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Identification of the *rpoB* gene in isolates of *Mycobacterium tuberculosis* multi-drug resistant (*MDR*) in the province of Papua by the method of *Polymerase Chain Reaction*

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ABSTRACT

Tuberculosis (TB) is an infectious disease the second highest cause of death worldwide after HIV. The World Health Organization in Annual Report on Global TB Control 2015 is still set Indonesia, among 22 other countries (high-burden countries), in third place as the country with people with Tuberculosis (TB) is the highest after India and China. The purpose of this study was to detect rpoB gene as a marker of resistant strains of MDR TB in Papua by PCR. From 81 sempel in get mikrroskopis Negative results: 64 samples, Scanty: 2 samples, Positive 1: 3 Sample, Positive 2: 4 and Positive Sample 3: 8 Samples. Of the 19 positive samples of microscopic done rpoB gene identification method GenXpert in get 100% positive and are resistent to the antibiotic rifampicin. Of the 19 positive samples of microscopic test and test GeneXpert proceed to Culture as a gold standard method and obtained 100% positive. And antibiotic resistance test terhadap4 obtained the following results: antibiotic Streptomycin: 7 resistant and 12 sensitive, Izoniasin 14 resistant and 5 sensitive, rifampicin: 17 resistance and 2 sensitive and resistant to the antibiotic Ethambutol 8 and 11 sensitive. From the electrophoresis test in getting DNA band with 300bp size so that it can be concluded that 19 samples contained entirely rpoB gene.

Keywords: Mtb, *rpoB* gene, PCR, MDR, Papua Province of Indonesia

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INTRODUCTION

Tuberculosis (TB) is an infectious disease the second highest cause of death worldwide after HIV. The high incidence of tuberculosis is becoming more complex due to the emergence of resistance to anti-tuberculosis drugs. WHO has been defining the multi-drug resistance, MDR-TB, is a disease which is caused by tuberculosis strains resistant *M.tuberculosis* and at least to isoniazid and rifampicin, which is the first-line drugs are most effective [1].
World Health Organization, in Annual Report on Global TB Control 2015 is still set Indonesia, among 22 other countries (*the high-burden countries*), in third place as the country with the most people with tuberculosis after India and China. Every year in the estimated eight million new infections occur and give rise to 2.5-3 million deaths, of all cases of TB in the world, 38% are in Southeast Asia and more than 95% of the cases are in developing countries, such as India, Indonesia, Bangladesh , Thailand, and Myanmar. In Indonesia is estimated cases of MDR-TB among new cases of pulmonary tuberculosis ternotifikasi as many as 5,700 cases. In 2014 the reported cases of MDR-TB in 1755 than 9580 suspected cases of MDR-TB, and there were 1291 cases treated.

More than 90% of isolates were resistant to rifampicin are also resistant to isoniazid (INH), so resistant to rifampicin is a sign that represents the occurrence of MDR [2]. It is known also that more than 96% were resistant to rifampicin caused by mutations in the segment of 81-bp *rpoB* gene from codons 507-533 were referred to as rifampicin resistant [3].

The diagnosis of MDR-TB with drug sensitivity test (DST) provides results that are very sensitive and specific but requires a long-time (3-12 weeks) given the rate of growth of *M. tuberculosis* is very slow [4]. Currently the use of a variety of molecular methods based on *Polymerase Chain Reaction* (PCR) has been designed to speed up the detection of mutations associated antimicrobial agent resistance problem [5]. Limitations of the current biomolecular test is only designed to detect gene polymorphisms of *rpoB* that appears most frequently in isolates *M.Tuberculosis* ie at codon 531, 526, and 516. This makes this method can not be applied universally as dots mutations in *M.tuberculosis* very influenced by the geographic [6].

Development of PCR method for detection of *M. tuberculosis* has been developed with the aim to improve the sensitivity is by *nested* PCR. The amount of geographical influence causes the need for a database on the conservative region mutations in *M. tuberculosis.* Identification of *rpoB* gene mutation in several regions in Indonesia has been done. However, for this kind of research Papua region have never been done. In this study used real-time PCR method for detecting the type of *rpoB* gene mutations causing resistance as well as an initial step in the gathering of conservative mutation sequence regions in Papua. Data produced will help the development of molecular techniques in the diagnosis of MDR-TB in Indonesia.

MATERIALS AND METHODS

The location of research conducted in the laboratory of tuberculosis BSL type 2, Center Health Laboratory, Jayapura, Papua Province, and TB Laboratory BSL type 2 Health Research Institute, Jakarta and Papua Biomedical Health Research Institute. The population in this study all samples of suspected MDR TB from November 2015-May 2016, Health Laboratory in Jayapura, Papua Province. The sample in this study is positive samples Mtb resistant to rifampicin and MDR were taken by purposive sampling.

Research procedure includes: a microscopic examination method Zielh Neelsen, coloring the background (*counter staining*), reading the microscopic preparations sputum, PCR (*GeneXpert*), Inspection culture BTA, test resistance anti tuberculosis drugs, the PCR process, procedure electrophoresis, purification of PCR products made with *Big Dye XTerminator Purification Kit.* PCR sequencing reaction products were centrifuged for 1 min, 1000 x g. Mix 10 mL and 45 mL Solution XTerminator SAM Solution added about 55 mL into each container. Homogenization is performed for 30 min with vortex or plate shaker (1800 rpm) and centrifuged for 2 min, 1000xg. Product is placed into the sequencing machines/Genetic Analyzer.

RESULTS AND DISCUSSION

Patients with MDR-TB suspect that refer to Health Laboratory Jayapura, totaling 81 patients in November 2015-April 2016, patients are requested to collect sputum for laboratory examination, each of which consists of 3 samples. Samples in getting the examination include: Microscopic, GeneXpert, Culture, and PCR.

Based on the results obtained shown that patients with suspected TB is obtained with negative microscopic examination as many as 64 samples, Scanty as much as 2 samples, Positive 1 as much as 3 sample, Positive 2 is 4 samples and Positive 3 are 8 samples. The diagnosis of pulmonary tuberculosis, especially tuberculosis, can be established by clinical examination, analysis of patient complaints and physical examination, laboratory tests and radiological examination. Third examination results were united for the diagnosis of tuberculosis. One of the laboratory examination is to detect *M. tuberculosis* as the cause. In general, the method used is the conventional method such as acid-fast bacilli by microscopic examination and culture examination. Microscopic examination is quite fast and economical but still lack sensitivity and specificity [7].

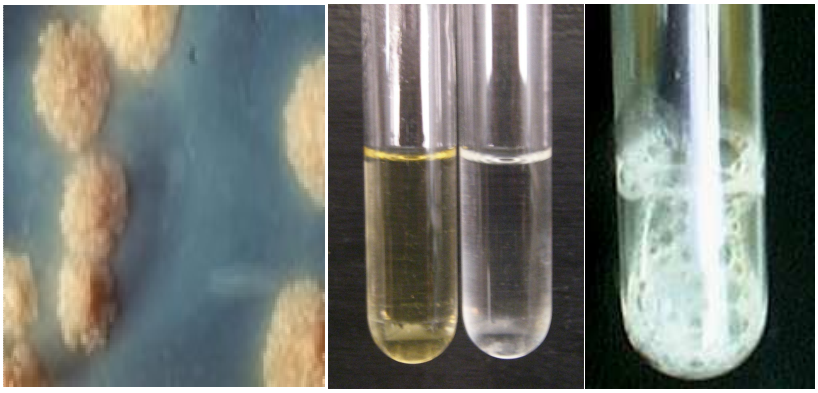

Fig 1. (A) The culture of M. tuberculosis Löwenstein-Jensen medium, colonies of *M. tuberculosis* aged 4 weeks to grow on the media; (B) The test results niacin *M. tuberculosis*. Test niacin is the main biochemical reactions that must be done for the identification of *M. tuberculosis*; and (c) at the M. tuberculosis catalase test. The enzyme *catalase-peroxidase* is one of the virulence factors of *M. tuberculosis* because the enzymes help the defense system against peroxide *M. tuberculosis* inside the macrophage cell

Microscopic examination of sputum is still considered important in diagnosis using the Ziehl-Neelsen method [8]. Sputum microscopy is highly dependent on the concentration of *M. tuberculosis* in sputum. To get a positive acid-fast bacilli under a microscope the number of M. tuberculosis at least 5,000 stems / 1 mL of sputum. As for checks on culture/culture of *M. tuberculosis* is the number of stem 50-100/mL of sputum. Sputum good to be inspected are those of the discharge brochus by 5-10 ml with minimum content of nasal or oral secretions. Taken a series three times in succession, wherever possible third is phlegm morning of the first day because it is estimated bronchial secretions accumulate in the lungs while sleeping at night.

Acid-fast stain is generally used to identify *Mycobacterium*, including *Mtb* and *M. leprae*. This coloring including differential staining, because it can distinguish acid-resistant bacteria (resistant acid leaching), and acid-resistant bacteria (not resistant to acid leaching). Tinting is also named Acid fast staining. This procedure uses the primary colorant with heating and Loeffler methylene blue, a dye counter. The engineering modifications then replace the heat treatment with the use of wetting agent (detergent to reduce the voltage) to ensure penetration of surface fat; dyes containing a wetting agent called dye kinyoun.

Ziehl Neelsen coloring genus Mycobacterium designated certain bacteria, actinomycetes, and the genus Nocardia containing a large amount of lipids in the cell wall, looks like a layer of wax. The lipid content is very high, in some species can reach 60% of the weight of the cell wall. Very high lipid content is causing the bacterial cell is difficult to be colored, because the dye can not penetrate the wax coating. If the acid-resistant bacteria fuksin tinged with a solution of carbolic acid, the dye was not easy dilunturkan by the solution. Both bacterial genus contains species are pathogenic in humans. For example, *Mycobacterium tuberculosis* causes *tuberculosis* and *Mycobacterium leprae* causes leprosy.

The nature of the acid-resistant bacteria associated with lipid content in the cell wall. With the help of heating the dye can penetrate into bacterial cell that is covered by the lipid. The dye is used first is carbolic fuksin; which is fuksin base dissolved in larutanfenol 5%. Phenol is used as a solvent to help perasukan dye into a bacterial cell during the heating process. Metilin blue dye as the latter does not cause discoloration of the red in acid-resistant bacteria. At the bacteria are not resistant to acid, blue methylene be tied by a bacterial cell that is colorless.

Organisms that can withstand the dye is said to be resistant to acid and appear red. In the cell walls of organisms that are not lipoidal, fuksin carbolic acid dyes that color cells can be easily dipucatkan by acid alcohol, and because it is called is not acid resistant. Alcohol acid is a highly intensive pemucat not to be confused with alcohol and acetone in Gram staining. Washed carbolic fuksin can be demonstrated by absorption of blue dye counter metilin by cell so that the bacteria appear blue.

Data results of the *GeneXpert*

GeneXpert examination done on samples with positive results in microscopic examination. GeneXpert examination results obtained shown that of the 19 positive samples with microscopic showed 19 samples were positive by nature resistant to rifampin. PCR GeneXpert recommended for: diagnosis of tuberculosis, for the detection of resistance to the drug rifampicin (resitensi rifampicin is a proxy for MDR-TB), then all results resiten of rifampicin should be confirmed by culture and sensitivity phenotypically plastic against Anti Tuberculosis first-line and second-line.

The results of acid-fast bacilli culture examination

Culture for *Mycobacterium sp* bacterium is a conventional method, and is the gold standard in diagnosis. Examination of cultur has kesenstifan higher compared geneXpert microscopic examination and, therefore, the culture is done to confirm the results of microscopic examination and geneXpert, but has drawbacks such as culture examination time is needed for bacterial growth longer.

The results of PCR

The results of the analysis of 19 samples tested in this study contained 5 samples continued to PCR, the samples used as PCR template derived from DNA isolated from *M. tuberculosis* MDR. Phase isolation of DNA is done by using a lysis buffer solution that causes lysis of the cells in the sample. Lipid layer of very thick cell wall of this bacteria caused needed strong lysis, amplification of gene *rpoB* conventional method using a primer rpoB CCACCCAGGACGTGGAGGCGATCACAC, rpoB CGTTTCGATGA ACCCGAACGGGTTGAC do with stage predenaturasi 94 °C for 5 min, denaturation 94 °C for 1 min, annealing 57 °C for 1 min, the extensions 72 ° C for 1 min, then final extension 74 °C for 10 min and hold 4 °C, after all stages of PCR elapsed, continue the process of electrophoresis, with the following results: amplification fragmmaen *rpoB* gene have been successful in done with conventional methods to produce 370 bp PCR as shown in the figure below while the 100 bp DNA marker.

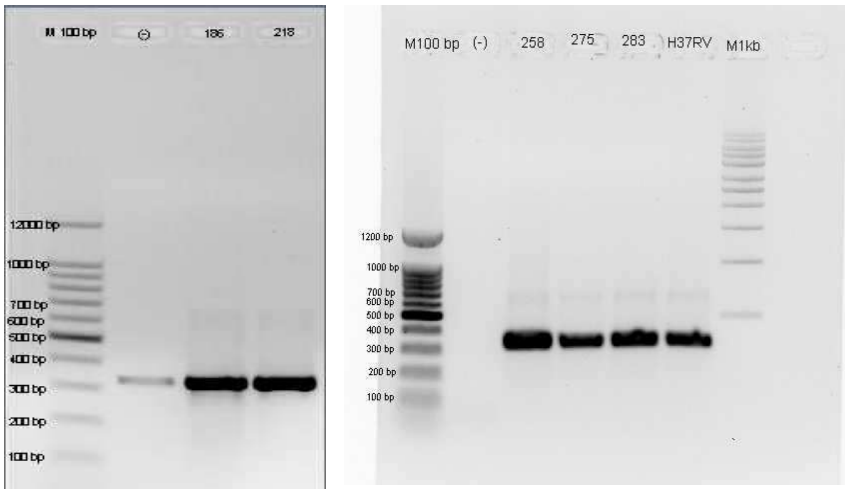

Fig 2. *Mycobacterium tuberculosis* DNA electrophoresis results

The diagnosis of MDR TB by drug sensitivity test or drug susceptibility testing, DST provides results that are very sensitive and specific but require a long time of 6-9 weeks given the pace of growth of *M. tuberculosis* is very slow. Therefore, the required tests more quickly and accurately as PCR-based molecular diagnostic test. Molecular diagnostic tests outline is done by taking samples from phlegm (sputum), pleura, or an enlarged gland biopsy, DNA isolation, amplification of DNA by PCR and gel electrophoresis. Limitations of the current molecular test is only designed to detect gene polymorphisms *rpoB* that appears most frequently in isolates of M. tuberculosis that is at codons 531, 526, and 516 [9-15].

Changes to *rpoB*526 and *rpoB*531 is a mutation in the region RRDR which is widely recognized as the most dominant place in the RIF resistant isolates. The results of the earlier study of 103 RIF resistant isolates showed as many as 53, 31, and 20 isolates respectively for *rpoB*531, *rpoB*526, and other *rpoB* mutants. Meanwhile, only katG315 mutations known to cause resistance to INH, and this study only got 16 isolates katG315. The results of the *rpoB* gene genotype characterization performed this study was lower than that reported by other researchers who conduct research in Italy, which amounted to 56.7% rpoB526 and 24.3% of mutations rpoB531 mutations occur in MDR-TB isolates. The study, conducted research in China gained 41% isolates were MDR-TB is *rpoB*531, and 40% is *rpoB*526. [16-18]

RIF-resistant nature of the relationship that is caused by mutations in *rpoB* not much is revealed. Publications that have been there mostly just shows the frequency data or rpoB mutation that occurs in *M. tuberculosis*. Meanwhile, the data obtained from these studies suggest a link between the phenotype of MDR-TB, which is the level of RIF resistance in *rpoB* genotypes. The results of this study pave the way for further research: examining the relationship of DNA mutation to the emergence of certain diseases, and diseases associated with pathogenic bacterial infections in Papua dan Indonesia [19-24].

CONCLUSION

The results of this study revealed that 81 samples get negative microscopic results is 64 samples, Scanty as much as 2 samples, Positive 1 is 3 samples, Positive 2 is 4 samples and Positive 3 is 8 sample. In the 19 positive samples of microscopic done *rpoB* gene identification method *GenXpert* in get 100% positive and are resistent to the antibiotic rifampicin. The analysis showed that of 19 positive samples of microscopic test and test *GeneXpert* proceed to the culture as a gold standard method and obtained 100% positive. Research reveals that the test of resistance to four antibiotic obtained the following results: the antibiotic streptomycin: 7 resistant and 12 sensitive, Izoniasid 14 resistant and 5 sensitive, rifampicin: 17 resistant and 2 sensitive and resistant to the antibiotic ethambutol 8 and 11 sensitive. While the results of electrophoresis in getting the DNA band with 300bp size so that it can be concluded that 19 samples contained entirely rpoB gene.

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