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STUDI PENDAHULUAN *NONTUBERCULOUS MYCOBACTERIA* (NTM): PEMBENTUKAN BIOFILM, MOTILITAS GESER, DAN POLA KEPEKAAN ANTIBIOTIK

PRELIMINARY STUDY OF NONTUBERCULOUS MYCOBACTERIA (NTM): BIOFILM FORMATION, SLIDING MOTILITY, AND ANTIBIOTIC SUSCEPTIBILITY PATTERN

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ABSTRAK

Nontuberculous mycobacteria (NTM) adalah mikrorganisme yang banyak dijumpai di lingkungan, namun, baru-baru ini dianggap patogen karena kejadian infeksinya meningkat secara signifikan. Penelitian ini bertujuan untuk mengetahui kemampuan pembentukan biofilm isolat NTM, korelasinya dengan sifat motilitas geser, dan untuk menganalisis pola kepekaan antibiotik. Strain NTM yang dipakai dalam penelitian ini adalah 10 isolat klinis NTM yang diperoleh dari laboratorium TB, Departemen Mikrobiologi, Fakultas Kedokteran UGM Yogyakarta. Kemampuan pembentukan biofilm dideteksi dengan menggunakan uji mikrotiter dan pewarnaan dengan kristal violet 1%. Uji motilitas geser dilakukan pada medium motilitas, terdiri dari 0,3% Middlebrook 7H9-agar tanpa suplemen. Pola kepekaan antibiotik diteliti dengan teknik dilusi sesuai metode CLSI. Dari penelitian ini menunjukkan bahwa 7 dari 10 isolat NTM merupakan penghasil biofilm kuat, sementara 1 isolat sebagai strain penghasil biofilm moderat, dan 2 isolat tidak menghasilkan biofilm. Sementara itu, strain pembentuk biofilm mampu melakukan motilitas geser pada agar semisolid, dan 2 isolat NTM yang tidak memiliki kemampuan pembentukan biofilm tidak dapat melakukan motilitas geser. Sifat pembentukan biofilm berkorelasi dengan kemampuan isolat NTM untuk melakukan motilitas geser pada media agar semisolid. Klaritromisin merupakan antibiotik yang paling efektif terhadap isolat NTM yang diuji (poten terhadap 50% isolat uji), diikuti oleh gentamisin (40%), sedangkan kanamisin, levofloxacin, dan ofloxacin menunjukkan tingkat potensi yang sama (30%). Ceftriaxone hanya mampu menghambat pertumbuhan isolat NTM sekitar 20%. Selanjutnya, kotrimoksazol dan amoksisilin memiliki aktivitas in vitro yang buruk terhadap isolat NTM karena tidak ada isolat NTM yang sensitif terhadap kedua antibiotik ini.

Kata kunci: kemampuan pembentukan biofilm, motilitas geser, NTM, pola kepekaan antibiotik

ABSTRACT

Nontuberculous mycobacteria (NTM) are ubiquitous organisms commonly found in the environment. However, recently it is considered as emerging global interest since the incidence increase significantly. This study aimed to investigate the biofilm forming ability of NTM isolates, correlated with the sliding motility properties, and to analyze their antibiotic susceptibility pattern. NTM strain included in this study were 10 NTM clinical isolates obtained from TB laboratory, Microbiology Departement, Faculty of Medicine UGM Yogyakarta. Biofilm forming capability was detected by using biofilm development assay in microtiter plate and staining with 1% crystal violet. Sliding motility assay was performed on motility medium, consisting of Middlebrook 7H9-0.3% agar without supplements. Antibiotic susceptibility pattern was investigated by macrobroth dilution technique according to CLSI methods. Our study revealed that 7 out of 10 NTM isolates produced biofilm strongly, while 1 isolate demontrated as moderate biofilm former strain, and the remaining 2 isolates did not produce biofilm on polysterene substrate. Meanwhile, biofilm-former strain are able to slide on semisolid agar, and 2 non-adherent NTM isolates did not have ability to perform sliding motility. A good correlation was found between mycobacterial sliding and biofilm assembly of NTM isolates. Clarithromycin has been shown as the most effective antibiotic against NTM isolates tested, which was active against 50% of all isolates, followed by gentamycin (40%), while kanamycin, levofloxacin, and ofloxacin showed the same level of potency (30%). Ceftriaxone was only able to inhibit the growth of NTM isolates about 20%. Furthermore, cotrimoxazole and amoxicillin had poor in vitro activity against NTM species..

Keywords: antibiotic susceptibility, biofilm forming ability, NTM, sliding motility

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INTRODUCTION

Nontuberculous mycobacteria (NTM) are ubiquitous organisms commonly found in the environment, such as water, soil, dust and diverse animals. For this reason, their isolation in clinical samples was regarded as contamination or colonization for a long time (Sousa, *et al.*, 2015). Although they are traditionally not considered a major public health issue as with tuberculosis, NTM is of emerging global interest and concern as its pathogenic potential becomes more apparent and diseases caused by NTM are increasingly prevalent (Chee, *et al*., 2012). Incidence rates of 7.2 to 13.6 cases per 100 000 persons were recently reported in Singapore (Tang, *et al*., 2015). In Indonesia, data on the epidemiology of lung disease due to NTM is still very limited. In contrast to *Mycobacterium tuberculosis*, there is no systematic reporting of the disease due to NTM infection either slowly growing NTM o rapidly growing mycobacterium, thus, precise incidence data are lacking (Groote, *et al*., 2006). Ley *et al*., reported that 4% of sputum samples grown in culture from Papua, one of Indonesia island, are nontuberculous mycobacterium (Ley, *et al*., 2015).

In addition, an increasing number of NTM cases from sputum samples examined by culturing in TB Laboratory of Department of Microbiology, Faculty of Medicine UGM through 2013-2014 has demonstrated, i.e.18 and 45 cases per year, respectively (3.4 and 5.0 %) (unpublished data).

Despite this fact, NTM are recognized as etiological agents of healthcare-associated infections (HAIs), which are a major public health concern (Bandeira, 2015). These bacteria are often responsible for respiratory tract colonization; however infections might develop related to medical procedures and disseminated infections in immunocompromised patients. Treatment of these infections is increasingly problematic because of the resistance of clinical isolates to an increasing number of antimicrobial agents and more importantly, due to its ability to grow as a biofilm. Biofilm formation may be a contributing factor to their mode of transmission and their resistance to antimicrobial agents (Martín-de-Hijas, *et al*., 2009).

It has been shown that sliding motility and biofilm-producing capacity were linked among clinical strains of NTM isolates, and seems to be related to the capacity of the strains to cause human disease (Maya-hoyos, *et al.*, 2015). Biofilm formation is a successful survival strategy for these ubiquitous organisms found in the environment. The ability to form biofilm has been associated with chronic bacterial infection (Sousa, *et al.*, 2015). Despite their importance as human pathogens, there are only a few in vitro studies about NTM. Since it is plausible that the ability to establish a biofilm can be associated with the difficulty to eliminate the infection, we investigate the biofilm forming ability of NTM isolates on polystyrene substrate, as well as to check the presence of sliding motility among these strains, and to determine their antibiotic susceptibility pattern.

MATERIALS AND METHODS

A total of 10 clinical isolates of NTM were included in this study. The strains were recovered coincidently from sputum specimen obtained from TB Laboratory, Microbiology Department, Faculty of Medicine Universitas Gadjah Mada Yogyakarta. The strains were identified according to the recommended schemes biochemical assay, i.e niacin and p-nitro benzoic acid (PNB) assay (Tortone, *et al*., 2018). The organism were cultured in either Middlebrook 7H11 agar and/or Middlebrook 7H9 broth containing 10% of oleic acid, albumin, dextrose, and catalase. Before used, the strains were stored in skimmed milk at -20 ^{0}C.

Biofilm formation assay

Biofilm formation was determined as described previously, by seeding 200 µl of 7H9 broth containing $1x10^7$ bacteria in a 96-well sterile flat-bottom tissue-culture treated polystyrene microtiter plates. It was assayed by determining the ability of cells to adhere to the wells as reported previously (O'Toole, *et al*., 2011; Martín-de-Hijas, *et al*., 2009). Briefly, after inoculation, plates were incubated at 37°C for 7 days. The used media then washed off by using PBS to remove the planktonic cell. Afterwhich 50 µl of a 1% crystal violet solution was added to each well (this dye stains the cells but not the polystyrene plates). The plates were incubated at room temperature for 30 min, rinsed vigorously three times with water, blotted on paper towels and scored for biofilm formation. The crystal violet was solubilized in 5% acid isopropanol (5% v/v 1M HCl in 2 propanol) and the absorbance at 570nm wavelength (A570) was determined as described previously (O'Toole, *et al*., 2011; Martín-de-Hijas, *et al*., 2009).

Results are presented as the median value of the triplicates. Biofilm formation was classified as non-adherent, weakly, moderate or strongly adherent. The cut off OD (Odc) for the microtiter plate test was defined as three standard deviation above the mean OD of the negative

control, which used non-biofilm producer strain (*S. epidermidis* ATCC 12228). Isolates were classified as follow : ODc<OD = non adherent, ODc<OD (2xODc) = weakly adherent, (2xODc)<OD<(4xODc) = moderately adherent, and OD> (4XODc) = strongly adherent (Stepanovic, *et al*., 2007; Hassan, *et al*., 2011).

Sliding motility test

Sliding motility properties has been reported are pivotal for pathogenic mycobacterial species for colonizing surfaces in the environment and host under nutrient depletion conditions. One colony of each mycobacteria was put in the centre of a plate of motility medium, consisting of Middlebrook 7H9 with 0.3% agar without supplements. The inoculated media were incubated at 37°C in a 5% CO2 atmosphere during 7 days. The diameter of the bacterial growth was measured at day 7 using a digital caliper. We considered a motility test to be positive if the diameter of the colony was >7 mm, as defined previously (Martín-de-Hijas, *et al*., 2009).

Antimicrobial Susceptibility Testing

The Minimum inhibitory concentrations (MICs) were determined by a broth microdilution technique using Middlebrook 7H9 broth (CLSI, 2014). All tests for each strain were carried out at least in duplicate. The isolates were grown on glass tubes. The inocula were prepared from actively growing bacteria collected from Lowenstein-Jensen slants. The strains were then adjusted with saline to a bacterial cell density of 10^8 CFU/ml (McFarland 0.5 standard), and then adjusted to a 1:20 dilution with Middlebrook 7H9 Supplement (7H9-S) (7H9 broth +10% ADC + 0.5% glycerol). Antibiotics were serially diluted twofold in 100 mL of 7H9-S. The MIC was defined as the lowest drug concentration that prevented bacterial growth indicated by no turbidity shown on the tubes. Each MIC was read on the 3rd to 6th days. The MIC breakpoints of the drugs were interpreted according to the approved guidelines established by the CLSI (CLSI, 2014).

RESULTS AND DISCUSSION

Identification of NTM

Our NTM isolates represented as acid fast bacilli under microscopic observation. In addition, they demonstrated as fast growth mycobacteria on Lowenstein-Jensen slants medium at 37°C within 2 weeks, not inhibited by PNB, and showed a negative niacin test.

Biofilm formation

To determine the ability of NTM isolates to produce biofilm, microtiter plate assay was performed that represent quantitative biofilm assay. Reproducibility of biofilm assay was confirmed in triplicate assay from three independent experiments. Seven out of 10 NTM isolates produced biofilm strongly, while 1 isolate demontrated as moderate biofilm former strain, and the remaining 2 isolates did not produce biofilm on polysterene substrate (Fig.1).

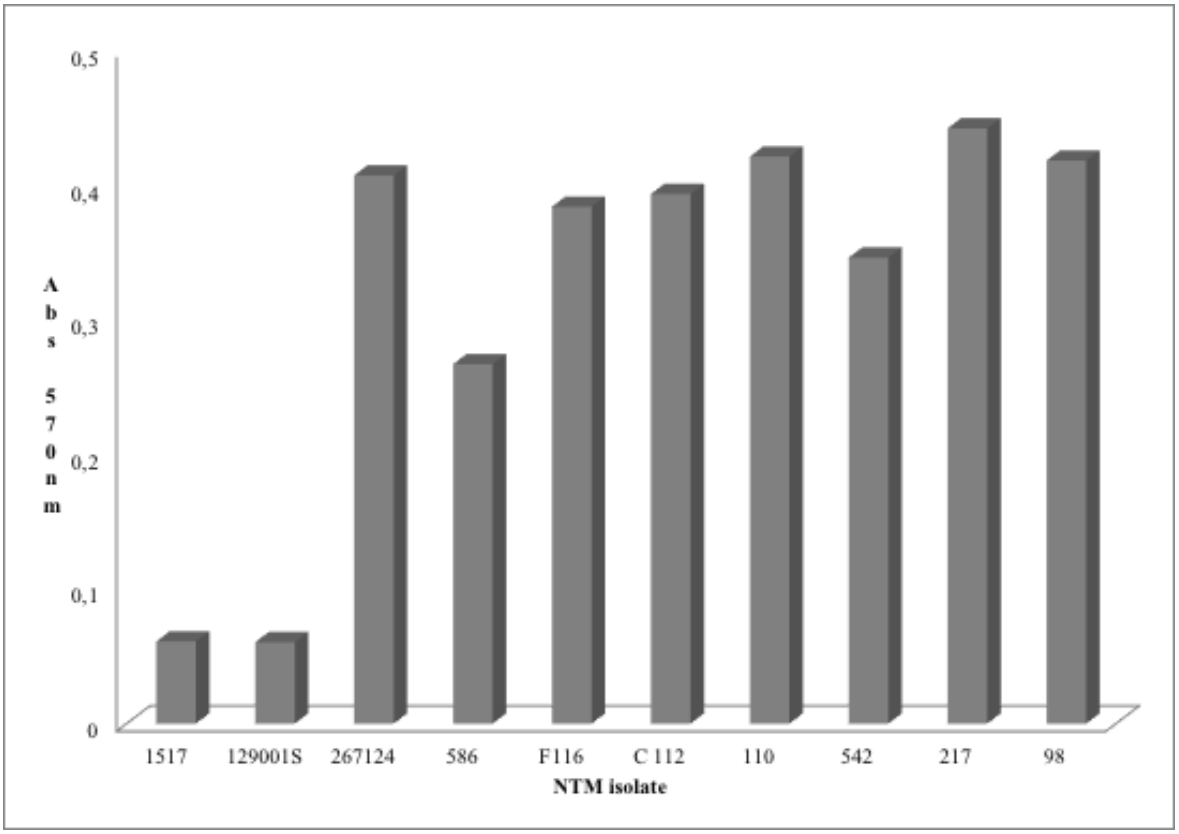

Figure 1. Biofilm formation of 10 NTM isolates performed using microtiter plate assay and stained with 1% crystal violet. Absorbance values were obtained from triplicate of three independent experiments, using at least 8 replications for each experiment.

Sliding motility

Seven out of 10 NTM isolates used in this study showed sliding motility on Middlebrook 7H9-0.3% agar plates at the day 7th (Fig 2). In addition, there were differences in the speed of sliding motility among strains, represented by the length of sliding motility (Table I).

Table I. The size of sliding motility of NTM isolates on Middlebrook 7H9 with 0.3% agar

NTM isolate	Motility	Length (mm)
C112	+	20
F116	+	20
586	+	6
267124	+	22
129001S	-	-
1517	-	-
110	+	22
542	+	16
217	+	18
98	+	18

A significant correlation was found between mycobacterial sliding and biofilm assembly of NTM isolates (Pearson correlation r=0.945). NTM isolate spread on the surface of the semi-solid media generating centrifuge finger-like structures originated at the inoculation point. The maximum length achieved by these structures was of 22 mm, while a different growth pattern was observed for other isolates, which grew around the inoculation point only reaching a maximum length of 6.5 mm.

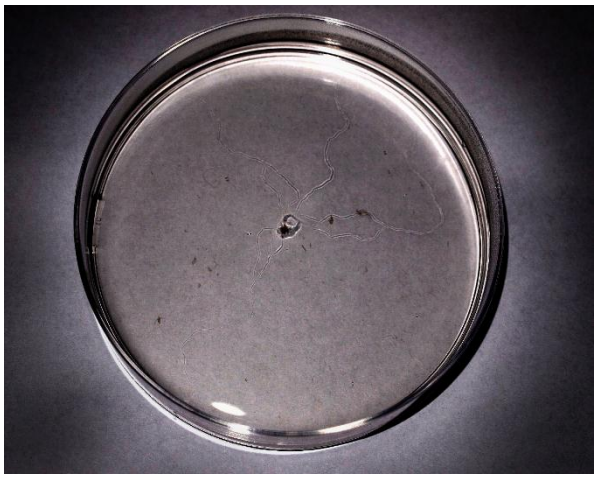

Figure 2. Macroscopic morphology of NTM (isolate C112), spreading on the surface of Middlebrook 7H9-0.3% agar plates, and considered as sliding motility. A single colony was transferred with a toothpick to the center of a agar plate. The plate was sealed with parafilm and incubated at 37°C for 7 days.

Antibiotic Susceptibility assay

Table II. Range of MIC of NTM isolates against 8 antibiotics tested

Antimicrobial agent tested	No. (%) of isolates			MIC range (µg/mL)
	Susceptible	Intermediate	Resistant	
Kanamycin	3 (30)	0 (0)	7 (70)	2.5 - >20
Gentamycin	4 (40)	3 (30)	3 (30)	2 - >64
Levofloxacine	3 (30)	1 (10)	6 (60)	<0.125 - >16
Ofloxacine	3 (30)	1 (10)	6 (60)	0.3 - >80
Amoxycillin	0 (0)	0 (0)	10 (100)	>64
Clarithromycin	5 (50)	4 (40)	1 (10)	0.16 - >10
Ceftriaxone	2 (20)	0 (0)	8 (80)	0.125 - >32
Cotrimoxazole	0 (0)	0 (0)	10 (100)	>64

Table II summarises the antibiotic susceptibility profiles of NTM isolates used in this study. Clarithromycin has been shown as the most effective antibiotic against NTM isolates, which was active against 50% of all isolates tested, while gentamycin, kanamycin, and levofloxacin showed the same level of potency against 40% of all isolates. In addition, ofloxacin, and ceftriaxone were only able to inhibit the growth of NTM isolates, about 30% and 20%, respectively. Furthermore, cotrimoxazole and amoxicillin had poor in vitro activity against NTM

species, since all of NTM tested were resist against both antibiotics. Additionally, antibiotic resistance of NTM isolates correlates significantly with biofilm forming capacity (Pearson correlation r=0.841), as well as with sliding motility properties (Pearson correlation r=0.903).

Recently, we coincidentally recovered NTM isolates from retrieved sputum, that aimed to examine the presence of *Mycobacterium tuberculosis*. All of the sputum specimen obtained from patient suffering respiratory tract infection complain. Our study investigated the biofilm forming ability of 10 NTM isolates, correlated with the sliding motility properties, and analyze their antibiotic susceptibility pattern. Mycobacteria is a nonflagellated microorganisms, able to perform sliding motility which is produced by the expansive forces of the growing bacterial population and cell surface properties that favor reduced friction between the cells and substrate, and the result is the slow movement of a uniform monolayer of cells as a unit (Martín-de-Hijas, *et al*., 2009). It has been known this sliding motility correlates with the presence of glycopeptidolipids and mycolyl-diacylglycerols (Nan, *et al*., 2015). Therefore the ability of mycobacteria to slide over the surface of motility plates is related to biofilm formation, which is in line with our finding, revealed that 7 strong biofilm-former and 1 moderate biofilm-former strains in this study were able to slide on semisolid agar, and significantly correlated with biofilm forming capacity (Pearson correlation r=0.945). In addition, 2 non-adherent NTM isolates did not have ability to perform sliding motility.

Regardless of their conventional characteristic as a non-pathogenic bacteria, non tuberculous properties have been recognized as emerging pathogens in recent years, mainly for immunodeficient individu (Faria, *et al*., 2015). In addition, the identification of NTM as pathogenic agent is probably underestimated because they are not included in the group of organisms that are looked for in many samples. However, as it is found in this study, NTM were shown to be able to form biofilm on surfaces as well as to form sliding motility, and were associated with their involvement as an responsible agent of respiratory infection.

Infections caused by these organisms were investigated in biofilm-related disease, such as device related infections or chronic respiratory tract infections.[1] These data revealed the role of biofilm formation as a virulence factor among NTM. Several studies have analysed biofilm development among NTM isolates (Sousa, *et al.*, 2015; Nan, *et al*., 2015; Williams, *et al*., 2009). In our study, a significant relationship between biofilm development ability and clinical infection has been demonstrated, indicating that biofilm development could be an important pathogenic factor for NTM. This fact is of great importance because biofilms are a well-known form of bacterial resistance against antibiotics, hence, the ability to develop these structures can explain treatment failures of some of these infections (Martín-de-Hijas, *et al*., 2009). Considering the comensal characteristic of non tuberculous mycobacterium, biofilm formation is one factor that, in combination with others, may contribute to development of human infections.

Another interesting result of our study concerns the relation between sliding motility and biofilm development. Sliding motility has been defined as a kind of surface translocation produced by the expansive forces in a growing culture in combination with special surface properties of the cells resulting in reduced friction between cell and substrate (Sousa, *et al.*, 2015; Schorey, *et al*., 2008). This property has been considered to be related to the glycopeptidolipid content of the mycobacterial cell wall and to other aspects of lipid metabolism (Schorey, *et al.*, 2008). There are several reports of a relationship between sliding motility and biofilm development ability in *M. smegmatis* and *M. abscessus* (Sousa, *et al.*, 2015 Schorey, *et al.*, 2008). However, other reports state that this relationship is not uniform (Martín-de-Hijas, *et al*., 2009). Moreover, in our report a statistical relationship among sliding motility, biofilm formation and antibiotic resistance was detected (Pearson correlation, r>0.8).

Most of biofilm-producing NTM isolates observed in this study were resistant to antibiotic tested, which are the drug of choice for NTM according to the guideline from ATS (American Thoracic Society) document delivered the information about the drug choice for NTM infection Griffith, *et al*., 2007; Brown-Elliott, *et al*., 2012). It has been demonstrated that clarithromycin was the most potent antibiotic, followed by gentamycin, kanamycin, levofloxacin which showed the same level of potency; ofloxacin, and ceftriaxone, respectively. Meanwhile, our NTM isolates resisted against cotrimoxazole and amoxicillin. It was suggested that biofilm formation play an important role in antibiotic resistance development of our NTM isolates, as there is significant correlation between these two parameters (Pearson correlation, r=0.841).

NTM isolates used in this study was analysed based on genus level, since species identification was not yet performed. Therefore it will be included as limitation of this study. Meanwhile, species identification is important step before determining empirical antibiotic therapy. However, our finding revealed preliminary data reporting antibiotic susceptibility pattern of NTM causing respiratory infection.

CONCLUSION

Sliding motility and biofilm-producing ability were associated among clinical strains of NTM isolates. Biofilm development of NTM is not evenly present among clinical isolates, however, it seems to be related to their involvement in pathogenesis of human disease. Most our NTM isolates resisted to antibiotic, and clarithromycin was the most potent antibiotic. Further study of NTM susceptibility assay involving more subjects is warranted.

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