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Original Article

Combination effects of DNase I and tobramycin on survival and biofilm architectures of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolated from chronic rhinosinusitis patients

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Abstract

This study aimed to investigate the effects of DNase I in combination with tobramycin (TOB) on drug susceptibility and biofilm architectures of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Biofilm formation, eDNA production and drug susceptibilities of *P. aeruginosa* and *K. pneumoniae* in both planktonic and biofilm forms were evaluated under different nutrient concentrations. The biofilm architectures of both bacteria were evaluated using confocal laser scanning microscopy. The results showed that the biofilm formations and eDNA quantities of both bacteria were reduced in a nutrient-limited condition. The eDNA concentrations in the biofilms were correlated with the amount of biofilm formed. Moreover, a combination with DNase I can enhance the bactericidal activity of TOB and decrease biofilm formation of both bacteria compared with TOB alone. These results suggest that the combined effects of DNase I and TOB allows prevention of biofilm formation and subsequent penetration of the drug resulting in an increased anti-biofilm effect.

Keywords: DNase I, eDNA, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, tobramycin

1. Introduction

A biofilm is the complex aggregation of microbes encased in a multifaceted matrix, and is more tolerant to conventional antibiotics than the planktonic bacteria. During biofilm formation, various unfavorable environmental conditions such as a nutrient-limited environment can enhance survival and tolerance of bacterial pathogens (Anderl, Zahller, Roe, & Stewart, 2003; Nguyen *et al.*, 2011). Several studies have highlighted that over 70-80% of chronic rhinosinusitis (CRS) patients were found to have biofilms on scanning electron micrographs (Dlugaszewska *et al.*, 2016; Sanclement, Webster, Thomas, & Ramadan, 2005). Evidence of biofilm producing organisms contributing toward the pathogenesis of CRS, and to disease severity, failure of effective clinical treatment and recurrent symptoms, has been reported (Jung, Cha, Kang, & Kim, 2015). Using culture-based methods revealed an overrepresentation of Gram-negative species, e.g. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in CRS patients (Mantovani *et al.*, 2010). Growth of *P. aeruginosa* and *K. pneumoniae* as biofilms exhibited resistance to multiple antimicrobial agents such as tobramycin (TOB),

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gentamicin, amikacin, ciprofloxacin (CIP) and ampicillin (AMP) (Anderl, Zahller, Roe, & Stewart, 2003; Poole, 2005). In addition, biofilms formed by *P. aeruginosa* and *K. pneumoniae* under nutrient limitation conditions were less susceptible to extermination by AMP and CIP (Anderl, Zahller, Roe, & Stewart, 2003; Zhang *et al.*, 2012). Biofilm formation and susceptibility of planktonic and biofilm forms of *P. aeruginosa* and *K. pneumoniae* to TOB under different nutrient concentrations, however, has not been reported. Therefore, the aim of this study was to give more information on those aspects.

The extracellular DNA (eDNA) is a crucial component of biofilms, involved in cell-to-cell interconnection for biofilm formation and stabilization of biofilm maturation (Okshevsky & Meyer, 2015). Due to its anionic character, eDNA can bind to positively charged antibiotics, such as aminoglycosides. As a consequence, eDNA seems to play a pivotal role in the tolerance of *P. aeruginosa* biofilms toward TOB (Wilton, Charron-Mazenod, Moore, & Lewenza, 2016). Enzymatic degradation of eDNA, particularly by DNase I, can decrease biofilm stability leading to increased drug penetration to the biofilm architecture that is important for improving the bactericidal efficacy of various drugs (Tetz, Artemenko, & Tetz, 20**09**). Moreover, recombinant human DNase administered as an aerosol was found to be safe and effective in reducing surface adhesivity and viscosity of cystic fibrosis (CF) sputum (Shak, Capon, Hellmiss, Marsters, & Baker, 1990; Wagener *et al.*, 1998).

TOB is a commonly used topical antibiotic and was used to treat CF patients (Rosenfeld *et al.*, 2001). Local administration of drugs offers a **"**site-specific**"** approach that has several advantages; primarily, it can be localized to infected sites at high concentrations without the side effects seen with systemic administration. Treatment of patients with CRS includes intranasal steroids, saline lavage and long-term macrolide antibiotics; however, topical medications used in CRS have not been well studied. The use of nasal irrigation with antibiotics was reported to be a potentially effective treatment for CRS patients who remain symptomatic after appropriate medical and surgical intervention (Elliott & Stringer, 2006; Lim, Citardi, & Leong, 2008). To the best of the authors' knowledge, however, there is no information about using DNase I in combination with TOB as a topical medication for CRS patients. To investigate a possible new strategy for prevention or control of *P. aeruginosa* and *K. pneumoniae* biofilms after appropriate medical and surgical intervention in CRS patients, the effects of DNase I in combination with TOB on biofilm architectures and survival of *P. aeruginosa* and *K. pneumoniae* compared with TOB alone are elucidated in this study.

2. Materials and Methods

2.1 Bacteria and cultures

P. aeruginosa and *K. pneumoniae* were isolated from nasal lavage of patients who presented to the otolaryngology clinic with CRS according to the criteria of the 2003 Chronic Rhinosinusitis Task Force (Hwang, Irwin, Griest, & Caro, 2003) at the Faculty of Medicine, Khon Kaen University (KKU), Khon Kaen, Thailand. The study was approved by the Human Ethics Committee of KKU (HE561075). These 2 bacterial strains displayed a biofilm-positive phenotype on the surface tissue from CRS patients in our previous study (Wongkaewkhiaw *et al.*, 2020). The bacteria were grown on MacConkey agar (MAC, HiMedia) at 37°C overnight, then liquid cultures were obtained by inoculating 10 ml of modified Vogel and Bonner's medium (MVBM) (Lam, Chan, Lam, & Costerton, 1980) with a single colony of bacteria. The bacterial cultures were incubated overnight at 37°C and used as the inoculum in the following experiments.

2.2 Drug susceptibility testing of bacteria under different nutrient concentrations

Drug susceptibility of *P. aeruginosa* and *K. pneumoniae* in MVBM and 10-fold diluted MVBM (0.1×MVBM) media was determined using transferable solid phase (TSP) pin lids, which resemble the Calgary biofilm device as previously described (Ceri *et al.*, 1999) with some slight modifications. To grow biofilms, a final volume (100 µl) of bacterial suspension in each medium (OD_{540} = 0.1) was added into each well of a 96-well plate and incubated at 37°C in a 100 rpm shaker-incubator for 24 h. Bacterial-free wells served as controls. Biofilms formed on the pegs of the TSP pin lid were transferred to a new plate which contained 100 µl/well of the 2-fold serial dilution of TOB (Amresco, Ohio, USA) in each medium. The final concentrations of TOB ranged from 0.024 to 500 µg /ml. Antibiotic-free wells were included as growth controls. The plates were incubated for 24 h. Then minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of shedding planktonic bacteria and minimum biofilm eliminating concentration (MBEC) values were determined as previously described (Ceri *et al.*, 1999). All experiments were repeated in triplicate on three separate occasions.

2.3 Quantification of biofilm growth and eDNA production in biofilms

Biofilm formations of *P. aeruginosa* and *K. pneumoniae* in MVBM and 0.1×MVBM were estimated using a crystal violet staining method as previously described (Taweechaisupapong *et al.*, 2005). Briefly, each bacterial strain was adjusted to give an optical density (OD) at 540 nm of 0.9. Two hundred microliters of starter culture were dispensed into each well of a 96-well plate. Wells containing only the medium served as negative control. The plates were incubated at 37°C for 3 h to allow adhesion. To remove non-adherent bacteria, the supernatant fluid of each well was gently aspirated and replaced with 200 µl of each fresh medium. The 1-day and 2-day old biofilms were obtained after incubation for an additional 21 and 45 h. Thereafter, the attached bacteria were fixed with 200 µl of 99% methanol for 15 min. The adherent cells were stained with 200 µl of 2% Hucker crystal violet for 5 min. Excess stain was removed with running tap water. The dye bound to the adherent cells was solubilized with 33% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 620 nm using a microplate reader. The ability of each isolate to produce biofilm was determined in three independent experiments and the results reported are the averages from these three independent experiments.

The eDNA was quantified in 96-well black plates concurrent with the biofilm quantification using QuantiFluor® dsDNA System kit (Promega, USA). After 1-day and 2-days, biofilms were washed twice in saline solution, 200 µl of freshly prepared QuantiFluor dsDNA dye in TE buffer was added in each well and incubated for 5 min before measuring the fluorescence intensity (excitation 504 nm/emission 531 nm) using a fluorometer (Varioskan Flash Multimode Reader, Singapore) with SkanIt Software 2.4.3 RE for Varioskan Flash. Lambda DNA was used to generate a standard curve for each run. All experiments were repeated in triplicate on three separate occasions.

2.4 Effect of DNase I on bacterial adhesion

To determine the effect of DNase I on the initial attachment of *P. aeruginosa* and *K. pneumoniae*, the Amsterdam Active Attachment (AAA) model (Exterkate, Crielaard, & Ten Cate, 2010) was used. The AAA model consists of a stainless steel lid with clamps that contain glass coverslips which were used as substratum for adhesion of *P. aeruginosa* and *K. pneumoniae*. Bacterial suspensions were adjusted to OD_{540} of 0.9 in phosphate buffered saline (PBS). Then, 1 ml of each bacterial suspension was added into a well of a sterile polystyrene 24-well plate (Corning, USA). To evaluate the effect of DNase I compared with proteinase K and RNase A on bacterial adhesion, 100 µg/ml of each enzyme was mixed with a bacterial suspension. A well with only PBS served as control. Then the AAA lid containing the glass coverslips was placed on the 24-well plate to allow bacterial adhesion on the glass surface. A plate was incubated at 37°C for 30 min. Then the coverslips were washed twice before staining with a solution of the Live/Dead BacLight Bacterial Viability kit (Molecular Probes Inc., USA) for 15 min at 4°C. Thereafter, the sessile cells were fixed with 1% glutaraldehyde for 3 h. The bacterial adhesion was examined using a confocal laser scanning microscope (CLSM, Zeiss LSM 800, Zeiss, Germany). The quantitative estimation of bacterial cells was determined from 16 random areas for each sample and analyzed using the ZEN 2.1 (blue edition) image analysis wizard software (Zeiss, Germany).

2.5 Effect of DNase I on *P. aeruginosa* and *K. pneumoniae* biofilm-forming capacity under different nutrient concentrations

To evaluate the effect of DNase I on *P. aeruginosa* and *K. pneumoniae* biofilm-forming capacity in MVBM and 0.1×MVBM, various concentrations of DNase I (0.1 – 10 µg/ml) were added to biofilm cultures at 0, 24 and 48 h. The biofilm-forming capacities of all tested bacteria were evaluated at 51 h using a crystal violet staining method as described above. All experiments were repeated in triplicate on three separate occasions. Then the optimal condition of DNase I treatment was selected for further study.

2.6 Effects of DNase I combined with tobramycin on biofilm structure, quantity of eDNA and viability of bacteria

To evaluate the effects of DNase I combined with TOB on the 2-day old biofilm structures, quantities of eDNA and the viability of *P. aeruginosa* and *K. pneumoniae* in MVBM, 0.1 µg/ml DNase I and 125 µg/ml TOB were used. Briefly, overnight cultures of bacteria were re-suspended in fresh MVBM media to give an OD_{540} of 0.9 and transferred to each well of the 24-well plate. Then 0.1 µg/ml DNase I was added to the wells of the treatment group. The wells without DNase I served as controls. The plates were covered with the AAA lid containing the coverslips and incubated at 37˚C for 3 h to allow adhesion. After 3 h, the lids were transferred to new 24-well plates containing fresh media with or without 0.1 µg/ml DNase I and incubated for an additional 21 h. Thereafter, the 1-day old biofilms were transferred to a new 24-well plate containing fresh MVBM media (control), 0.1 µg/ml DNase I alone, 125 µg/ml TOB alone, or a combination of 0.1 µg/ml DNase I and 125 µg/ml TOB and incubated for another 24 h to obtain 2-day old biofilms.

To determine the viability of *P. aeruginosa* and *K. pneumoniae* in 2-day old biofilms, the coverslips were stained with a Live/Dead BacLight Bacterial Viability kit for 15 min at 4°C. Then, the sessile cells were fixed with 1% glutaraldehyde for 3 h and examined using CLSM. The quantification of bacterial viability was determined from 16 random areas of each sample and analyzed using the ZEN 2.1 (blue edition) image software.

To evaluate the effects of all tested agents on biofilm structures and quantities of eDNA, 2-day old biofilms on the coverslips were stained with 50 µg/ml fluorescein isothiocyanate-concanavalin A (FITC-Con A) (Sigma, Missouri, USA), and 2 µM TOTO-3 (Thermo Fisher Scientific, Oregon, USA), for 15 min at 4˚C. Thereafter, the 2-day old biofilms were fixed with 1% glutaraldehyde for 3 h. The coverslips were examined using CLSM at 630× magnification. Data on the structure of the biofilms were collected and 3-dimensional images were reconstructed. The fluorescence intensity of extracellular polysaccharides and eDNA were analyzed using the ZEN 2.1 (blue edition) image software.

2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). Two-way ANOVA with Bonferroni post-test was used to compare the effects of various concentrations of DNase I on biofilm-formation. Kruskal-Wallis analysis and Dunn's multiple comparison tests were used to compare the live/dead ratio of bacteria and the differences among the extracellular polysaccharides and eDNA after DNase I and TOB treatment. *P*-value < 0.05 was considered significant.

3. Results

3.1 Drug susceptibility of bacteria

The P-MBC determinations of planktonic *P. aeruginosa* and *K. pneumoniae* in both MVBM and 0.1×MVBM revealed that *P. aeruginosa* was less susceptible to TOB than *K. pneumoniae* (Table 1). Interestingly, *P. aeruginosa* and *K. pneumoniae* were able to grow at higher TOB concentrations when incubated at high compared to low

Table 1. Susceptibility of planktonic and biofilm bacteria to tobramycin under different nutrient concentrations

Medium	*P. aeruginosa*			*K. pneumoniae*		
	P-MIC[a] (µg/ml)	P-MBC[b] (µg/ml)	MBEC[c] (µg/ml)	P-MIC[a] (µg/ml)	P-MBC[b] (µg/ml)	MBEC[c] (µg/ml)
MVBM	7.81	62.5	250	7.81	15.62	500
0.1×MVBM	0.98	7.81	250	0.49	0.49	0.49

[a]P-MIC: shedding planktonic minimum inhibitory concentration
[b]P-MBC: shedding planktonic minimum bactericidal concentration
[c]MBEC: minimum biofilm eliminating concentration

nutrient levels. *K. pneumoniae* biofilm in MVBM was less susceptible to TOB than *P. aeruginosa* (MBEC results), while the MBEC value of TOB against *K. pneumoniae* in 0.1×MVBM was lower than that of *P. aeruginosa*. The drug susceptibilities of both biofilm bacteria (MBEC results) in MVBM were much higher than those of planktonic cells (P-MBC results).

3.2 Quantities of eDNA correlated with biofilm formation

P. aeruginosa and *K. pneumoniae* produced significantly more biofilm when incubated at high (MVBM) vs low (0.1×MVBM) nutrient level (Figure 1). In addition, the biofilm formations and eDNA quantities of both bacteria in MVBM at 48 h were higher than those at 24 h. Both bacteria in 0.1×MVBM, however, appeared to produce slightly more biofilms at 48 h than at 24 h. Surprisingly, the eDNA quantity of *P. aeruginosa* biofilm in 0.1×MVBM at 24 h was higher than that at 48 h while the eDNA quantity of *K. pneumoniae* biofilm in 0.1×MVBM at 24 h was less than that at 48 h.

3.3 DNase I reduces bacterial adhesion and biofilm formation

It was clear that treatment with DNase I had a significant impact on the initial attachment of both *P. aeruginosa* and *K. pneumoniae* compared to Proteinase K and RNase A (Figure 2). The effects of DNase I on *P. aeruginosa* and *K. pneumoniae* biofilm-forming capacities were further evaluated by adding various concentrations of DNase I (0.1, 1 and 10 µg/ml) to biofilm cultures at different time points (0, 24 and 48 h). The results showed that addition of DNase I up to 48 h after inoculation had a pronounced effect in reducing biofilm formation of both isolates in MVBM compared to controls (Figure 3). The biofilm-forming capacities, however, of both isolates in 10-fold diluted medium (0.1×MVBM) of all conditions (with and without DNase I) were much lower than those in MVBM. No significant reduction of biofilm in 0.1×MVBM was found after DNase I treatment compared to controls.

3.4 DNase I in combination with TOB enhances bactericidal activity and decreases biofilm formation

Since the lowest concentration of DNase I (0.1 µg/ml) significantly reduced biofilm formation of both isolates in MVBM, this condition was selected for further

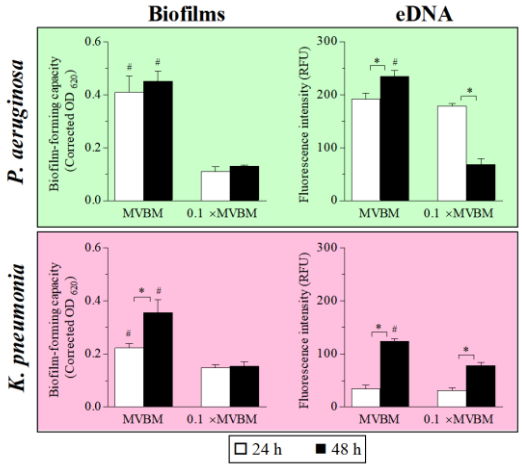

Figure 1. Biofilm-forming capacity and eDNA production by *P. aeruginosa* (green) and *K. pneumoniae* (pink) at 24 and 48 h. Data are presented as the mean and standard deviation of three independent experiments performed in triplicate. *$p < 0.05$ compared between MVBM or 0.1×MVBM at the different time points. $^{\#}p < 0.05$ compared between MVBM and 0.1×MVBM at the same time point.

study of the effects of DNase I combined with TOB on 2-day old biofilm structures, quantities of eDNA and viability of both isolates in biofilms using CLSM. Confocal laser scanning micrographs of the control and with DNase I alone treated *P. aeruginosa* biofilm showed a greater amount of green fluorescence indicating more adherence of alive bacteria. In contrast, a decrease in green fluorescence with an increased red fluorescence was seen both for TOB alone treated and for the combination treated *P. aeruginosa* biofilm (Figure 4A). Quantification of bacterial viability showed that live/dead ratio of *P. aeruginosa* after exposure to the combination of DNase I and TOB was significantly lower than in control and with either agent alone ($P < 0.01$). For *K. pneumoniae*, the live/dead ratio after exposure to the combination of DNase I and TOB was significantly lower than control and DNase I alone treatment ($P < 0.01$) (Figure 4B).

CLSM images and Zen 2.1 imaging analysis of 2-day old *P. aeruginosa* and *K. pneumoniae* biofilms after exposure to the tested agents are shown in Figures 5 and 6. Biofilms treated with DNase I appeared thinner and less dense than in untreated controls. Moreover, the biofilms of both isolates that underwent treatment of the combination of DNase I and TOB showed a decreased green fluorescence area and

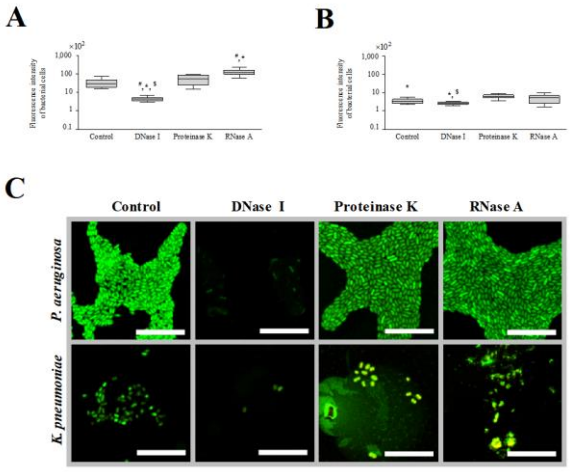

Figure 2. *P. aeruginosa* (A) and *K. pneumoniae* (B) attached cells on glass coverslips after 30 min incubation in phosphate buffer saline (no-treatment control), 100 µg/ml of DNase I, Proteinase K and RNase A. The fluorescence intensity of attached cells was calculated from 16 random areas of 2 coverslips for each sample. $^{\#}p < 0.05$ compared with control. $^{*}p < 0.05$ compared with Proteinase K treatment. $^{\$}p < 0.05$ compared with RNase A treatment. Confocal laser scanning micrographs of *P. aeruginosa* and *K. pneumoniae* attached cells stained with LIVE/DEAD BacLight Bacterial Viability kit (C). Green color indicates live bacteria and red color indicates dead bacteria. Images were viewed at 630x magnification. The scale bar is for 10 µm.

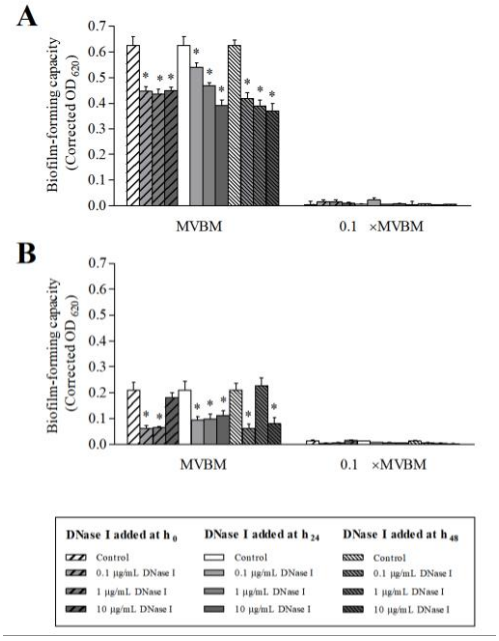

Figure 3. Biofilm-forming capacity of *P. aeruginosa* (A) and *K. pneumoniae* (B) after treated with 0.1, 1 and 10 µg/ml of DNase I at 0, 24 or 48 h. Data are presented as the mean and standard deviation of three independent experiments performed in triplicate. ($^{*}p < 0.05$ compared with control).

thinner biofilm architecture than control or with the agents alone (Figures 5A, 5C, 6A and 6C). Zen 2.1 imaging analysis for biofilm quantification showed that among the 4 groups initially tested, the combined DNase I and TOB case had significantly less biomass than controls or when treated with the agents alone ($P < 0.05$). Moreover, the combination of DNase I and TOB treated *P. aeruginosa* biofilm showed a significant decrease in the quantity of eDNA compared with TOB alone case (Figure 5B), while the quantity of eDNA in *K. pneumoniae* biofilm after exposure to the combination decreased significantly compared with TOB or DNase I alone (Figure 6B).

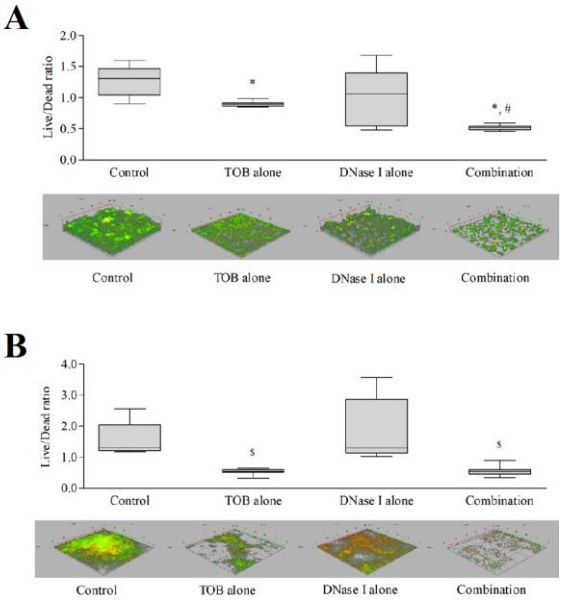

Figure 4. Live/Dead ratios and CLSM 3-D reconstruction of 2-day old *P. aeruginosa* (A) and *K. pneumoniae* (B) biofilms after exposure to 125 µg/ml TOB, 0.1 µg/ml DNase I and combination of 125 µg/mL TOB and 0.1 µg/ml DNase I. The biofilms were stained with LIVE/DEAD BacLight Bacterial Viability kit. Green color indicates live bacteria and red color indicates dead bacteria. Images were viewed at 630x magnification and the quantification of bacterial viability was determined from 16 random areas for each sample. ($^{*}p < 0.01$ compared with control, $^{\#}p < 0.01$ compared with TOB and DNase I treatments and $^{\$}p < 0.01$ compared with control and DNase I treatment)

4. Discussion

Several studies have determined the effect of nutrient concentration on biofilm formation. Some authors suggested that an increase in nutrient concentration enhances biofilm formation (Rochex & Lebeault, 2007), while others have shown that a high nutrient medium inhibits biofilm growth (Dewanti & Wong, 1995). The correlation between biofilm formation and eDNA production of *P. aeruginosa* and *K. pneumoniae* under different nutrient concentrations, however, is still mostly unknown. In this study, it was found that under different nutrient concentrations, both *P. aeruginosa* and *K. pneumoniae* produced less biofilm in 0.1×MVBM than those in MVBM. Since the MVBM is a chemically defined medium with a large amount of glucose (Lam *et al.*, 1980), the high glucose concentration in MVBM could contribute to higher levels of biofilm production

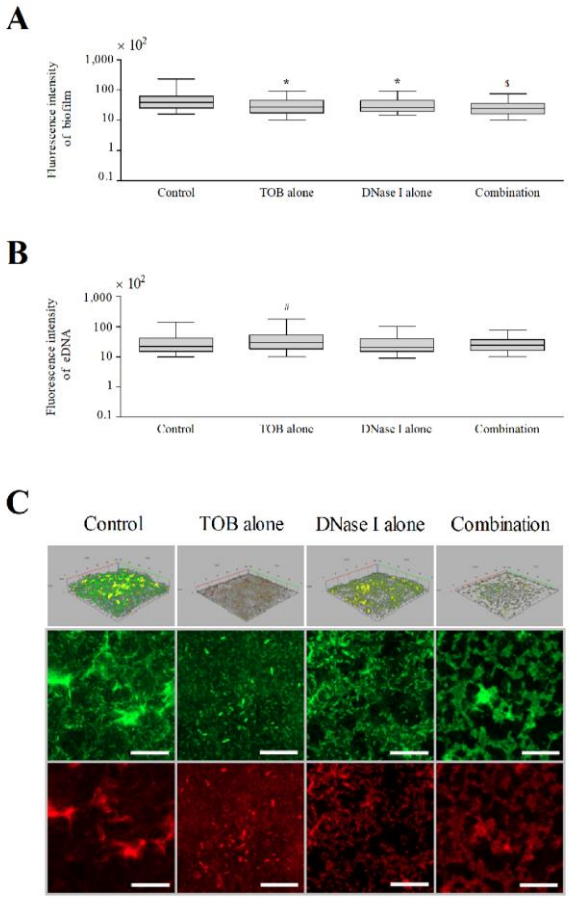

Figure 5. Effect of DNase I and tobramycin on biofilm structure of *P. aeruginosa* by CLSM. Quantification of biofilm stained with FITC-conA (A). Quantification of eDNA stained with TOTO-3 (B). Data are the value calculated from 16 random areas. $*p < 0.05$ compared with control. $^{\$}p < 0.05$ compared with control and DNase I alone. $^{\#}p < 0.05$ compared with control, DNase I alone, and the combination. The CLSM 3-D reconstruction of 2-day old *P. aeruginosa* biofilm (C). FITC-conA showing biofilm in green and TOTO-3 showing eDNA in red. The scale bar is for 10 µm.

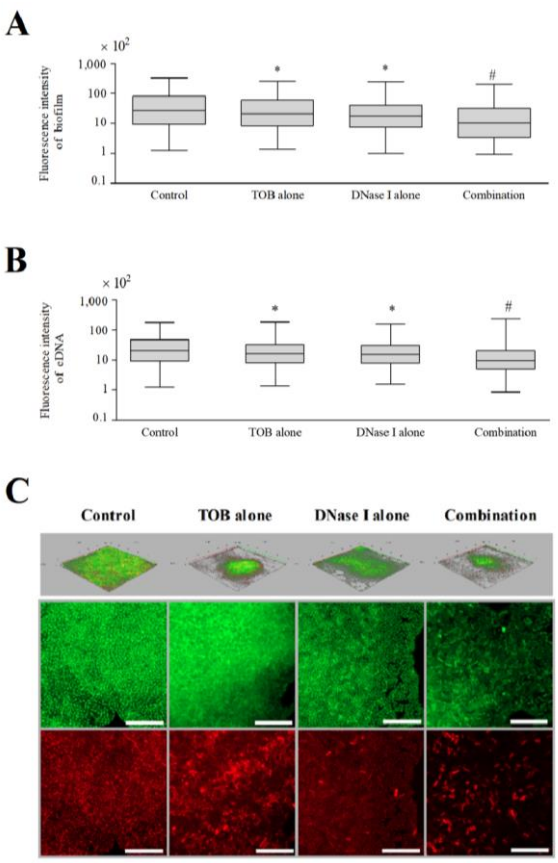

Figure 6. Effect of DNase I and tobramycin on biofilm structure of *K. pneumoniae* by CLSM. Quantification of biofilm stained with FITC-conA (A). Quantification of eDNA stained with TOTO-3 (B). Data are the values calculated from 16 random areas. $*p < 0.05$ compared with control. $^{\#}p < 0.05$ compared with control, TOB alone, and DNase I alone. The CLSM 3-D reconstruction of 2-day old *K. pneumoniae* biofilm (C). FITC-conA showing biofilm in green and TOTO-3 showing eDNA in red. The scale bar is for 10 µm.

compared to 0.1×MVBM. These results are in accordance with several previous reports, which showed the influence of glucose concentration on biofilm formation by other bacteria (Kyoui, Hirokawa, Takahashi, Kuda, & Kimura, 2016; Waldrop, McLaren, Calara, & McLemore, 2014). Moreover, the results in this study showed that both *P. aeruginosa* and *K. pneumoniae* in MVBM exhibited less susceptibility to TOB than under the low-nutrition concentration (0.1×MVBM) (Table 1). The increased biofilm production of both bacteria in MVBM may make the bacteria more resistant to TOB than those in 0.1×MVBM. Greater susceptibility to antibiotics under the low-nutrition concentration was also found in the study of beta-lactamase producing bacteria. Wang and colleagues demonstrated that with a high growth rate in 100% LB, the higher production of beta-lactamase to degrade ceftriaxone compared with the 2% or 1% LB cases was observed leading to the less susceptibility of beta-lactamase producing bacteria in rich medium (Wang, Ran, Wang, Ouyang, & Luo, 2015). A possible factor inducing TOB susceptibility under low-nutrition concentration (0.1×MVBM) in this study may have been the lower growth rate of both *P. aeruginosa* and *K. pneumoniae* (supplementary figure) that subsequently reduced their antibiotic-inactivating enzymes when antibiotic stress was encountered.

The eDNA quantities in biofilms of both bacteria were different but correlated with the amount of biofilm production (Figure 1). This may be due to different mechanisms involved in eDNA release of both bacteria. Numerous species of bacteria are able to release eDNA by different mechanisms, such as autolysis and active secretion, as well as through its association with membrane vesicles (Ibanez de Aldecoa, Zafra, & Gonzalez-Pastor, 2017). Since eDNA is thought to be important for the structural integrity and the antibiotic resistance of many bacterial biofilms, destabilization of biofilms by removing eDNA using exogenous DNase enzymes is an attractive option for biofilm control. The results obtained in this study showed that

treatment with DNase I had a significant impact on initial cellular attachment and biofilm-forming capacity of both *P. aeruginosa* and *K. pneumonia* (Figures 2 and 3). Neither treatment with RNase A nor treatment with proteinase K had a significant impact on attachment. These results are consistent with previous studies which demonstrated that DNase I can inhibit the initial attachment and biofilm formation by other bacteria (Harmsen, Lappann, Knochel, & Molin, 2010; Pakkulnan *et al.*, 2019).

Topical medications are an alternative for the treatment of recalcitrant infections after endoscopic sinus surgery (ESS) in CRS patients. Topical therapy with drugs such as TOB for patients with CRS can be effective in reducing mucosal edema, pain, secretions and postnasal drip, especially after ESS (Chiu, Antunes, Palmer, & Cohen, 2007). Although TOB is a commonly used topical antibiotic, TOB irrigation resulted in the eradication of viable bacteria within the lumen of the sinus but did not eradicate the *Pseudomonas* biofilm (Chiu *et al.*, 2007). In this study evidence is provided that a combination of DNase I can enhance the bactericidal activity of TOB toward biofilms of *P. aeruginosa* and *K. pneumoniae* compared with TOB alone (Figure 4). Moreover, *P. aeruginosa* and *K. pneumoniae* biofilms were thinner and less dense when growing from the beginning with DNase I, and after 24 h growth, TOB was added for 24 h with DNase I compared with control or the agents alone (Figure 5 and 6). In addition, it was found that DNase I has no inhibitory activity on bacterial cell growth (data not shown). Therefore, differences in the amount of biofilms at the end of the 48 h incubation reflect the effects of preventing biofilm development in the presence of DNase I, preventing further biofilm growth after 24 h due to TOB, and possibly removal of biofilms during combined DNase I and TOB treatment.

Nasal blockage/congestion/obstruction or nasal discharge (rhinorrhea or postnasal drip) are symptoms of CRS (Fokkens *et al.*, 2020). Recombinant human DNase was reported to decrease the viscosity of purulent CF secretions and improve primary nasal symptoms in CRS (Mainz *et al.*, 2014; Shak *et al.*, 1990; Wagener *et al.*, 1998). In addition, Recombinant human DNase therapy also reduced the costs of treating respiratory tract infections with low frequency adverse reactions (Oster, Huse, Lacey, Regan, & Fuchs, 1995). Thus it is worth mentioning that DNase I in combination with TOB might have great potential for use as topical medication for CRS patients.

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