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The detection of Mycobacterium tuberculosis using in vitro granuloma tuberculosis model intracellular specimens

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ABSTRACT
The Granulomas are a group of innate and adaptive immune cells that are highly organized, and during tuberculosis infection caused by the bacterium *Mycobacterium tuberculosis.* Estimated as many as 1.6 million cases of death in 2017 according to World Health Organization. Tuberculosis ranks in the top 10 causes of death worldwide and it is predicted that more than 1.7 billion people (around 25 percent of the world's population) are estimated to be infected by *Mycobacterium tuberculosis*. The purpose of this study was to confirm the Mtb by examining a new method by modifying Ziehl-Neelsen stain using an intracellular specimens for tuberculous granuloma in vitro. This study used the isolate of the bacterium *Mycobacterium tuberculosis H37Rv* obtained from the Microbiology Laboratory of the Institute of Tropical Disease Universitas Airlangga, Surabayaa, using Peripheral Blood Mononuclear Cells to make model granuloma tuberculosis. The result is Ziehl-Neelsen staining was modified, it was found that the Mycobacterium tuberculosis H37Rv as detection of bacterial confirmation is said to be successful.

INTRODUCTION

Tuberculosis (TB) is a significant and substantial burden of global disease that is very interesting in development and research.[1] Although it is a classic and curable disease, TB remains the leading cause of death in the world from infectious agents, more than HIV/ AIDS. Estimated as many as 1.6 million cases of death in 2017 according to WHO (World Health Organization). Tuberculosis ranks in the top 10 causes of death worldwide and it is predicted that more than 1.7 billion people (around 25 percent of the world's population) are estimated to be infected by Mycobacterium tuberculosis.[2] The global incidence of TB peaked around 2003 and appears to be declining, with a deceleration of around 2 per cent per year. Ending the TB epidemic by 2030 is among the health targets of the newly adopted Sustainable Development Goals. WHO has gone one-step further and set a 2035 target of 95% reduction in deaths and a 90% decline in TB incidence similar to current levels in low TB incidence countries today.[2] Whereas in Indonesia as many as 1,000,000 TB cases and as many as 110,000 cases are estimated to die each year so that the main problems of TB mortality and morbidity are difficult to

eliminate and eradicate.[3] The conventional detection of MTB in tuberculosis-endemic countries is through microscopic examination for acid-fast bacilli (AFB), usually by Ziehl–Neelsen (ZN) staining, in smears of respiratory specimens, most commonly sputum.[4] So far, the gold standard inspection is the conventional method, namely Ziehl-Neelsen (ZN) staining, but the results are less specific.[5]

The Granulomas are a group of innate and adaptive immune cells that are highly organized, and during tuberculosis infection caused by the bacterium *Mycobacterium tuberculosis (Mtb).*[6] When the host cell of the immune system goes down, it will affect the Mtb bacteria to infect and enter the cells in the lung organs. The existence of this granuloma serves as a protector and locks the Mtb bacteria from spreading and infecting so that the granuloma is the earliest natural defense system. In the composition of the granuloma there are various immune cells clustered together to form granulomas, one of which is macrophage cells. we know that macrophage cells are natural defense cells that function to phagocytose antigens in the body. therefore macrophage cells along with other immune cells unite to form granuloma clusters so that the Mtb bacteria do not spread and infect sufferers and do not get worse.

The purpose of this study was to confirm the Mtb by examining a new method by modifying Ziehl-Neelsen (ZN) stain using an intracellular specimens for granuloma tuberculosis in vitro. A Granuloma is the best defense mechanism against the Mtb bacteria so that in the early phase it can be immediately detected later it will affect the initial diagnosis and the best treatment.[4]

MATERIALS AND METHODS

This type of research is a pure experiment with the treatment of making granuloma tuberculosis models similar to humans. The purpose of this study is to determine the presence or absence of Mtb bacteria in the Tb granuloma model in vitro using the modified Ziehl-Neelsen (ZN) staining method. This examination was carried out as many as 2 specimens with four repetitions with a systematic sampling method so that the total specimen was obtained as many as 8 specimens. The number of specimens was 8 specimens carried out by the systematic sampling method.

A. Isolation of *Mycobacterium tuberculosis H37Rv*

This study used the isolate of the bacterium *Mycobacterium tuberculosis H37Rv* obtained from the Microbiology Laboratory of the Institute of Tropical Disease Universitas Airlangga, Surabaya.

B. Peripheral Blood Mononuclear Cells (PBMC)

Centrifuge a specimen of blood cells for 10 minutes at 2000 rpm, then the blood plasma is transferred to a 1.5 ml tube (store at -80º C), after storage, 3 ml PBS is added once to the conical tube containing the specimen. Then add 5 ml of histopague to the new conical tube 3 times and carefully move the 4th time. Then centrifuged for 30 minutes at 1700 rpm. Discard the top liquid (plasma) and move the middle liquid (buffy coat) into a new conical tube. PBS was added 1x to 15 ml into a conical tube containing a middle liquid (buffy coat) and then centrifuged for 5 minutes at 1500 rpm. Then the top liquid (plasma) is removed and added PBS 1x to 15 ml then centrifuged 1300 rpm for 5 minutes. Then the liquid is removed and added 600 µl PBS 1x and mixed using a pipette. Then transfer it to a 1.5 ml tube and store and keep it at -30º C.

C. Model Granuloma Tuberculosis

PBMC media that had been cultured was added with Roswell Park Memorial Institute (RPMI) and then inoculated with *Mycobacterium tuberculosis H37Rv* for 5 days.

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After that, 10 µl is harvested and placed in a glass object polielisin and observed under a microscope to observe the morphological shape of granuloma TB.

D. The Modification of Ziehl – Neelsen (ZN) Staining

An intracellular specimen for granuloma tuberculosis in vitro was taken with a 10 µl micropipette placed in a glass object polielisin then done painting. Preparations flooded with carbol fuchsin 0.3% solution to the full. Then it is heated but don't boil it. Then let stand for 5 minutes, wash with running water. The next step is decolorization with 3% acid alcohol (alcohol + 3% HCl concentration) until the fuchsin red color disappears, wash with water. Then the latter is flooded with a 0.3% Methylen Blue solution for 10-20 seconds, rinse with running water after that dry and then the preparations are ready to be observed under a microscope at 1000x magnification.

RESULTS AND DISCUSSION

The results of the examination refer to the International Union Against Tuberculosis and Lung Disease (IUATLD) and World Health Organization (WHO).[7]

Table 1. Acid Fast Bacilli (AFB) Result of Test Using The Modification Of Ziehl-Neelsen (ZN) Staining

The Modification Of Ziehl-Neelsen (ZN) Staining	Result	Acid Fast Bacilli (AFB) / Field
Specimen 1	+	1-9 AFB/ 100 field
Specimen 2	+	1-9 AFB/ 100 field
Specimen 3	+	1-9 AFB/ 100 field
Specimen 4	+	1-9 AFB/ 100 field
Specimen 5	+	1-9 AFB/ 100 field
Specimen 6	+	1-9 AFB/ 100 field
Specimen 7	+	1-9 AFB/ 100 field
Specimen 8	+	1-9 AFB/ 100 field

Grading of AFB density on the slide was as follows: negative (0 AFB/300 fields), scanty (1-8 AFB/300 fields), 1+ (3-9 AFB/100 fields), 2+ (1-9 AFB/10 field), 3+ (1-9 AFB/ field), 4+ (≥ 10 AFB/ field).[8] Interpretation of results from Specimens 1, 2, 3, 4, 5, 6, 7, and 8 obtained observations of AFB density grading on a slight slide (1-8 AFB / 300 fields) indicating the presence of Mtb bacteria in the ganuloma specimen in vitro. The results above show that there is fast acid bacilli / field even though only 1-2 are found. This identifies that in the granuloma there are live or dormant Mtb bacteria. Modified ZN method is more sensitive and specific than the conventional ZN method. Furthermore, it is simple and easy. The modified ZN method greatly improves the diagnostic value of microscopic examination, and it is comparable to fluorescence technique in identifying patients with low-density bacilli.[9]

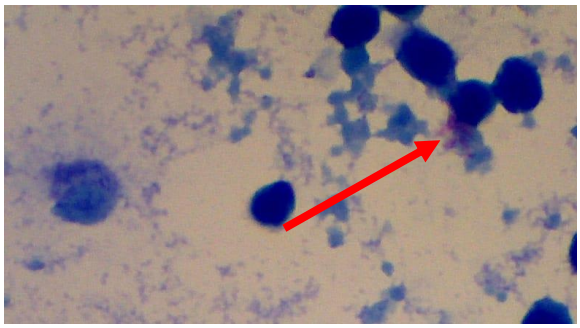

Figure 1. *Mycobacterium tuberculosis H37Rv* bacteria is seen to be covered in granulomas

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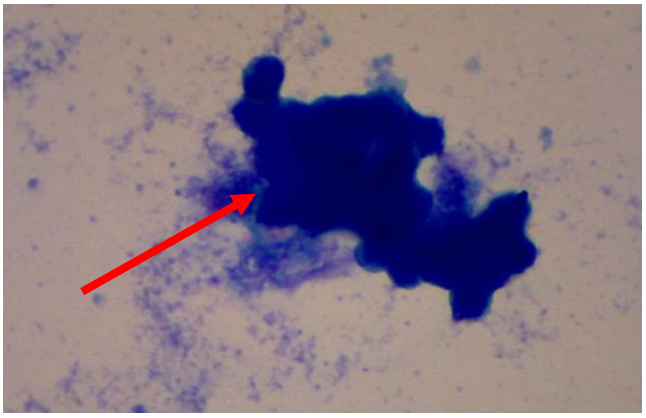

Figure 2. *Mycobacterium tuberculosis H37Rv* bacteria looks red lancet closed granuloma hordes

After Ziehl-Neelsen (ZN) staining was modified, it was found that the Mycobacterium tuberculosis H37Rv was seen in Figure 1 and Figure 2, which appeared marked by a red arrow that red-colored bacteria were surrounded by a group of cells called granulomas, the color was somewhat faint because it was covered in granuloma so that the test was shown with red arrows. early detection of bacterial confirmation is said to be successful. The method of modification of ZN is still not used in clinical examination. Mostly to see the severity of TB sufferers using conventional ZN staining. New highly sensitive, affordable diagnostic methods are urgently required to improve TB early phase outcomes.[6] Detection of bead bacilli (the more common observed in the necrotic zone) by Ziehl-Neelsen stain the intracel of granulomas shows a relationship between tissue reactions and mycobacterial infection. However, the Ziehl Neelsen stain has a relatively low sensitivity using sputum specimens to detect Mtb for this infection. Considering the mechanism of Ziehl-Neelsen staining and its relatively low sensitivity and specificity, this staining technique has limited and insufficient diagnostic value. Ability for pathophysiological assessment of M.tb antigen in tissue. Look for mycobacterial antigens using IHC is a well-known technique with special applications in research project.[10] Key factors for the control of tuberculosis are a rapid diagnosis, effective treatment, and preventing transmission with scanning. Te gold standard method for laboratory diagnosis of tuberculosis is culture method. However, getting the results takes a few weeks long. Moreover, sensitivity of the microscopic examination following the Ziehl–Neelsen staining is low. For preventing tuberculosis spread, it is critical to diagnose the disease within 1 or 2 days and start the treatment immediately. Terefore, there is a need for quick, sensitive, reliable, point-of-care, and economical methods for the laboratory diagnosis of tuberculosis. Whereas for after cell intracelluler is harvested the granuloma morphology observed under an inverted microscope with a magnification of 400x can be seen that dense and clustered aggregation can be seen in Figure 3 on the yellow arrows so that confirmation of the granuloma TB model for 5 days can be said to be successful.[11]

Identification of Mycobacterium tuberculosis in Granuloma in vitro stained with Hematoxylin and Eosin (H and E) stain and Zeihl-Neelsen stain. The advantage of this method is that it is of low cost, simple in technique, and rapid.[12] The Ziehl Neelsen's staining modification test. This examination aims to confirm the presence or absence of bacteria Mycobacterium tuberculosis in an in vitro granuloma model. After BTA was stained with a variation of the day observed under a binocular microscope with a magnification of 1000x, the results were obtained in the presence of bacteria Mycobacterium tuberculosis is a red langsit surrounded by hordes various cells that make up the granuloma. Fixed smears were stained with Papanicolaou stain and air dried smears were stained with Giemsa and Ziehl-Neelsen-stain. Smear study showed scant cellularity, cells composed of neutrophils, lymphocytes, plasma cells and macrophages.

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Background was necrotic. Giemsa stained smears showed, in addition to cells mentioned, negatively stained ghostly rod shaped structures in the cytoplasm of macrophages. Ziehl-Neelsen-stain showed numerous acid fast bacilli. Both the aspirates were signed out as tuberculous lymphadenitis.[13]

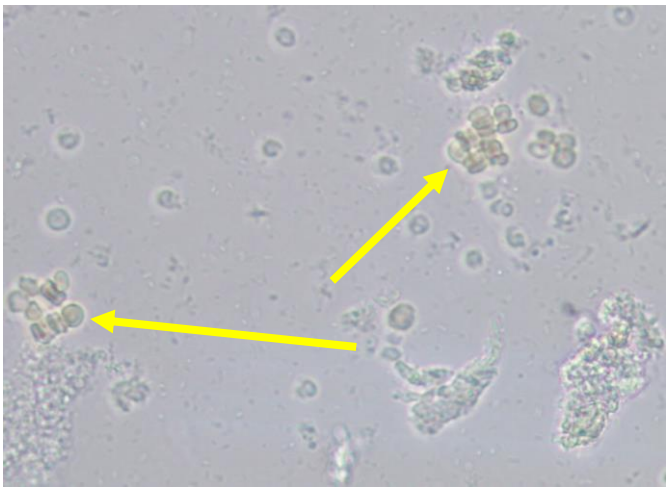

Figure 3. Model form of Granuloma Tuberculosis In Vitro

The main cause of tuberculosis is the bacterial pathogen Mtb is a global health problem. When this pathogen infects the lungs and induces host cells in the tissue, remodeling creates a chronic inflammatory lesion called granuloma. The structure that forms these granulomas serves as the control and development of disease.[14] This granuloma plays a dual role as a balance as preventing the spread of infection in host cells as well as protecting the spread of M.tb pathogens as the body's immune response.[15] This modification of Ziehl-Neelsen (ZN) staining gives an idea of the severity of Tb disease sufferers by morphological form coordination and confirmation of how fast the development of M.tb bacteria in infecting host cells especially in the defense mechanism of the body during the initial infected phase is latent Tb.[4] So, it is expected to be appropriate in the diagnosis and treatment of drug administration will be right on target. Granulomas are inflammatory lesions that contribute to bacterial containment, but their progressive formation can cause lung organ damage. Mouse BCG sarcoid granulomas are similar in cell composition and cytokine environment to sarcoid granulomas in human. The dominant cell type in these lesions are monocytes and monocyte-derived macrophages, but dendritic cells, T cells, and B cells are also present. These inflammatory effector cells have a limited lifespan, and so continuous cell replacement and granuloma reformation is needed to maintain granulomas long-term. In fact, more than a third of monocytes are replaced within in a week in a granuloma transplantation model, and dendritic cells and lymphocytes have a similar turnover rate. The recruitment of granuloma-forming cells is regulated by many functionally overlapping chemokines and cytokines after infection, including MCP-1, MIP-1a, MIP-1b, RANTES, and IL-8, to name a few [1] and Interleukin-6 (IL-6) is one of the main mediators of inflammation in immune response initiated by infection.[16] Infection occurs when the inhaled Mycobacterium tuberculosis are phagocytized by resident lung alveolar macrophages. Infected cells recruit mononuclear phagocytes to the infection site, forming a nascent granuloma.[17] During the subclinical stage of infection, the granuloma provides the immune environment required for the containment of bacteria.[18] Mycobacterium tuberculosis-specific T cells are crucial for the granuloma maturation, maintenance, and control of the bacterial spread.[19] However, if due to impaired immunity the integrity of the granuloma is lost, reactivation of

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Mycobacterium tuberculosis leads to the destruction of the lung structure and to the transmission of Mycobacterium tuberculosis to other human.[20]

The initial stage of granuloma formation occurs when monocyte and macrophage cell aggregation occurs, this is due to the ability of Mycobacterium tuberculsosis bacteria to secrete a compound called ESAT-6.[21] The study said that at the time of granuloma formation there was a role for the neutrophil cells that live in experimental animals carried out in vivo in zebrafish. Cytokine IL-6 is fully involved in the differentiation process of macrophage and cytotoxic T lymphocytes. Then CD + T lymphocytes were induced IFN-γ by cytokine IL-12 in Th1 effectors. CD4 + and CD8 + T lymphocytes will be induced by immune cells such as lymphocytes, monocytes, and neutrophils when infected, directed by cytokines which function to strengthen macrophage antimicrobial phagocytosis. Potential ESAT-6 protein filtrate culture 10 (CFP-10) in an experimental animal model after infection with Mycobacterium tuberculosis is a spectacular finding as a candidate for the TB vaccine because T lymphocytes are induced by immune cells.[22] Difficulty in Mtb infection when using a model in human lung biopsy specimens when observing granuloma formation, with this study it is hoped to be able to study and understand the dynamic processes in Mtb granulomas so that using intracellular TB granuloma specimens is the first step applied to the treatment and potential infection caused by Mtb is not getting worse.[23] In vitro tb granuloma research has weaknesses in the natural micro-tissue structure in the human body, but it is sufficient to provide insight into the interaction of host cells with pathogens of Mtb bacteria, how when it infects until the formation of body protection, namely forming granulomas in humans.

CONCLUSIONS

The results of this study can be concluded that the modification of Ziehl-Neelsen (ZN) staining in the detection of Mtb bacteria in the granuloma model in vitro is confirmed so that it can be applied. In the future, this research will lead to granuloma in vivo using the mice model.

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