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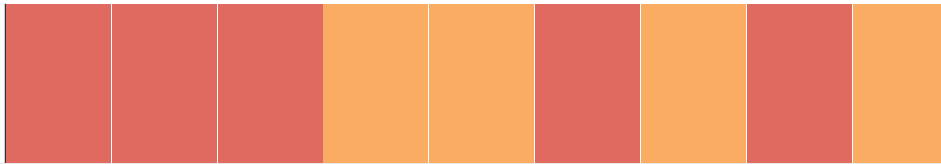
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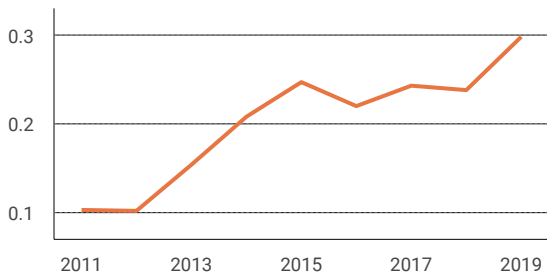
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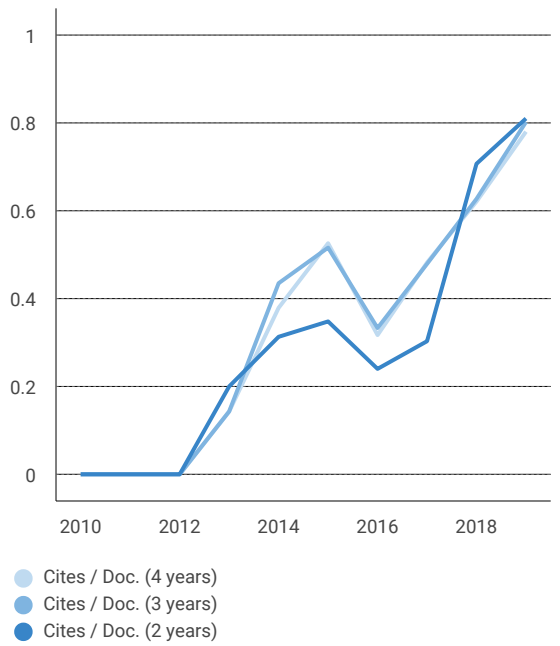
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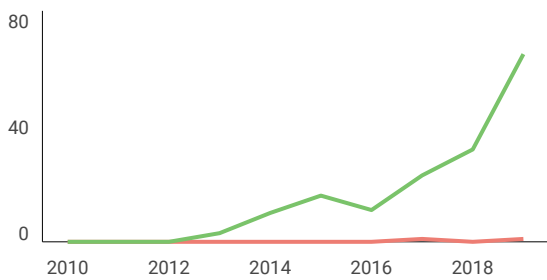
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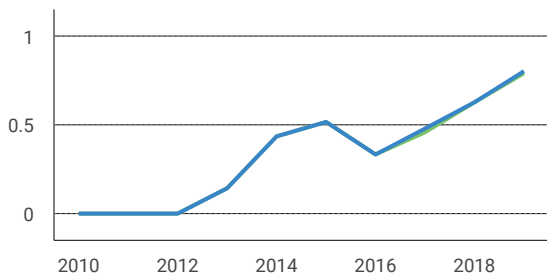
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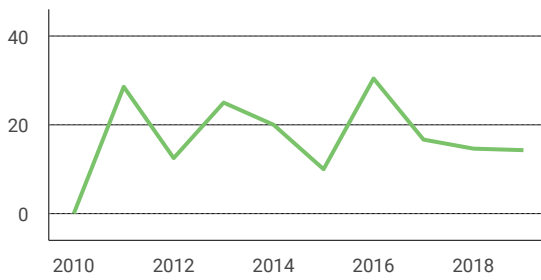
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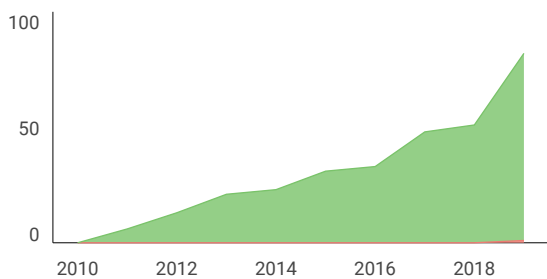
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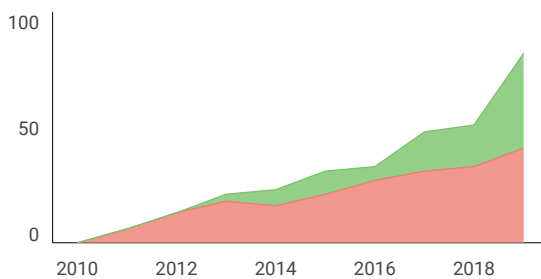
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ACANTHAMOEBA SP.S-11 PHAGOCYTOTIC ACTIVITY ON *MYCOBACTERIUM LEPRAE* IN DIFFERENT NUTRIENT CONDITIONS

Sepling Paling^{1*}, Ratna Wahyuni², Ni'matuzahroh¹, Dwi Winarni¹, Iswahyudi², Linda Astari³, Dinar Adriaty², Indropo Agusni^{2,3}, Shinzo Izumi²

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Abstract

Background: *Mycobacterium leprae* (*M. leprae*) is a pathogenic bacterium that causes leprosy. The presence of *M. leprae* in the environment is supported by microorganisms that act as the new host for *M. leprae*. *Acanthamoeba*'s potential to be a host of *M. leprae* in the environment. *Acanthamoeba* sp. is *Free Living Amoeba* (FLA) that classified as holozoic, saprophytic, and saprozoic. The existence of nutrients in the environment influence *Acanthamoeba* ability to phagocytosis or pinocytosis. This study is aimed to determine *Acanthamoeba* sp.S-11 phagocytic activity to *Mycobacterium leprae* (*M. leprae*) which cultured in non-nutrient media and riched-nutrient media.

Materials and Methods: This research conducted by culturing *Acanthamoeba* sp.S-11 and *M. leprae* on different nutrient media conditions. *M. leprae* intracellular DNA were isolated and amplified by *M. leprae* specific primers through Real Time PCR (Q-PCR).

Result: The results showed that *Acanthamoeba* co-cultured on non-nutrient media were more active to phagocyte *M. leprae* than on rich-nutrient media.

Conclusion: The use of non-nutrient media is recommended to optimize *Acanthamoeba* sp. phagocytic activity to *M. leprae*.

Keywords: *Acanthamoeba* sp., *Mycobacterium leprae*, Nutrient, Phagocytosis, Real Time PCR

Introduction

Mycobacterium leprae (*M. leprae*) is a pathogenic bacterium to humans and causes leprosy. Leprosy is one of the tropical diseases in Indonesia that causes permanent disability in the sufferer if treated late. Leprosy in Indonesia ranks third after Brazil and India in 2014. Leprosy prevalence rate has decreased, but new cases of leprosy continue to emerge even though the World Health Organization (WHO) Multidrug Therapy (MDT) program has been implemented (Ministry of Health of the Republic of Indonesia, 2013).

The emergence of leprosy new cases suspected on environmental factors that play a role in the leprosy transmission, especially in leprosy endemic areas. The big question is how the bacteria *Mycobacterium leprae* (*M. leprae*) can survive in the environment. While as known, *M. leprae* is a bacterium which transmitted through direct contact with the patient and can only live in the cell (intracellular obligate). Based on this, it is possible that *M. leprae* survives in the environment with the support of microorganisms as a new host for *M. leprae*.

Some research results reveal *Acanthamoeba* sp. is the most likely candidate to host *M. leprae* in the environment (Lahiri and Krahenbuhl, 2008; Agusni *et al.*, 2009; Turankar *et al.*, 2012; Wheat *et al.*, 2014). *Acanthamoeba* sp. is a *Free Living Amoeba* (FLA) which is holozoic, saprophyte, and saprozoic as well as live freely in the environment. *Acanthamoeba* is *Free Living Amoeba* (FLA) which live freely in various environments (Khan, 2015) either on the ground (Sawyer, 1989; Tsvetkova *et al.*, 2004; Lorenzo-Morales *et al.*, 2005), various water environments (such as freshwater,

seawater, soft lens cleaning water, and textile industry wastewater), dialysis units, coolers, artificial media (Marciano-Cabral et al., 2000; Maghsood et al., 2005; Astorga et al., 2011; Wink et al., 2011; Siddiqui and Khan 2012; Trabelsi et al., 2012; Geisen et al., 2014; Mahmoudi et al., 2015; Thammaratana et al., 2016), and on air conditioning (AC) (Ithoi et al., 2011; Tanveer et al., 2013).

Acanthamoeba acts as a reservoir for several pathogenic bacteria that are resistant to the phagocytosis mechanism such as *Mycobacterium leprae*, *Mycobacterium avium*, *Mycobacterium abscessus*, *Mycobacterium fortuitum*, *Mycobacterium ulcerans*, *Arcobacter butzleri*, etc. (Gryseels et al., 2012; Medina et al., 2014; Drancourt, 2014; Wheat et al., 2014; Behera et al., 2016)

The nutrients existence in the environment affect *Acanthamoeba* ability to perform phagocytosis or pinocytosis. *Acanthamoeba*'s ability to perform phagocytosis related to its life cycle. The active phase of *Acanthamoeba* is the trophozoite form. In this phase, *Acanthamoeba* is active to perform various physiological activities such as phagocytosis and digest various phagocytosed nutrients sources. While the *Acanthamoeba* silent phase is the cyst form. Phase of trophozoites and cysts according to Paling et al., 2016 influenced by the nutrients presence and incubation time.

This research observed *Acanthamoeba* Sp.S-11 phagocytic activity to co-cultured *M. leprae* cells on Page's Modified Neff Amoeba Saline (PAS) media and Peptone Extract Yeast Glucose (PYG) media with varying incubation time. PAS media is a buffer media that contains no nutrients. While PYG media is the rich nutrients media.

Materials and Methods

This study was conducted at the Leprosy Study Group Laboratory, Institute of Tropical Disease (ITD) Airlangga University Surabaya. Isolat *Acanthamoeba* sp.S-11 and isolat *Mycobacterium leprae* used are the collection of ITD Leprosy Laboratory Airlangga University Surabaya.

Investigations were conducted using factorial experimental design with two replications. Before used *Mycobacterium leprae* (*M. leprae*) suspended with PAS and the cell count calculated by Zhiel Neelsen (ZN) staining and observed microscopically. Cell count is calculated based on the Breed method (Waluyo, 2010). Isolate *Acanthamoeba* sp.S-11 dari -70°C and cultured in 10 mL of PYG media in the 12 mL flask. After incubation for 3 days, *Acanthamoeba* sp.S-11 sub cultured as much 50 $\mu\text{L}/8$ mL media ATCC 712 PYG (Peptone, Yeast extract, Distilled water, 0,4 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0,05 M CaCl_2 , 0,1 Sodium citrate. 2 H_2O , 0,005 M $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0,25 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0,25 M KH_2PO_4 , 2 M glucose) in 12 mL flask and incubated for 2 days. Initial and final number of subculture incubation calculated using hemocytometer which observed microscopically with 200X magnification.

4×10^4 cells/ μL *Mycobacterium leprae* and 2×10^3 cells/ μL *Acanthamoeba* sp.S-11 co-cultured each in 1 mL of media PAS (NaCl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, Na_2HPO_4 , KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Aquades) and PYG, then incubated for 0, 7, 14, 21, and 28 days at temperature 30°C . Co-culture centrifuged at low speed 5000g for 10 minutes. After that, the supernatant and pellet formed are separated. The pellet is suspended in 10x PAS volume, then spread made on the object through Ziehl Neelsen (ZN) staining by fast fixation of fire, then put solution of carbol fuchsin 0,3 % to cover the entire preparation surface. The next step is the preparation incubated for 30 minutes. Then the preparations are rinsed in succession with running water and alcoholic acid (HCl 3 % in alcohol 70 %) until the red fuchsin disappears. The preparation rinsed with running water and then stained with methylene blue 0,3 % until covering the entire surface for 1-2 minutes. Finally, the preparation rinsed with running water and kept to dry. Observations were recorded using 1000x magnification microscope.

Acanthamoeba sp.S-11 phagocytosis activity confirmed with Real Time PCR (qPCR) check by observing the amount of copy DNA *M. leprae* concentration. The first step is to isolate DNA *M. leprae* as template for qPCR. DNA *M. leprae* were isolated using Qiagen miniprep kit which is based on its manual's instructions. Once DNA obtained, then performed detection of DNA *M. leprae* by using qPCR (Amersham Biosystem 7300). The main component needed in detecting *M. leprae* DNA using qPCR are a pair of specific primers *M. leprae*, the primary forward Lp33F (5'-AGGTGTCGGCGTGGTCAAT-3'), and primary reverse Lp33R (5'-AATGGTGCAAGGGATAACATCAG-3'), and require SyBrGreen Reagent. The detection performed on the operational standard of the qPCR user manual. The standard concentrated *M. leprae* series is included in each reaction for the quantity results consistency. The standard used are 100 pg/ μL , 10 pg/ μL , 1 pg/ μL , 0,1 pg/ μL , dan 0,01 pg/ μL .

Results

Observation of *Acanthamoeba* sp.S-11 phagocytosis activity to *M. leprae* shown by the following figure:

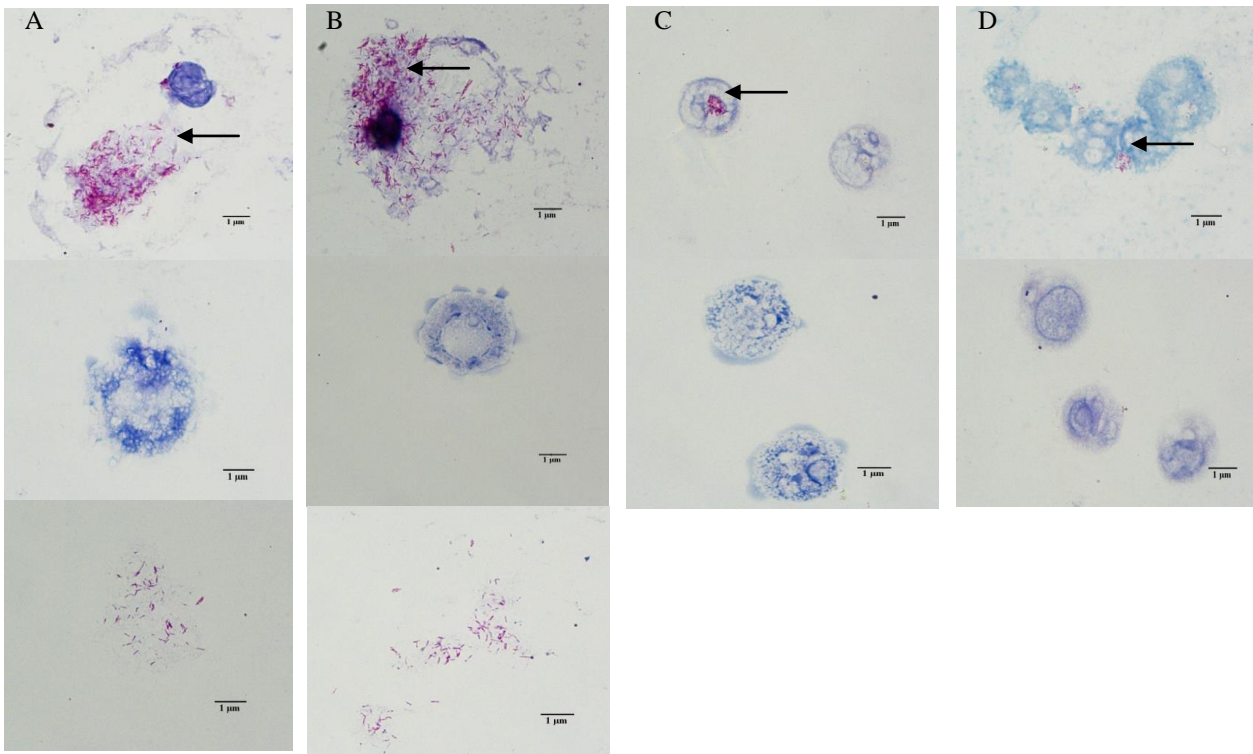


Figure 1: *Acanthamoeba* sp.S-11 phagocytosis activity to *M. leprae*. The black arrow shows *M. Leprae* cells that successfully phagocytosed by *Acanthamoeba* sp.S-11. A – B : Co-Culture on PAS media, C – D : Co-Culture on PAS media PYG, E – F : *Acanthamoeba* sp.S-11 control on PAS media, G – H : *Acanthamoeba* sp.S-11 control on PAS media PYG, I : *M. leprae* control on PAS media, J : *M. leprae* control on PAS media. ZN staining.

Confirmation of *Acanthamoeba* sp.S-11 phagocytosis activity, The real time PCR (qPCR) examination was performed. The results of qPCR examination can be seen in the following figure:

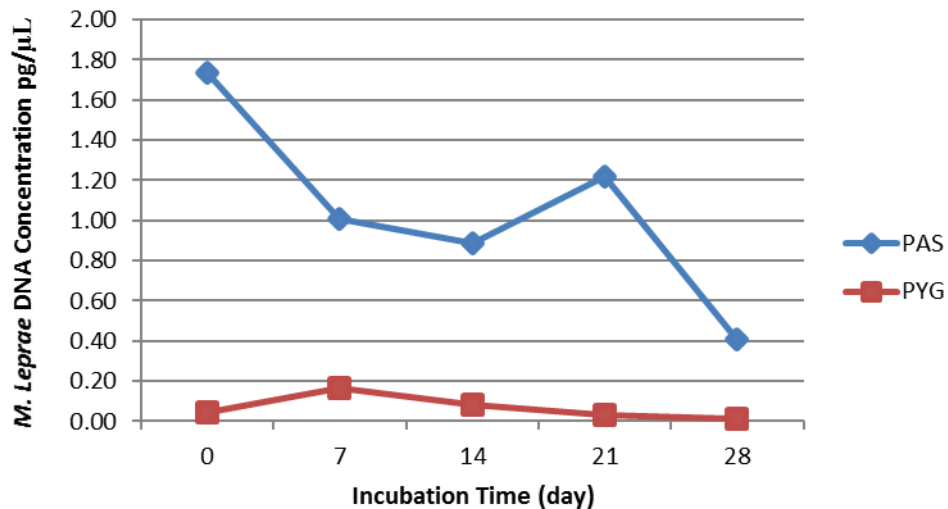


Figure 2: Concentration of *M. leprae* Intracellular DNA copy after Real Time PCR examination.

Discussion

Based on figure 1, *Acanthamoeba* sp.S-11 phagocytosis activity to *M. leprae* that co-cultured on PAS media is much more effective than PYG media. Because PAS media used as co-culture media is a non-nutrient media thus forcing *Acanthamoeba* sp. S-11 to phagocytosis to *M. leprae* cells. *Acanthamoeba* sp.S-11 suppose that *M. leprae* as the nutrients source used as the energy source to perform its activities. Unlike PYG media that used as co-culture media showed *Acanthamoeba* sp.S-11 was not able to phagocytosis optimally to *M. leprae* cells around. *Acanthamoeba* sp. The S-11 prefers to pinocytosis to the nutrients contained in the PYG media. This supported by the qPCR results that shows the amount of *M. leprae* intraseluler copy DNA concentration that co-cultured on PAS media are higher than co-culture on PYG media.

The result of qPCR in figure 2 shows the growth pattern of *M. leprae* co-culture on PAS media and co-culture on PYG media. *M. leprae* cell grows on the 7th day of co-culture on PYG media and grows on the 21st day of co-culture on PAS media. Thus it can be said that *M. leprae* cells have the ability to multiply in *Acanthamoeba* sp. S-11 cells. However, further research on the mechanism of growth of cultured *M. leprae* cells with *Acanthamoeba* sp.S-11 is required.

Pinocytosis is the process of nutrients absorption in the form of small molecules or small particles such as amino acids, ions, and glucose through *Acanthamoeba* cell membranes by cytoplasmic invagination that curved inward to form the pinocytosis bubble called pinosomes. Pinocytosis performed by *Acanthamoeba* sp.S-11 caused by the nature of nutrients molecules contained in the PYG media which always moves to occupy the entire space in the media, so *Acanthamoeba* Sp.S-11 does not need to perform an active movement to get the nutrients since directly those nutrient molecules will be attached themselves to *Acanthamoeba* sp.S-11 cell membrane, due to the presence of protein receptors and ions around and inside the cell resulting in invagination mechanism in the *Acanthamoeba* Sp.S-11 cell membrane until the molecules absorbed in *Acanthamoeba* sp.S-11 cytoplasm. In addition, *Acanthamoeba* prefers to pinocytosis since the small molecules such as amino acids, glucose, and ions contained in PYG media are the simple-form molecule making it easily absorbed and digested by *Acanthamoeba* sp.S-11 cells compared to solid particles with still very complex shape, such as bacteria, algae, bits of plants and animals that have died, or other solids particles either detrimental or beneficial to the *Acanthamoeba* cells thus requiring a certain mechanism to digest the solids particles into simpler molecular components. Digestive mechanism or so-called degranulation done through a fusion of the phagosome and enzyme produced by lysosome. The lysosome is a membrane organelle that originates from the Golgi body and contains acid and hydrolytic enzymes (Ruppert *et al.*, 2004). PYG media is an artificial medium that can support the growth of *Acanthamoeba* (Hereder-Bermejo *et al.*, 2012, Peretz *et al.*, 2015) So when PYG media used as co-culture medium, *Acanthamoeba* cells will multiply rapidly.

Conclusion

The nutrients presence in the environment can inhibit *Acanthamoeba* sp.S-11 phagocytosis activity to *M. Leprae*. Meanwhile, the absence of nutrients in the environment will increase the *Acanthamoeba* sp.S-11 phagocytosis activity to *M. Leprae*.

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