ANTIMICROBIAL TOLERANCE OF *MYCOBACTERIUM ABSCESSUS* IN DUAL SPECIES BIOFILM FORMED WITH *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Mycobacterium abscessus (MABSC) can cause chronic lung disease in patients with immunocompromised status or structural lung diseases. *M. abscessus* infection was found with *Pseudomonas aeruginosa* infection or colonization. Moreover, both pathogens have the ability to produce biofilms, which increase the severity of infection. Therefore, this study aimed to investigate the effect of the *M. abscessus* and *P. aeruginosa* dual-species biofilm on the antimicrobial tolerance of *M. abscessus*. Co-culture and viability plate count methods were used to evaluate the effect of biofilm. As a result of this study, we found that at early phase of biofilm formation, *M. abscessus* cannot tolerate to antibiotics when co-culture with *P. aeruginosa*. However, at late phase, *M. abscessus* in dual-species biofilm can tolerate to antibiotics even high concentration.

Keywords: *Mycobacterium abscessus*, biofilm, antimicrobial tolerance

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INTRODUCTION

Mycobacterium abscessus belong to the rapidly grower non-tuberculous mycobacteria (NTM), which were commonly found in environments like water and soil. Nevertheless, *M. abscessus* also had the ability to be a pathogen in humans and animals (Pfyffer, 2015; Rastogi, Legrand, & Sola, 2001). MABSC could be classified into three subspecies based on their genome sequence; *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (Bryant et al., 2013; M.-R. Lee et al., 2015a). MABSC was one of the pathogenic NTMs that can be found in patients with chronic lung disease and also causes other infections such as the skin and soft tissues, the central nervous system, bacteremia, the eyes, and other infections (M.-R. Lee et al., 2015b; Syrmis et al., 2015). MABSC frequently caused severe lung infections in people with pre-existing structural lung illnesses such as CF, bronchiectasis, prior TB, and Chronic Obstructive Pulmonary Disease (COPD)(Fennelly et al., 2016; Qvist et al., 2015a). The bacterial cell population that formed a three-dimensionally attached polymeric matrix is known as a bacterial biofilm. Bacteria waer shielded by biofilm from the host immune system and antimicrobial medication. (Watnick & Kolter, 1999; Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002). Biofilm was one of virulence factor which enhanced the severity of infection. NTM could persist in biofilms, inside infected host cells like macrophages, or outside. Both sputum samples and alveolar walls of lungs from infected patients demonstrated NTM biofilm-forming (Qvist et al., 2015b; Zhang et al., 2018). The prevalence of MABSC co-infection with other bacteria in human lungs was reported in earlier investigations. (Bar-On et al., 2015; Cavalli et al., 2017; Lu et al., 2019; Martiniano, Sontag, Daley, Nick, & Sagel, 2014; Meoli, Deolmi, Iannarella, & Esposito, 2020), especially in CF patients. One of the main bacterial pathogens that co-infect MABSC in the lungs is *P. aeruginosa.* (Bjarnsholt et al., 2009; Chernenko et al., 2006; Sermet-Gaudelus et al., 2003; Takano et al., 2021), which caused acute and chronic lung infections with a high rate of death and disability. *P. aeruginosa* was highly resistant to antimicrobials and had ability to form biofilms (Wagner & Iglewski, 2008). Co-infection of MABSC with *P. aeruginosa* led to more severe disease, including impaired lung function and repeated hospital stays. (Brugha & Spencer, 2021). Additionally, there was a study reported that co-culture biofilm of MABSC and *P. aeruginosa* induced MABSC more tolerated to antibiotics (Rodríguez-Sevilla et al., 2019; Graciela Rodríguez-Sevilla et al., 2018). According to these problems, this study aimed to investigate the effect of dual-species biofilm of MABSC and *P. aeruginosa* on antimicrobial tolerance of MABSC. The results of this study may assist with patient management and MABSC treatment planning in situations when MABSC and *P. aeruginosa* are co-infected.

MATERIALS AND METHODS

Bacterial isolates and cultivation

Mycobacterium abscessus ATCC19977 and *P. aeruginosa* ATCC27853 were used in this study. The frozen stock of MABSC was grown on Middlebrook 7H10 agar or Lowenstein-Jensen media at 37 °C for 3-5 days, then subcultured to Middlebrook 7H9 supplemented with 0.2% glycerol and cultured at 37 °C shaking at 200 rpm until reaching to the stationary phase. The stationary culture were adjusted to 0.1 OD_{600} in Middlebrook 7H9 supplemented with 0.2% glycerol and cultured at 37 °C shaking 200 rpm until reaching to log phase. *P. aeruginosa* ATCC27853 from frozen stock was grown in LB broth at 37 °C, shaking at 200 rpm for 24 hours to reach the stationary phase. The stationary culture were adjusted to 0.1 OD_{600} in LB broth and cultured at 37 °C shaking 200 rpm, until reaching to log phase. And log phase cultures were used for experiments.

Amikacin and Clarithromycin preparation

10 mg of amikacin powder was dissolved in 1 mL of steriled distilled water to prepare a stock solution with a concentration of 10mg/mL, after dissolved, the solution was filtered through

0.22 µm syringe filter and stored at -20 ºC until use. For clarithromycin, 2 mg of powder was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to prepare a stock solution with a concentration of 2 mg/mL and stored at -20 ºC until use.

Antimicrobial testing of dual-species biofilm

The dual-species biofilm was performed, the turbidity of both bacterial cultures were measured by spectrophotometer at 0.5 *McFarland standard (McF)* and adjusted to approximately 10^5 CFU/mL in Sauton medium, then inoculated into flat bottom 96-wells plate, as co-culture using 100 µL of each microorganism per well (ratio 1:1). The plates were incubated at 37°C. Single species biofilm of MABSC and *P. aeruginosa* ATCC27853 were included as control (Izano, Amarante, Kher, & Kaplan, 2008). To investigate the antimicrobial tolerance of MABSC in dual-species biofilm. The amikacin and clarithromycin were used to determine an alternation of MABSC antimicrobial tolerance in biofilm (Daley et al., 2020; Porvaznik, Solovič, & Mokrý, 2017). After two and seven days of dual-species biofilm development, the excess media planktonic cell were discarded by pipetting and washed gently with Phosphate Buffer Saline (PBS) 2 times. Amikacin solution (1-64 µg/mL) and clarithromycin solution (0.06-16 µg/mL) were prepared in fresh Mueller Hinton Broth were added to dual-species biofilm in 96-well plate. Then the plates were incubated at 37°C for 2 days. Viability of MABSC biofilm were evaluated by viability counting (Li et al., 2022).

Statistical analysis

All the experiments were performed in biological triplicate. The results were analyzed by GraphPad Prism version 8.0.1 software. Categorical variables were compared using Data were analysed using two-way ANOVA, Sidak's multiple comparisons test (Alam, Catlow, Di Maio, Blair, & Hall, 2020). A *p*-value of <0.05 in a two-tailed test was considered to be a statistically significant difference.

RESULTS

Amikacin tolerance

Effect on amikacin tolerance were investigated by viable colony counting and compare viability of M. abscessus in dual-species condition with M. abscessus in condition without *P. aeruginosa*. The viability of *M. abscessus* biofilm at day 2 (fig.1-A), at amikacin concentration more than 1 µg/mL, *M. abscessus* from dual-species condition cannot survive (0 CFU/mL), while *M. abscessus* from single-species condition could survive even high concentration (64 µg/mL). Moreover, the result from *M. abscessus* biofilm at day 7 (fig.1-B) showed that viability of *M. abscessus* in dual-species biofilm has significant difference only in condition 0 µg/mL, when compared with single-species condition. In amikacin treated condition has no significant difference between dual- and single-species. However, at 32-64 µg/mL of amikacin, *M. abscessus* from dual-species cannot survive.

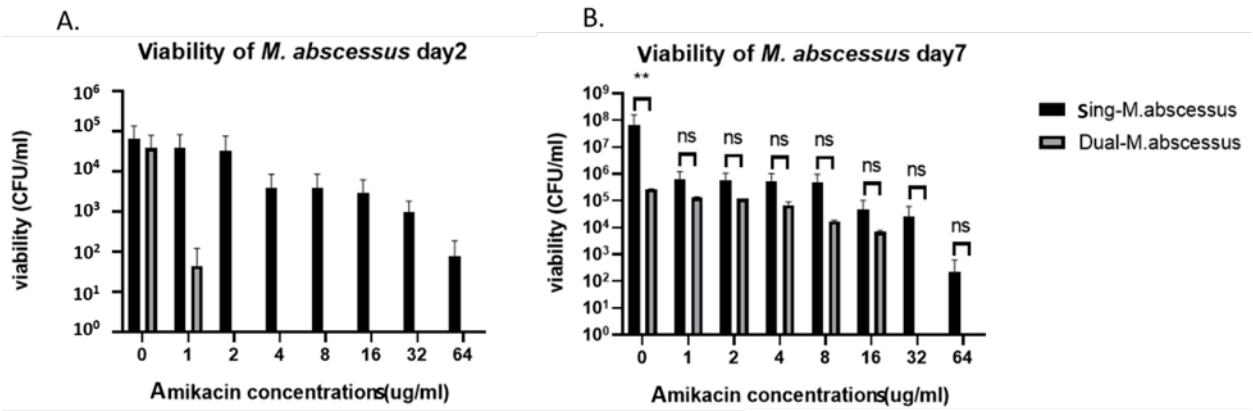

Figure 1 The Colony forming unit (CFU) of *M. abscessus* ATCC19977 biofilm when treated with each concentration of amikacin (0-64 µg/mL) at day 2 (A), and day 7 (B) of biofilm development. All experiments were technical and biological triplicates. Data are the mean ± SD from three biological replicates. Data were analysed using two-way ANOVA and Sidak's multiple comparisons test ($p < 0.05$), NS is no significant.

Clarithromycin tolerance

Viability of *M.abscessus* from dual-species condition at day 2 showed that cannot tolerate to clarithromycin at concentration more than 0.12 µg/mL. On the other hand, *M.abscessus* in single-species condition can tolerate to 1 µg/mL, anyway, there was no significant difference in 0.5 µg/mL and 1 µg/mL of clarithromycin between single- and dual-species condition (Fig.2-A). Furthermore, as a result of day 7 biofilm (Fig.2-B), at low concentration of clarithromycin (0-0.25 µg/mL), *M. abscessus* from dual-species biofilm showed lower viability than *M. abscessus* from single-species significantly. However, at concentration more than 0.25 µg/mL have no significant difference between conditions.

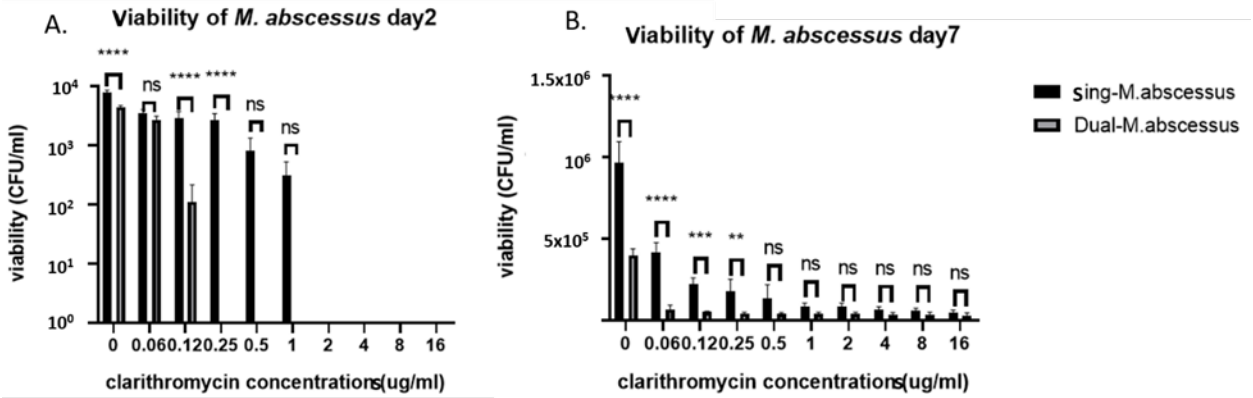

Figure 2 The Colony forming unit (CFU) of *M. abscessus* ATCC19977 biofilm when treated with each concentration of clarithromycin (0-16 µg/mL) at day 2 (A), and day 7 (B) of biofilm development. All experiments were technical and biological triplicates. Data are the mean ± SD from three biological replicates. Data were analysed using two-way ANOVA and Sidak's multiple comparisons test ($p < 0.05$), NS is no significant.

CONCLUSION & DISCUSSION

M. abscessus was commonly found co-infection with other pathogens especially, *P. aeruginosa* . (Bjarnsholt et al., 2009; Chernenko et al., 2006; Sermet-Gaudelus et al., 2003; Takano et al., 2021). Moreover, both pathogens had the ability to produce biofilm as a virulence factor (Qvist et al., 2015b; Zhang et al., 2018), (Wagner & Iglewski, 2008), (Rodríguez-Sevilla et al., 2019; Graciela Rodríguez-Sevilla et al., 2018). This study aimed to determine the effect of dual-species biofilm of both pathogens to antimicrobial tolerance. Biofilm at day 2 was represented as biofilm at early state, which was the initiation phase of biofilm production, and biofilm at day 7 was represented mature biofilm. From our result, at day 2 of biofilm development, in the co-culture condition, caused *M. abscessus* had lower survival ability than condition without *P. aeruginosa*, which may was caused by *P. aeruginosa* outcompetes over *M. abscessus*, which was a slower growing, which may affect the low viability of *M. abscessus* in biofilm cannot tolerate to antibiotics, resulting in a weaker biofilm of *M. abscessus* that can be killed by antibiotic (G. Rodríguez-Sevilla et al., 2018). Furthermore, result on day 7, we found that *M. abscessus* from the dual-species biofilm had the ability tolerate to antibiotics, both amikacin and clarithromycin, even at high concentrations, that showed no significant difference between two conditions, which may was caused *M. abscessus* in day 7 biofilm was maturity enough to tolerate to antibiotics, even though it co-cultured with *P. aeruginosa.* That may infer that co-infection of *M. abscessus* and *P. aeruginosa* caused *M. abscessus* has more ability to tolerate to antibiotics. Moreover, different results of *M. abscessus* in dual species between amikacin and clarithromycin at day 7, high concentrations of amikacin could eliminate *M. abscessus*, while *M. abscessus* could have viability in high concentration of clarithromycin, that was caused by amikacin presented bactericidal activity, while clarithromycin presented bacteriostatic activity (J. Lee et al., 2021).

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