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Identification of Mycobacterium Tuberculosis in Active Smokers with Ziehl–Neelsen Staining Method

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ABSTRACT

Tuberculosis is still a health problem for the people of Indonesia, especially for active smokers. There are many risk factors that can cause tuberculosis in active smokers including age at starting smoking, number of cigarettes consumed per day, and how long smoking has been done. The purpose of this study was to determine the results of the identification of mycobacterium tuberculosis in active smokers with the Ziehl–Neelsen staining acid resistance test method. This type of research is a descriptive analytic study that aims to determine the results of the identification of Mycobacterium tuberculosis in active smokers. The Ziehl–Neelsen staining acid resistance test was conducted at the Tourism Hospital Laboratory of the University of East Indonesia Makassar on September 22 to October 6, 2022. The population in the study this is 10 people. The sample was determined using a total sampling technique so that 10 samples were obtained. The research variables were active smokers. The data obtained are presented in the form of tables and narratives. The conclusion after laboratory testing using the Ziehl–Neelsen staining method was found that 1 out of 10 samples of active smokers was identified as Mycobacterium Tuberculosis**.**

Keywords:
Active Smoker, Ziehl–Neelsen, Mycobacterium Tuberculosis

1. INTRODUCTION

According to the World Health Organization (WHO) in the Global Tuberculosis Report 2020, Indonesia is the 2nd highest country with the largest number of TB cases in the world. The number of TB cases in the world by 55% are in five countries, namely India, Indonesia, China, the Philippines and Pakistan. The incidence rate for tuberculosis patients in Indonesia in 2018 was 316 per 100,000 population and the death rate for tuberculosis patients was 40 per 100,000 population. In 2019 the number of tuberculosis cases found was 543,874 cases, a decrease when compared to all tuberculosis cases found in 2018 which amounted to 566,623 cases. [1]

According to the Indonesian Ministry of Health (2017) 95% of tuberculosis patients are in developing countries and 75% of tuberculosis sufferers are in the productive age group (15-90 years) with low socioeconomic levels. The prevalence of tuberculosis (TB) in Indonesia, especially South Sulawesi is in 7th position which has a high prevalence rate in eastern Indonesia with the number of tuberculosis patients recorded from January 2018 to January 2019 as many as 23,427 people.[2]

Research presented by Meiliza et al 2019 states that the risk factors that can cause tuberculosis in active smokers include age at starting smoking, number of cigarettes consumed per day, and how long smoking has been done.

Smoking and tuberculosis (TB) are two major health problems in the world. Based on WHO data, Indonesia is the country with the 3rd largest cigarette consumption after China and India, followed by Russia and America [3]. Tuberculosis is the predominant infectious cause of mortality today, killing 3 million people annually.

Smoking is one of the bad habits that can lead to dependence such as dependence on certain drugs. The number of smokers in the world continues to increase from year to year, and currently WHO estimates

that there are around 1.1 billion smokers in the world. Tuberculosis (TB) is caused by Mycobacterium tuberculosis, which enters the body through the lungs and which, expectorated in sputum, can be seen as clusters or individually in stained sputum smears under a microscope. Two staining methods are used: auramine, which requires fluorescence microscopy; and Ziehl-Neelsen (ZN), which requires bright-field microscopy. The microscope is the cornerstone of TB screening in low- and middle-income countries (Steingart et al. 2006), as it is a relatively cheap piece of equipment. Positive sputum smear detection using a microscope makes up the largest fraction of total TB detections (WHO 2007). We concentrate on TB screening using a bright-field microscope, as this is the preferred method in developing countries, due to the low cost and ease of equipment maintenance compared to fluorescence microscopy; low-cost fluorescent microscopes have, however, recently become available (Hanscheid 2008). TB screening with a conventional microscope has variable sensitivity; values between 20% and 60% have been reported in some studies, while sensitivity above 80% has been reported in others (Steingart et al. 2006). The WHO recommends that a slide be declared TB-negative if no bacilli are seen in 100 high-power microscope view fields (WHO 2003); a negative diagnosis, however, requires the examination of two or three negative smears. A technician normally examines between 30 and 40 smears in a day (Toman 2004) and may diagnose a positive slide as negative because of sparseness of bacilli, or because too few fields have been examined. In addition, the workload in high-prevalence countries leads to technician fatigue, diminishing the quality of microscopy (van Deun 2002).[4]

Smoking tends to cause chronic cough which is the main symptom of tuberculosis, cough in smokers can decrease the specificity and therefore, predict it is lower. The diagnosis of tuberculosis can be delayed which can result in a higher probability of relapse. Smoking is not only a cause of comorbid diseases, such as chronic bronchitis, COPD, emphysema, and coronary heart disease, but is also a facility for the progression of tuberculosis infection, but smoking can also cause damage to lung function thereby worsening tuberculosis itself. Smoking causes excessive iron deposits in lung tissue macrophages as a direct effect of damage to immune response cells to fight microorganisms [5]

Research by Risa 2011, shows tuberculosis and smoking are two very significant public health problems and interrelated smoking can interfere with the effectiveness of some respiratory defense mechanisms. The results of cigarette smoke can stimulate mucosal formation and reduce ciliary movement resulting in mucosal accumulation and an increased risk of bacterial growth including Mycobacterium Tuberculosis which can cause infection [1]

Based on research conducted by O'Leary et al conducted in Dublin, Ireland, it was found that in the lung compartment of the smokers group, there was an increase in the number of alveolar macrophages, which indicated a decrease in specific immunity, which would decrease the immune response to Mycobacterium Tuberculosis (MTB) infection. One theory states that smoking can cause structural changes in Mycobacterium Tuberculosis (MTB) exposure. Lung fluid production function will increase both for normal people and those affected by tuberculosis. Smoking also causes changes in natural and acquired cell immunity which can affect macrophages and leukocytes.

A cross sectional study was conducted at Gondar University Referral Hospital, Northwest Ethiopia. Three sputum specimens were collected from consecutive TB suspects. Direct and concentrated sputum smears were air-dried, heat-fixed and stained by auramine O and Ziehl-Neelsen staining techniques respectively. The stained slides were examined for acid fast bacilli using direct Fluorescent Microscopy and Ziehl-Neelsen concentration techniques.[6]

To reveal the presence of intracellular M. tuberculosis and improve the detection of extracellular M. tuberculosis from a small volume of CSF specimens, we developed a highly efficient Ziehl-Neelsen stain involving the use of only 0.5-ml CSF specimens from TBM cases. The formed elements in the CSF, including the bacilli and cells, were compactly collected onto the slides by cytospinning followed by staining with acid-fast dyes containing the detergent Triton X-100. Using this modified staining method, AFB can be clearly revealed within the immune cells, and the detection rate of extracellular AFB was significantly improved as well.[7]

Microbiological examination using the Ziehl-Neelsen staining technique found that the sensitivity and specificity of staining were 77.8% and 100%, respectively. Pulmonary TB is more common in male sex 72.7%, age less than 50 years 81.8%, last education junior high school 63.6%, and work as a driver 45.5%. The avidin-biotin complex peroxidase (ABC-P) method was used to detect Mycobacterium bovis, and the results were compared with those obtained by the Ziehl-Neelsen (ZN) technique. Lesions were examined

from 18 cows and 24 goats with tuberculosis. All animals showed pulmonary lesions, which in the cattle were mainly minor (i.e. primary complex) but in the goats were sometimes minor and sometimes severe. Microscopically, typical granulomas were seen in the lungs and lymph nodes, with central necrosis and the cellular components of chronic inflammation, but mycobacteria were either seen in small numbers or were not detectable. The ABC-P technique was more sensitive than the ZN method, as shown by the number of positive animals detected, the intensity of staining, and the successful use of low magnification. Caprine lesions, although more severe than bovine lesions, appeared to contain fewer organisms. [8].

The cornerstones of diagnosis rest on microscopy of specimens using auramine and Ziehl-Neelsen stains followed by culture on Lowenstein-Jensen or alternative media. The long generation time of Mycobacterium tuberculosis means 2–8 weeks usually elapse before a result is available to the clinician. [9]

The presentation of tuberculosis is variable depending on the severity of the infection, the age of the patient, whether the infection is primary or secondary, and whether the manifestations are due to inhalation of organisms or hematogenous dissemination. A definitive diagnosis is made by culture of the organism; spontaneously expectorated sputum is the most suitable specimen for diagnosing pulmonary tuberculosis. Diagnosis of extrapulmonary tuberculosis frequently requires tissue biopsy. The classic staining method for demonstrating tubercle bacilli is the Ziehl-Neelsen technique. [10] Newer methods based on fluorescent dyes and phase-contract microscopy make rapid screening feasible, but false-positive identification is more frequent. Culture of tubercle bacilli is most successful when two media are used.[11] [12].

The applicability of the PCR directly to DNA extracted from Ziehl-Neelsen stained smears could become a valuable alternative approach for rapid identification of M. tuberculosis, and could be used to evaluate quality of the control of local laboratories in tuberculosis (TB) screening and solve the problem of specimen transportation. In addition, the method could be used in retrospective studies involving a wide range of PCR-based analyses, such as detection of rifampicin resistant gene in multidrug-resistant tuberculosis (MDR-TB) study.[13]. [14]

Based on this background, it is necessary to do a test using Ziehl - Neelsen staining on active smokers. Thus, the presence of Mycobacterium tuberculosis can be identified.

2. METHOD

This study is a descriptive analytical study that aims to identify Mycobacterium tuberculosis in active smokers with the Zeihl-Neelsen staining method. The sample in this study was selected with a saturated sampling technique where all selected samples will be examined for sputum. Patient inclusion and exclusion criteria: Active smokers are people who consume cigarettes regularly with the slightest even though it's only 1 cigarette a day. The slides were randomly picked up from boxes with stored slides from different years.[15].[16]

The working procedure in this study was:

1. Sputum sampling
   a. Tool: 1) Sputum pot, 2) Handscoon, 3) Shelf, 4) Name tag
   b. Ingredient: 1) Sputum sample
   c. Procedure: 1) Write the patient's name in the hospital register book, 2) Label the sputum sample according to the patient's name. 3) After that, the researcher brought the sputum sample to the laboratory for examination
2. Examination of sputum samples by the Ziehl-Neelsen method
   a. Tool: 1) Object glass, 2) Skewers, 3) Toothpick, 4) 2B Pencil, 5) Plastic contains disinfectant, 6) Bunsen and lighter, 7) Tweezers, 8) Painting rack, 9) Tissue paper
   b. Ingredient: 1) Carbol fuchsin 1%, Acid alcohol 3% and Methylene blue 0.1%, 2) Sputum sample, c. Procedure: 1) Preparation of preparations: a) Clean the glass object from dirt and grease, b) Write the identity on the frosted part using a 2B pencil, c) Make a smear by taking purulent sputum (phlegm) using a flat stick and making a size of 2 × 3 cm (oval). d) Evenly smear the sputum using a small stick with a spiral motion (coil type) and evenly, e) The sticks that have been used are disposed of in a plasticcoated container containing disinfectant
3. Drying: a) Leave at room temperature, b) If the preparation is dry, it is not allowed to make spiral movements again because there is a risk of damage to the preparatio
4. Fixation: a) After making a smear of the specimen and fixation, b) Clamp using tweezers, c) Pass the preparation over the blue Bunsen flame 2-3 times for 1-2 seconds. If heated too long can cause the preparation to spoil.

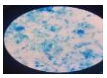
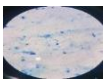
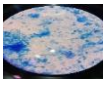
5. Staining: a) Immerse the preparation with carbol fuchsin, heat it on a painting rack using a Bunsen fire. b) Heating until steam appears and it is not allowed to boil because it will cause crystal deposits, c) Chill for about 10 minutes, d) Discard the remaining Carbol fuchsin, rinse with running water, e) Flood with acid alcohol for 10-20 seconds until the red color disappears (pale), f) Rinse with running water, g) Flood with methylene blue paint, leave for 1 minute, h) Remove the remaining methylene blue paint, rinse with running water. i) Dry the preparation on the drying rack
6. Reading the laboratory results: a) Look under the microscope from using the 10x magnification objective lens to determine the focus and field of view, then the 100x objective lens magnification by adding immersion oil, b) The reading is taken along the longest horizontal line from the left end to the right or vice versa. Minimum 100 fields of view, c) BTA will appear as red rod-shaped bacteria, either solitary or in groups.

3. RESULTS AND DISCUSSION

Based on the results of the laboratory using the Ziehl-Neelsen Staining Test at the tourist hospital, the University of East Indonesia, the following results were obtained:

Table 1: Evaluation of ZN, AO and ZN+AO microscopy against culture positive. [17]

Sl. No.	Measures	ZN vs Culture (%)	AO vs Culture (%)	ZN+AO vs Culture (%)
1	Sensitivity	59.72	97.22	97.22
2	Specificity	93.33	90	86.67
3	Predictive value of positive test	95.55	95.89	94.59
4	Predictive value of negative test	49.12	93.1	92.86
5	Percentage of false positive	6.66	10	13.33
6	Percentage of false negative	40.27	2.78	2.78
7	Efficiency	69.6	85.29	94.11

Sample Code	Age	long time smoking	Identification Results	Result Interpretation
01	51	±2 years		Negatif
02	55	±4 years		Negatif
03	48	±3 years		Negatif
04	39	±2 years		Negatif
05	25	±4 years		Negatif
06	27	±4 years		Negatif

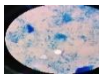
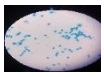
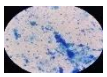
07	42	±5 years		Negatif
08	28	±3 years		Negatif
09	34	± 4 years		Negatif
10	58	±12 years		7 BTA Positif

Figure 1. The results of the Ziehl Neelsen staining test in the laboratory of the East Indonesia University Tourism Hospital

From the table above, it is known that for sample codes 01 to 09 the test results are negative (-) or there is no Mycobacterium Tuberculosis, while for sample code 10 the test results are 7 positive smears or Mycobacterium tuberculosis is present. Based on the results of the Ziehl – Neelsen staining test on 10 samples with an age range of 25 to 58 years, it was found that active smokers who had smoked for less than 12 years had negative smear results compared to active smokers who had smoked for more than 12 years, 7 positive smears were identified with In other words, the code 10 sample was positive for Mycobacterium tuberculosis.[11]. [18]

Smoking can cause tuberculosis because it can lower the immune system and interfere with respiratory defenses. there is a significant relationship between smoking habit and the incidence of tuberculosis and people who smoke for a period of > 10 years have a 2 times greater risk of developing tuberculosis, through interviews conducted by respondents who have a smoking habit and suffer from tuberculosis claiming that they cannot stop smoking because they are addicted Even though they already know the dangers of smoking, the smoking behavior of people with tuberculosis has been going on for years and they still don't stop smoking even though they have tested positive for tuberculosis, smoking with the incidence of tuberculosis is a double problem because smoking helps spread the infection and worsens the severity of tuberculosis.[19]. [20]. Smoking has become a very common and widespread habit in society. The dangers of smoking on the health of the body have been felt by many people and the effects are well known. Many studies have shown that smoking causes various diseases in our body, such as heart disease and blood vessel disorders, lung cancer, oral cavity cancer, laryngeal cancer, high blood pressure, impotence and pregnancy disorders and defects in the fetus [21]. The dangers of smoking are also shown not only for smokers (active smokers) but also for people who are not smokers inhaling cigarette smoke around smokers (passive smokers) and the effects received from passive smokers will be much more dangerous than active smokers.

Tuberculosis has been characterized as a disease of poverty, but this is an aspect of developing tuberculosis, not of relapse. Measures of low socioeconomic status, like low family income, illiteracy and low social class have been found to be associated with an increased risk of developing tuberculosis.Although socioeconomic variables have never been shown to be associated with an increase in tuberculosis relapse, inadequate treatment has, and it is possible that poverty may lead to inadequate treatment in some circumstances.[22]

Based on the results of laboratory tests with Ziehl-Neelsen staining, the researchers stated that there was a significant relationship between smoking habits and the incidence of tuberculosis in active smokers with a period of > 10 years and from the results of interviews, respondents who had a smoking habit and had tuberculosis admitted that they could not stop smoking because already dependent even though they already know the dangers of smoking, smoking behavior of tuberculosis sufferers has been going on for years and still does not stop smoking even though they have tested positive for tuberculosis, smoking with the incidence of tuberculosis is a double problem because smoking helps spread the infection and worsens the severity of tuberculosis.[23] The sensitivity of the Ziehl-Neelsen staining method needs to be considered, so that further research can obtain more significant results on the examination of AFB samples.

This is in accordance with the results of research conducted by Ira Elvi in 2019 which stated that the sensitivity of the Zielh-Neelsen Test was 77.8%.

The Ziehl-Neelsen staining guidelines need to incorporate a wider error margin for widespread application under field conditions. Quality assurance is of utmost importance, and needs more commitment from National Tuberculosis Programmes and other health authorities. In particular, allocation of sufficient resources for rechecking and integration of laboratory supervision must be ensured. Countries must make better investments in the purchase of high quality microscopes and laboratory supplies. To address the human resource crisis, personnel without specific laboratory schooling can, in principle, be trained to respond to immediate needs for TB diagnostic microscopy services. Periodic reporting on acid-fast smear examinations is highly desirable for regular monitoring and a more balanced provision of suppli [9]. New, reliable, rapid, and easy-to-use methods that display high specificity and sensitivity are required for an effective struggle against tuberculosis. [24]. [25]

4. CONCLUSION

Identification of Mycobacterium Tuberculosis with the Ziehl-Neelsen staining test obtained results from 10 samples, there was 1 sample identified as positive BTA so that the Ziehl-Neelsen test cannot be used as the main reference for performing sputum examination, other tests are needed so that the diagnosis gets more accurate results.

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