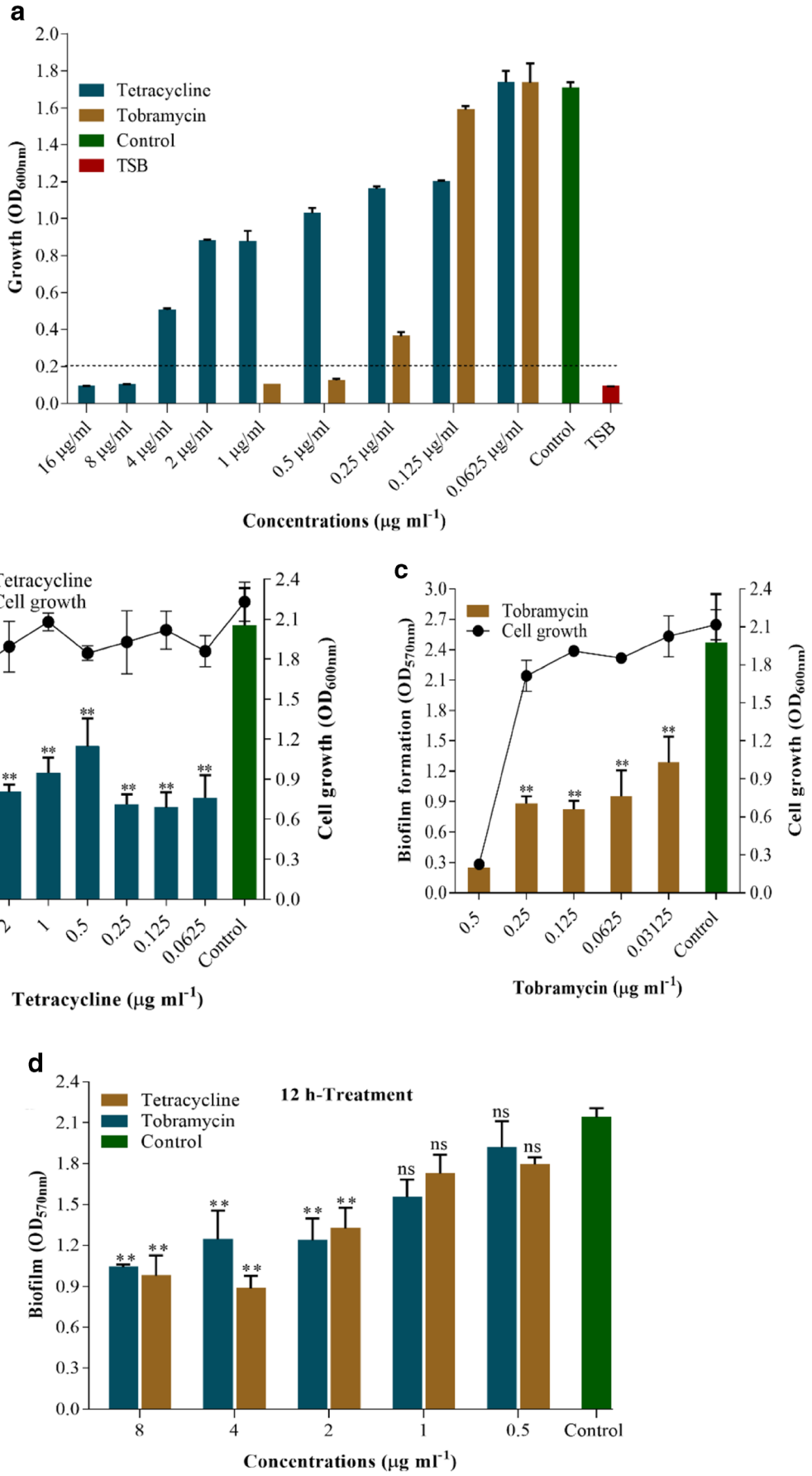
Fig. 4 Dispersion of established 24-h mature biofilm of *P. aeruginosa* PAO1 by various concentrations of streptomycin. **a** 24-h mature biofilm treated by streptomycin for 6 h. **b** 12-h treatment. **c** 24-h treatment. *$P < 0.05$ and **$P < 0.01$ were accepted as statistically significant

exhibited a concentration-dependent eradication towards 24-h mature biofilm of GNUH-NCCP 6039 when treated for 12 h (Fig. S3).

Streptomycin inhibits biofilm formation on the surfaces of urinary catheter

Urinary catheter is one of the commonly used medical devices, which may readily acquire the biofilm formation by pathogenic bacteria on either outer or inner surfaces when it is inserted into the patient (Al-Mathkhury et al. 2011). Reports have showed that *P. aeruginosa* commonly colonizes and establishes biofilms on the surface of urinary catheter, which have become one of its resistance properties against the anti-biofilm agents (Shigemura et al. 2006; Stickler 1996). For this reason, we have also studied the impact of sub-MICs of streptomycin on the biofilm of *P. aeruginosa* formed on the urinary catheter. The impact of streptomycin on biofilm was determined by viable cell counting and crystal violet staining of bacterial cells attached to the surface of urinary catheter. The results from crystal violet staining showed that the sub-MICs of streptomycin inhibited *P. aeruginosa* biofilm formation in a dose-dependent manner as shown in Fig. 6. Approximately 66% of biofilm biomass was inhibited at the sub-MIC concentration (2 μg ml^{-1}) of streptomycin (Fig. 6a). This result has also been confirmed by determining the bacterial cell count of attached biofilm cells (Fig. 6b). The result of the bacterial cell count was also relatively concentration-dependent in response to streptomycin.

Fig. 5 **a** MIC determination of tobramycin and tetracycline against *P. aeruginosa* PAO1 in TSB and pH 7.9. **b** Inhibitory effects of sub-MICs of tetracycline on *P. aeruginosa* PAO1 biofilm formation and cell growth without shaking condition. **c** Inhibitory effects of tobramycin at sub-MICs on *P. aeruginosa* PAO1 biofilm formation and cell growth without shaking condition. **d** Eradication of 24-h mature *P. aeruginosa* PAO1 biofilm by 6-h treatments of tobramycin and tetracycline using crystal violet staining assay **$P < 0.01$ was accepted as statistically significant and ns indicated non-significance


a
Tetracycline
Tobramycin
Control
TSB
Growth (OD600nm)
16 μg/ml
8 μg/ml
4 μg/ml
2 μg/ml
1 μg/ml
0.5 μg/ml
0.25 μg/ml
0.125 μg/ml
0.0625 μg/ml
Control
TSB
Concentrations (μg ml-1)
b
Tetracycline
Cell growth
Biofilm formation (OD570nm)
Cell growth (OD600nm)
4
2
1
0.5
0.25
0.125
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Control
Tetracycline (μg ml-1)
c
Tobramycin
Cell growth
Biofilm formation (OD570nm)
Cell growth (OD600nm)
0.5
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Control
Tobramycin (μg ml-1)
d
12 h-Treatment
Tetracycline
Tobramycin
Control
Biofilm (OD570nm)
ns
8
4
2
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Control
Concentrations (μg ml-1)

Streptomycin attenuates the virulence properties of *P. aeruginosa*

P. aeruginosa virulence properties which contribute to the bacterial pathogenicity were found by the hemolysis of RBCs and also production of several virulence factors (Allen et al. 2014). The impact of streptomycin on the hemolytic properties was studied at sub-MIC levels. Streptomycin at sub-MIC level (2 μg ml^{-1}) has reduced the *P. aeruginosa* hemolytic properties by about 54%, as compared to the control (Fig. 7a).

The biosynthesis of virulence factors such as pyocyanin, rhamnolipid, and siderophores such as pyoverdine was evaluated in the presence of streptomycin at sub-MICs. Under sub-MICs of streptomycin, approximately 77.8% of pyocyanin production was reduced in comparison with the control (Fig. 7b). Similarly, the production of rhamnolipid and pyoverdine was also reduced by about 65% and 88%, respectively, by streptomycin at sub-MIC (2 μg ml^{-1}) (Fig. 7c, d).

Synthesis of hydrolytic protease enzyme such as LasA protease is also crucial for *P. aeruginosa* pathogenicity towards the host cells (Andrejko et al. 2013; Chanda et al. 2017). The present study examined the effect of sub-MICs streptomycin (0.125–4 μg ml^{-1}) in attenuating the protease activity of the bacteria. Results from proteolytic assays using both azocasein protein digestion and casein agar plates showed that in comparison to the control, streptomycin treatment was able to reduce the protease activity (Fig. 8a, b). Thus, similar to the hemolytic and several virulence factors, *P. aeruginosa* protease can also be suppressed by applying treatment of sub-MICs streptomycin.

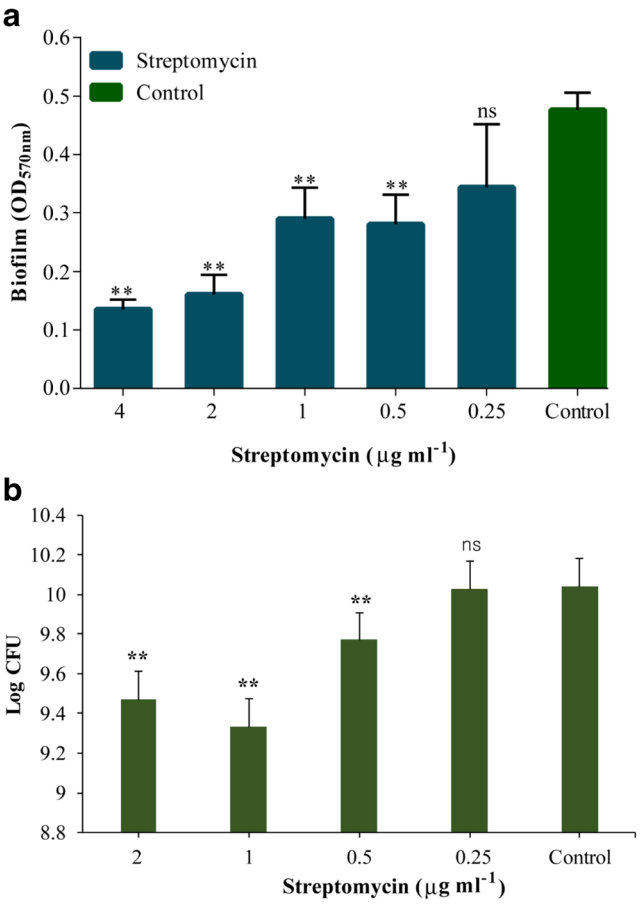

Fig. 6 Biofilm inhibition assays on urinary catheter strips in the presence of sub-MICs of streptomycin. **a** Crystal violet staining assays. **b** Viable cell count of the biofilm cells. $^{**}P < 0.01$ was considered statistically significant and ns indicated non-significance

It is well studied that the motility towards a biotic or abiotic surface is linked to the bacterial virulence and is governed by different mechanisms (Harshey 2003; Josenhans and Suerbaum 2002; Shi and Sun 2002). In the present study, we have checked *P. aeruginosa* swarming, swimming, and twitching movements in the presence of the sub-MIC of streptomycin. The results indicated that streptomycin at sub-MIC (2 μg ml^{-1}) inhibited these motions significantly, with 99% and 67% of bacterial swimming and swarming motions was reduced as compared to the control (Fig. 9a, b, c, d). Likewise, the bacterial twitching motility was also significantly inhibited as shown in Fig. 9e, f. Combining with the growth studies of *P. aeruginosa* at different concentrations (Fig. 1a, b), inhibition of motility at sub-MICs of streptomycin was not due to the defect in the bacterial cell growth.

Effect of sub-MIC of streptomycin on biofilm-associated and QS-related virulence genes expression

In *P. aeruginosa*, the establishment of biofilm and production of other virulence factors which contribute to the bacterial pathogenesis are majorly regulated by LasI/LasR and RhlI/RhlR QS systems associated with PQS (*Pseudomonas* quinolone sensing signal) and IQS (integrating quorum sensing signal) (Martinez et al. 2019). The LasI/LasR system which uses 3-oxo-C12-hemoserine lactone (3O-C12-HSL) autoinducer and the RhlI/RhlR system which uses butanoyl homoserine lactone (C4-HSL) autoinducer determine the production and expression of pyocyanin, siderophores, rhamnolipid, and protease enzymes as well as swarming motility (Holm and Vikstrom 2014; Rutherford and Bassler 2012). With promising final effects from virulence phenotypic assays performed earlier, the expression of each specific virulence gene of *P. aeruginosa* under sub-MIC of streptomycin (2 μg ml^{-1}) was then further studied using qRT-PCR. Results from qRT-PCR revealed that streptomycin at sub-MIC significantly suppressed the expression of genes encoding for virulence factors (pyocyanin—*phzE* and *phzC*; pyoverdine—*pvdA* and *pvcC*; elastase—*lasB*), exopolysaccharide (*algA*) and flagella (*flgG*), in comparison with the non-treated control (Fig. 10). A similar effect was observed in the group of QS-regulated genes (*lasI*, *lasR*, *rhlI*, and *rhlR*) in the presence of sub-MIC of

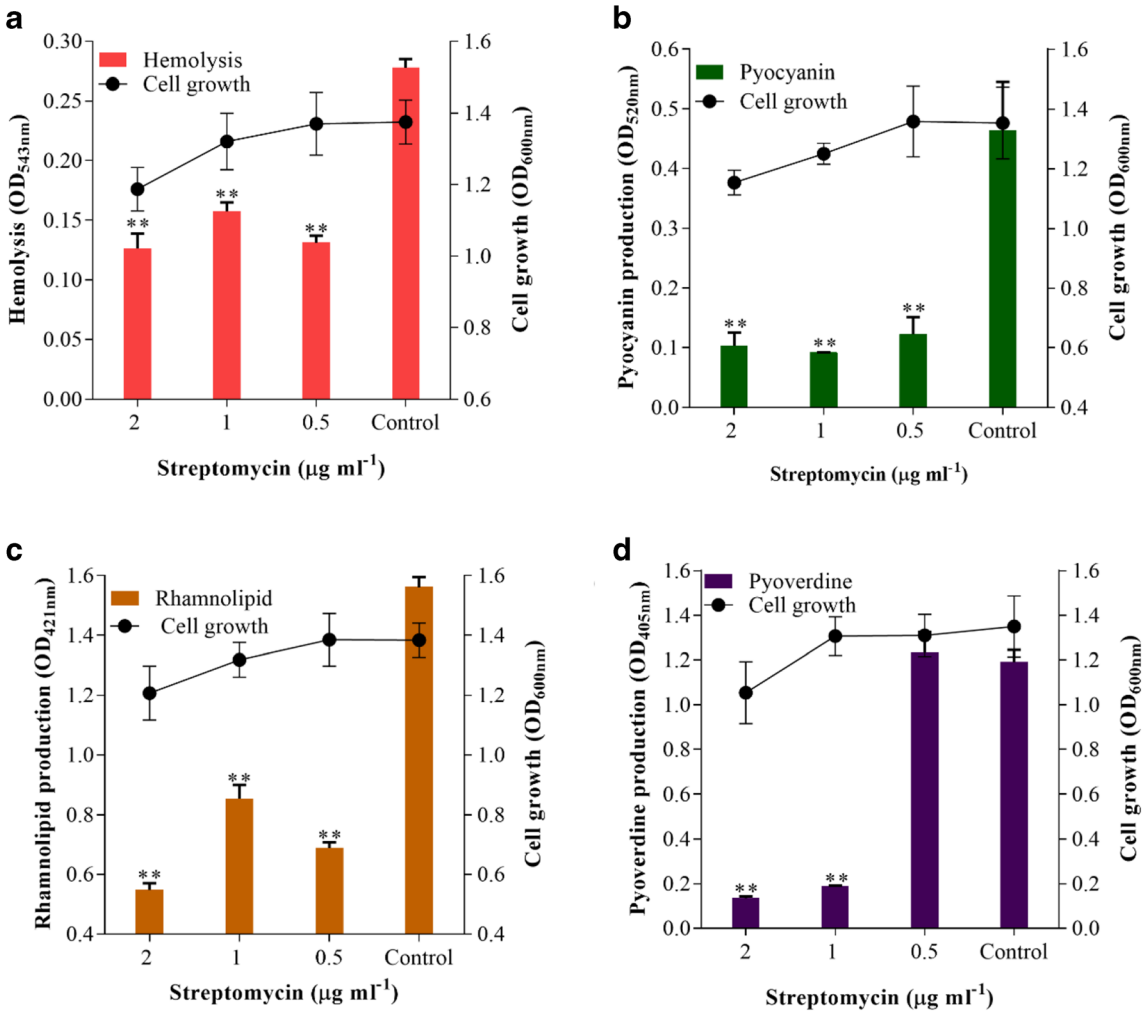

Fig. 7 Inhibitory effects of sub-MICs of streptomycin on hemolytic activity and production of virulence factors of *P. aeruginosa* PAO1. **a** Hemolysis assay, **b** pyocyanin production, **c** rhamnolipid production, and **d** pyoverdine production. The hemolysis and production of each virulence factor from streptomycin-treated sample are represented a relative value with respect to the control. All the experiments were performed in triplicates. $^{**}P < 0.01$ was considered statistically significant

streptomycin. In contrast, streptomycin at sub-MIC enhanced the expression of the remaining exopolysaccharide genes, which are *algU* and *pslM*. Combining the results from phenotypic and gene expression analysis of virulence properties, streptomycin at sub-MIC can be regarded as a promising anti-virulence agent against *P. aeruginosa*.

Discussion

Aminoglycoside is a common antibiotic family owing to the polycationic nature, which was previously hypothesized to limit them from penetrating through the bacterial cells without concentration loss (Li et al. 2015). In fact, these antibiotics have been reported to be unable to control the growth of several bacteria as their efficacy is encountered by multiple barriers, including bacterial cell wall permeability (Bansal-Mutalik and Nikaido 2014; Needham and Trent 2013; Sarathy et al. 2012), degrading enzymes (Ramirez and Tolmasky 2010), modified-binding target (Wilson 2014), and efflux pumps (Fernandez and Hancock 2012). However, for the complications of a bacterial community formed within a biofilm structure, along with varied results obtained in different bacteria, a general understanding about the resistant responses of *P. aeruginosa* towards aminoglycosides currently remained lacking (Garneau-Tsodikova and Labby 2016; Poole 2012). Although at present, fluoroquinolone is known as the most frequently used antibiotic against *P. aeruginosa*, the prevalence of resistance emergence had also been recorded in several studies (Guss et al. 2009; Wu et al. 2018; Yang et al. 2015), urging further exploitation for alternative approaches such as combination strategy and diversifying the available antibiotic options (Khan et al. 2019b, c, d; Kotra et al. 2000). For these reasons, streptomycin, which is a common

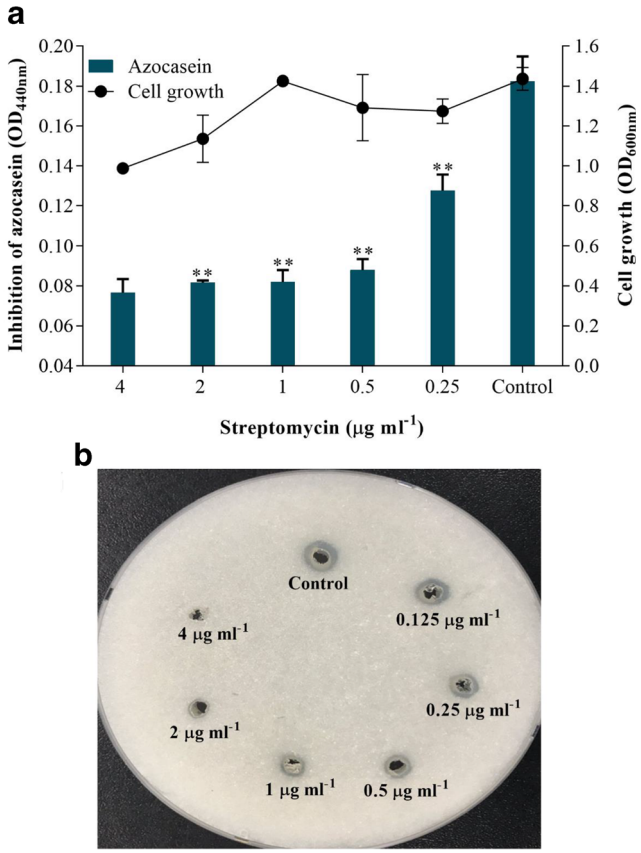

Fig. 8 Inhibition of protease activity in *P. aeruginosa* PAO1 by sub-MICs of streptomycin. **a** Effect of sub-MICs of streptomycin on azocasein degrading protease enzymes secreted by *P. aeruginosa*. **b** Effect of sub-MICs of streptomycin on protease activity on the casein agar plate. $^{**}P < 0.01$ was considered statistically significant

member of the aminoglycoside antibiotic family, has been selected in the current study to evaluate the effects on *P. aeruginosa* growth, biofilm formation, and virulence properties. In addition, owing to the unique polycationic nature of streptomycin, the pH value, the culture media, and temperature were also taken into consideration.

The sub-MIC values were selected as the working concentrations for subsequent tests, as no inhibition in *P. aeruginosa* growth was recorded at these concentrations. Previous studies have reported that the antimicrobial activity of some antibiotics such as aminoglycosides was hindered by their electrostatic interaction with the negatively charged components present in the biofilm extracellular matrix (Rabin et al. 2015, Stewart 2002). Therefore, the present study proposed one possible approach to improve aminoglycoside activity is by changing the bacterial culture environment, which included temperature, pH, and media type. Three levels of pH, temperature, and three culture media types were used to generate various culture conditions for *P. aeruginosa* biofilm formation under streptomycin treatment at sub-MICs. Results have shown that the biofilm inhibition was correlated to the environmental pH, temperature, and media type. Overall, streptomycin anti-biofilm activity against *P. aeruginosa* was maximized under the condition of 35 °C of temperature, alkaline pH, and TSB media. One of the purposes of forming biofilm in bacteria, particularly *P. aeruginosa*, is to resist and survive through extreme conditions both inside the host system and outside the environment system. Firstly, pH was known to affect biofilm formation and EPS synthesis (Henry-Stanley et al. 2014). Furthermore, previous studies have shown that the acidic pH even promoted the biofilm establishment of *P. aeruginosa* and reduced the efficacy of anti-biofilm agents (Schlessinger 1988; Wilton et al. 2016). A similar observation was also obtained from the present study, in which acidic pH when combined with all tested temperature and media types did not support streptomycin anti-biofilm activity. In contrast, under alkaline pH, gentamycin and its combination with L-arginine have reduced *P. aeruginosa* and *S. aureus* biofilm formation (Lebeuax et al. 2014). Secondly, temperature was known to affect biofilm structure, thickness, virulence, and genetic transfer in biofilm (LaBauve and Wargo 2012; MacFadden et al. 2018). In fact, high temperature (37 °C) could accelerate the maturation of *P. aeruginosa* biofilm (Kannan and Gautam 2015). Finally, media type could act as a nutrient source as well as the environment for movements and biofilm thickness as well as multiple physiochemical properties (Vrany et al. 1997). For instance, the biofilm inhibition activity of several aminoglycoside, including streptomycin against *Staphylococcus aureus*, was dependent on the culture media (Henry-Stanley et al. 2014). Of all three media tested, which are LB, MHB, and TSB, streptomycin performed the highest inhibitory activity against *P. aeruginosa* biofilm in TSB media. Along with alkaline pH and temperature of 35 °C, this culture condition can be used to improve the aminoglycoside penetration into *P. aeruginosa* biofilm. Interestingly, the anti-biofilm activity of streptomycin was also comparatively noteworthy in LB media with alkaline pH and 35 °C of temperature. Results have revealed that sub-MIC levels of streptomycin exhibited inhibitory activity on these biofilms under alkaline pH and in TSB media. Eradication activity of streptomycin was also shown at sub-MICs, as well as above MIC levels. Several reports showed that *P. aeruginosa* developed different types of resistance mechanisms against the action of antibiotics such as (1) modification and mutation of the antibiotic binding site, (2) modification of antibiotic chemical structure, (3) modification of the bacterial membrane, and (4) efflux pump (Al-Wrafy et al. 2017; Garneau-Tsodikova and Labby 2016). The effectiveness of sub-MIC streptomycin on *P. aeruginosa* biofilm was also supported by SEM and fluorescence microscopic

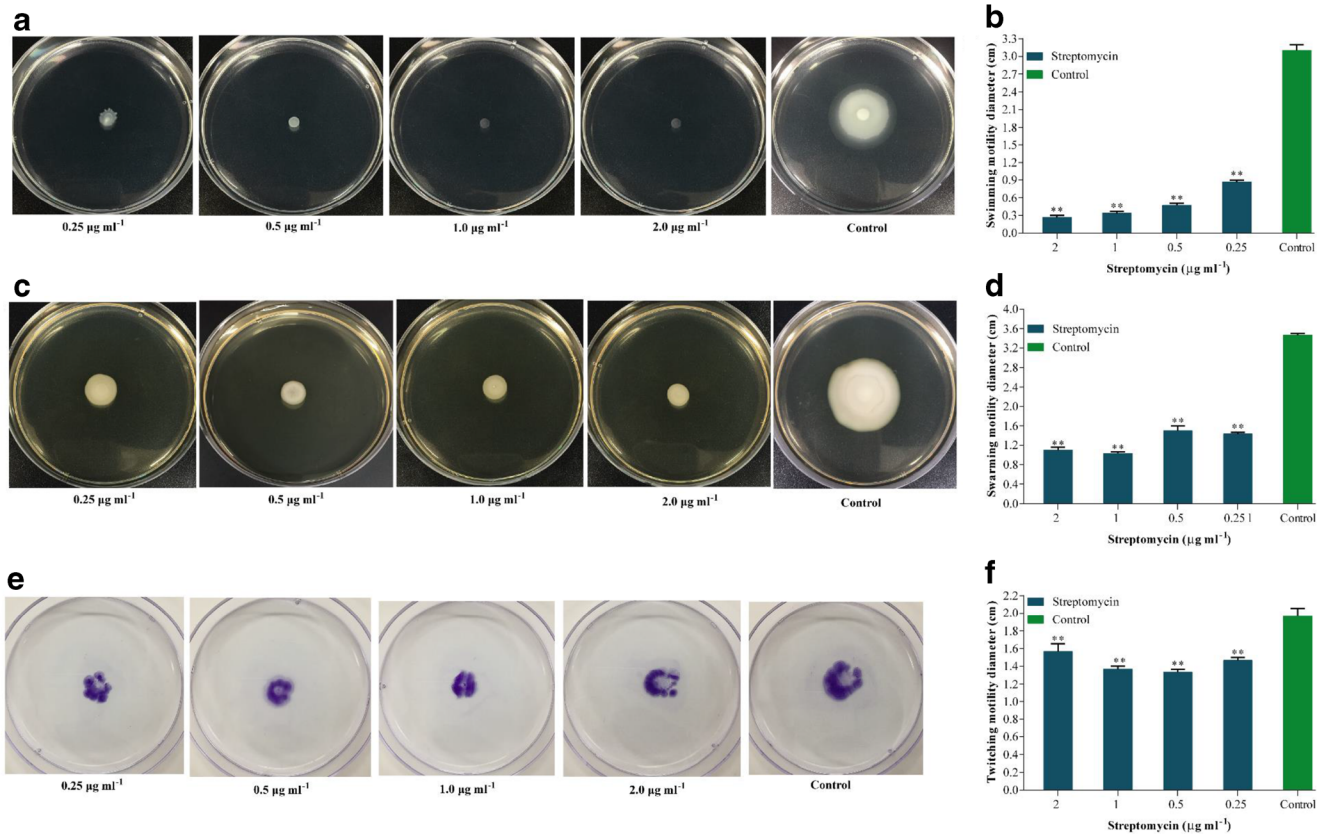

Fig. 9 Inhibition of motility properties of *P. aeruginosa* PAO1 by sub-MICs of streptomycin. **a** Swimming motility on agar plate. **b** Diameter value (cm) of swimming motility. **c** Swarming motility on agar plate. **d** Diameter value (cm) of swarming motility. **e** Twitching motility. **f** Diameter value (cm) of twitching motility. A representative image of each assay is presented and each experiment was carried out three times. $^{**}P < 0.01$ was considered statistically significant

visualization. The results from microscopic visualization showed that the biofilm structure genuinely appeared wrinkled and less dense in the presence of streptomycin at sub-MIC. Compared to the previous studies in which streptomycin was recognized as a weak biofilm inhibitor to *P. aeruginosa*, the sub-MIC levels of the antibiotic turned out to be limitedly studied (Mu et al. 2016; Zhang et al. 2013). Overall, the findings from the present

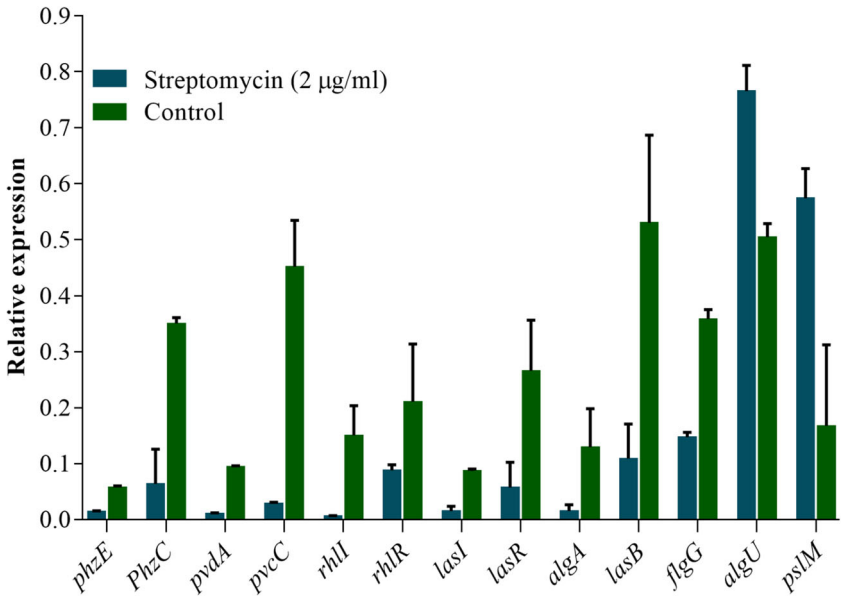

Fig. 10 qRT-PCR analysis for the determination of relative expression levels of biofilm-forming, virulence, and motility genes in the presence of sub-MIC of streptomycin. The relative expression of genes represents transcriptional levels after treatment with sub-MIC streptomycin versus non-treated control. The experiment was carried out in triplicate

study suggested a specific concentration range, environmental pH, and temperature, which is sub-MIC levels, pH 7.9, 35 °C, and TSB as bacterial culture media to improve the anti-biofilm function of streptomycin.

In order to compare the anti-biofilm effects of streptomycin with different antibiotics, tobramycin and tetracycline which also have similar inhibitory effect on protein synthesis as streptomycin were employed. Under similar culture conditions as proposed for streptomycin, both antibiotics were more active in eradicating the pre-formed mature biofilm formed by *P. aeruginosa* PAO1 strain. The modifications in terms of pH, temperature, and media type of the culture environment have possibly caused a noticeable change in the anti-biofilm activities of all tested antibiotics. According to previous reports by Kumar and Ting (2016) and Hoffman et al. (2005), the sub-MICs of streptomycin and tobramycin promoted *P. aeruginosa* biofilm formation. However, in these studies, different culture conditions were used such as [TSB, pH 7.0, and 37 °C] as for streptomycin and [MHB, 37 °C] as for tobramycin. Furthermore, the condition used for PAO1 strain was also tested on a clinical isolate (GNUH-NCCP 6039). Results indicated that streptomycin, tetracycline, and tobramycin showed a significant biofilm inhibition and a concentration-dependent dispersion towards the preformed mature biofilm.

Furthermore, the application of streptomycin at sub-MIC for the biofilm inhibition on medical devices such as urinary catheter has also been examined using similar media and conditions. An effective biofilm inhibition by streptomycin was found in a concentration-dependent manner as evaluated by crystal violet staining. The viable cell count of the cells which formed biofilm on the surface of urinary catheter also showed significant reduction in the presence of streptomycin at sub-MIC.

Along with biofilm formation, secretion of virulence factors also primarily characterizes for the pathogenicity, infection, and survival of *P. aeruginosa* in adverse environmental conditions (Luo et al. 2017; Meirelles and Newman 2018; Orgad et al. 2011; Poppe et al. 2018). Phenotypic studies revealed that as compared to the control and other sub-MIC levels, the concentration of 2 μg ml^{-1} of streptomycin has reduced the production of all QS-related virulence factors and inhibited the hemolytic activity of *P. aeruginosa*. Therefore, this sub-MIC of streptomycin was selected for genetic analysis of the virulence phenotypes, along with (1) their QS regulation genes, (2) exopolysaccharide genes, (3) protease-encoded gene, and (4) flagella-encoded gene. Except *algU* and *pslM*, the expression of all tested genes was downregulated by sub-MIC of streptomycin, which reasonably explained for the reduction in these phenotypes as observed earlier. Similarly, in previous studies, the sub-MICs of ciprofloxacin and azithromycin had also reduced biofilm formation by attenuating these factors (Bala et al. 2011; Gupta et al. 2016; Imperi et al. 2014). In fact, recently, the sub-MIC level of common antibiotics such as quinolone and beta-lactam has gained more attention in the modern anti-biofilm therapies against a variety of biofilm-forming bacteria (El-Mowafy et al. 2017; Haddadin et al. 2010; Otto et al. 2013; Vidya et al. 2005; Viedma et al. 2018). At sub-MIC levels, these antibiotics most likely targeted the virulence factor production as well as the virulence-regulated system, which is termed "quorum sensing." As aforementioned, the virulence factors play an equally important role as biofilm formation in characterizing the *P. aeruginosa* pathogenesis; thus, using antibiotics as anti-virulence agents can be considered a promising alternative strategy for combating the bacterial biofilm formation (Gupta et al. 2016).

The present study also investigated the effects of sub-MIC of streptomycin on inhibiting *P. aeruginosa* motility, including swimming, swarming, and twitching (Davies et al. 1998; Glessner et al. 1999; O'Toole and Kolter 1998). Results have shown that sub-MIC of streptomycin could impair the flagella-mediated swimming, swarming and type IV pili-regulated twitching motility of *P. aeruginosa* (Fig. 9). This result was supported by the suppression of flagella-encoded gene (*flgG*) expression under streptomycin at sub-MIC treatment using qRT-PCR (Fig. 10). Similar observations were reported by Saroj and Rather (2013), in which sub-MIC levels of streptomycin effectively inhibited the genes encoded for motility in *Acinetobacter baumannii*, which was also regulated by QS system. However, further research is required to identify the mechanism behind the anti-motility effect of streptomycin at sub-MIC levels.

In conclusion, this study aimed to revitalize the potentials of a common antibiotic at sub-MIC levels, where streptomycin showed the inhibitory effects against *P. aeruginosa* biofilm formation, eradicated the mature biofilm and attenuated various bacterial virulence properties. The alkaline pH and 35 °C of temperature as well as TSB culture media were proposed to play a crucial role in improving the anti-biofilm activity of streptomycin. Moreover, the ability of streptomycin at sub-MIC level to reduce the bacterial biofilm formation on the surface of urinary catheter proposed its potential application in preventing the infections associated with this medical device. Overall, from the obtained results, streptomycin can be considered as a promising candidate for the up-to-date anti-biofilm approaches which employ the sub-MIC levels of common antibiotics to target the bacterial virulence properties. Nevertheless, future research is in demand in order to understand the specific molecular interactions between streptomycin antibiotic and the virulence compounds, as well as the drug pharmacokinetics and optimal environmental conditions for the antibiotics to perform its inhibitory activity against *P. aeruginosa* biofilm.

Author contributions FK, JWL, JHL, and DTNP performed the experiment. FK, HWK, YKK, and YK designed the experiment and analyzed the data. All authors were involved in the writing and correction of the manuscript.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals.

Conflict of interest The authors declare that they have no conflict of interest.

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