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Making the collection genome of *Mycobacterium tuberculosis* that are resistant to streptomycin in pUC19 vector: A model of search for genes responsible for resistance properties

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ABSTRACT

Tuberculosis caused by *Mycobacterium tuberculosis* (Mtb), which gives the infection is chronic and systematic, by spreading the human body through the lymphatic, vascular, respiratory, so it can attack any organ or tissue of the body. The purpose of this study to conduct a study on the gene responsible for resistance to antibiotics in bacteria Mtb that can be developed methods of detection of bacteria. This initial phase was the production of a collection of clones of *E. coli* bacteria that bring partial DNA (genome) Mtb bacteria that are resistant to the antibiotic streptomycin (*str^R*) (partial genomic library). It is expected that the collection of the genome can be used as a model search for genes responsible for resistance to antibiotics, especially streptomycin. This study has been successfully created collection Mtb genome of bacteria that are resistant to streptomycin in the vector pUC19. The number of colonies that grew from a transformed bacterium *E. coli* JM101 was about 7000 colonies. Comparison of the number of colonies of white and blue is 1:4. Plasmid DNA isolation method based on alkaline lysis showed that of 41 recombinant plasmid isolated from white colonies, only 33 are visible containing DNA inserts. The data provides results ligation efficiency of 16.10%. The success of the manufacture of recombinant DNA is influenced by many things including comparisons between DNA ligation of vector and insert DNA, dephosphorylation of the ends of the DNA fragments to be ligated to avoid self-ligated. While the collection is influenced by its competent genome of the host cell used. The more competent host cell will provide much better results.

Keywords: *Mycobacterium tuberculosis*, partial genomic library, antibiotic streptomycin (*str^R*), Papua Isolates

INTRODUCTION

TB disease caused by the bacterium *Mycobacterium tuberculosis* are on the infection is chronic and systematic, by spreading the human body through the lymphatic, vascular, respiratory, so it can attack any organ or tissue of the body [1-2]. The usual method for the diagnosis of TB disease takes between 2-8 weeks, sometimes even can reach three months, with the exception of microscopic methods Basil Hold acid of sputum patients who require less than one day. However, it is unfortunate the latter method is not specific for acid resistant properties possessed by all mycobacteria, so it is not only positive for Mtb bacteria. Conventional lab now can actually do the breeding, identification, and susceptibility test bacteria. However, because it takes a very long time (6-8 weeks), eventually most laboratories only perform microscopic examination can be completed in minutes. The consequence is the eradication program is not adequate. The length of time required for the diagnosis of TB is not only detrimental to the patient, but also raises the problem of transmission of TB bacteria into the surrounding environment [3-5].

The purpose of this study was to conduct a study on the gene responsible for resistance to antibiotics in bacteria Mtb that can be developed methods of detection of bacteria. This initial phase was the production of a collection of clones of *E. coli* bacteria that bring partial DNA (*genome*) Mtb bacteria that are resistant to the antibiotic streptomycin (*str^R*) (*partial genomic library*) [6]. It is expected that the collection of the genome can be used as a model search for genes responsible for resistance to antibiotics, especially streptomycin. With obtained a collection of *M. tuberculosis* genome, the genes known to be responsible for the nature of resistance, and a method for detecting the gene using *Polymerase Chain Reaction* (PCR), is expected to speed up the diagnosis of TB disease with quick and precise therapy. The use of *M. tuberculosis* detection methods based on PCR process is a sensitive and specific method, which only takes one or two days.

MATERIALS AND METHODS

Propagation of *M. tuberculosis* that resistant to streptomycin

M. tuberculosis strains that are resistant to streptomycin (*str^R*) is obtained from the Laboratory of Biochemistry, University of Cenderawasih. The bacteria were obtained in the form of culture on solid media Lowenstein-Jensen (LJ). For the purposes of this research, back in bacteria cultured in a liquid medium sterile Mycobacterium. It consists of a liquid medium (NH₄)₂SO₄ 1,0 g, Na₂HPO₄ 0,5 g, KH₂PO₄ 0,5 g, MgSO₄ 0,2 g, FeSO₄.7 H₂O 0,005 g, MnSO₄ 0,002 g, and paraffin solution 5.0 mL which is then diluted with distilled water until a final volume of 1.0 L. The solution was shaken until homogeneous. By using a needle Ose, a single bacterial colonies on solid medium (LJ) in aseptic inoculated into 10 mL liquid medium (ATCC) containing antibiotics streptomisin 100 mg/mL. Stages of the research was conducted in the Safety Cabinet. Cultures were incubated for four weeks at 37 °C, 175 rpm agitation using a shaker bath. During incubation volume of water in the incubator to be maintained in order not diminished. The next process is the total isolation of chromosomal DNA and bacterial cell harvest [2, 7-8].

Bacterial cell lysis, DNA purification chromosomes, and DNA concentration determination

Allowed to thaw frozen cell buffer. Cells are broken down by adding lysozyme to a final concentration of 500 ug / mL and incubated for one hour at 37 °C on a shaker-bath. Further suspension (cell debris) was added to 500 mL solution containing 100 mM Tris-HCl pH 8.0, 400 mg/mL proteinase K, 1.0% sodium dodecyl sulfate (SDS) newly created (freshly) [9]. The crude extract was incubated for two hours at 55 °C in the incubator. Then added 200 µL of NaCl 5M To separate the DNA from the protein extraction was performed three consecutive sorts, with phenol, followed by chloroform, and finally with ether. Each extraction is done two times and the volume ratio of aqueous phase to the organic phase is 1: 1. On each extraction is taken from the water phase.

DNA concentration was measured by measuring the absorbance at ultraviolet light using a spectrophotometer. DNA concentration was measured by absorption at λ_{260} nm, while the purity of DNA obtained by comparing the absorption at λ_{260} nm with λ_{280} nm.

Preparation of vector DNA

DNA vector used is a plasmid, pUC19. Plasmid pUC19, which had been isolated before this, were cut using *Bam*HI enzyme that is one of the enzymes in *polycloning site*, so we get a linear plasmid vector DNA. Perfect cutting pUC19 vector DNA is done as follows: in one eppendorf made the reaction mixture DNA PUC 10 µL, 12 µL ddH₂O, 10x buffer 2.5 µL, and 0.5 µL *Bam*HI, so as obtain a total volume of 25 µL. The mixture was incubated at 37 °C for 2.5 h. To test cutting results, then 2.5 µL of the total volume of the reaction product is added bifer loading as much as 2 µL, put in a 0.8% agarose gel wells and the re-run on 70 volts for 1.5 h. The next stage followed by ligation of the chromosomal DNA and the linear vector DNA into a host cell transformation and characterization of clones transformed.

Collection of partial genome

In this study, a partial genome collections created by collecting the white colonies on the LB medium containing as much as 20 mL ampicillin 100 mg/mL. Created overnight culture at 37 °C, with shaking at 150 rpm on a shaker. Furthermore, from 20 mL of fresh overnight culture is divided into 19 mL eppendorf 2.0 which already contains glycerol sterile. Comparison between the volume of cell culture and glycerol is 85% and 15%. Eppendorf containing the culture-glycerol was then immersed in dry ice-ethanol (-100 °C temp.) more or less for 5 min. After that the eppendorf storage at freezer kept at -70 °C as glycerol stocks.

RESULTS AND DISCUSSION

Culturing bacteria from the isolation of total DNA

str^R Mtb bacteria obtained from the Laboratory of Biochemistry, University of Cenderawasih, cultured in mycobacteria media ATCC (*American Type Culture Collection*) for 4 weeks. Results obtained in the form of

bacteria, white, shiny and very slow growth. Chromosomal DNA concentration of total isolation of chromosomal DNA, obtained through UV spectrophotometry, at a wavelength of 260 nm, the absorbance value (A) = 1.0 correspond to double-strand DNA concentration of 50 µg/mL. While the DNA purity is measured based on the ratio A_{260}/A_{280} . The results obtained for the measurement of chromosomal DNA concentration by spectrophotometry are as follows:

Table 1. The results of measurement of the concentration of *M. tuberculosis* chromosomal DNA with a spectrophotometer

No.	Measurement	Data (dilution, 250x)
1.	A (260 nm)	0.133
2.	A (280 nm)	0.080

From the above data can be determined the concentration and purity of DNA chromosomes were obtained, namely, the concentration of DNA = 0.275 µg/µL and DNA purity 1.66.

Ligation of DNA fragments of chromosomes and DNA pUC19 and transformation

The recombinant plasmid was made by ligating the chromosomal DNA fragment and pUC19 DNA which have both been cut with restriction enzymes. Ligation using T4 ligase enzyme unit 1 to the state of 1.5 µg chromosomal DNA fragments with 390 ng plasmid DNA that had been cut, incubated at 14 °C for 18 h. Results ligation is not analyzed by gel electrophoresis, but by the nature of the cell phenotype transformant carrying the recombinant plasmid DNA or pUC19 after the transformation process.

Data transformation result on day one, namely: control of competent cells of living in LB medium without ampicillin; control transformation with plasmid DNA intake as much as 10 ng produce blue colonies around 300 that growing on an agar medium ampicillin. While the results of the transformation on day-2 showed: control efficiency by as much as 10 ng of DNA PUC (the same with day 1) to produce blue colonies about 1000 colonies; control transformation with pUC19 cut as much as 97.6 ng provides the number of blue colonies around 30-40 pieces; control transformation with pUC19 DNA *religated* (ligated back) as much as 97.6 ng provides the number of blue colonies about 1500 pieces; transformation with recombinant DNA with the amount of 212.4 ng plasmid DNA provides the number of colonies around 7000 pieces, with white and blue colonies ratio is 1:4. All cell transformant day 2 was grown on an agar medium ampicillin.

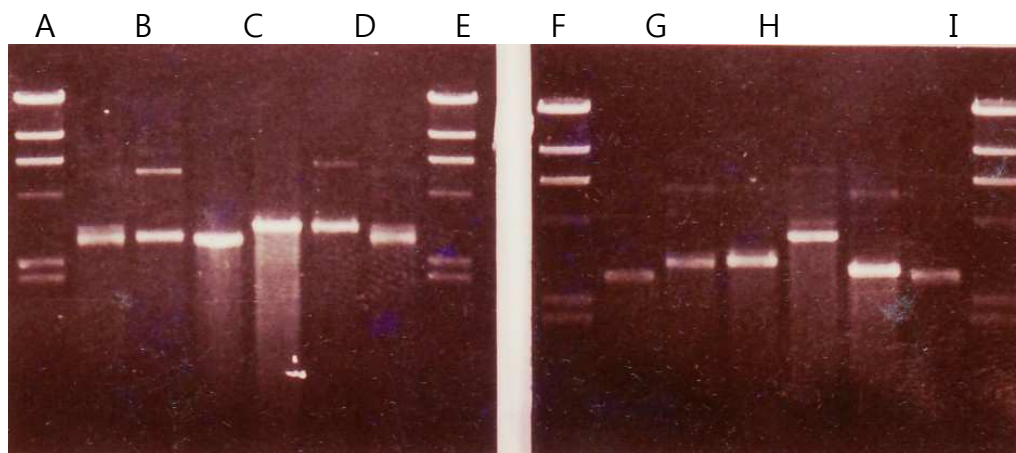


Fig. 1. The result of a 0.8% agarose gel electrophoresis. Perfect cuts recombinant DNA plasmid and pUC19 plasmid from transformant cells. Column A, H, I, and P: *HindIII* cut lambda DNA; Column B, G, J, and O: The plasmid isolated from blue clones were cut *HindIII*; and Column C-F and K-N: The plasmid isolated from white clones were cut *HindIII*

Further characterization transformant cells containing recombinant plasmids from white colonies grown on ampicillin media, and as a comparison was also held on the characterization of blue colonies containing only DNA pUC19.

Restriction enzyme analysis

A total of 10 plasmids were analyzed with restriction enzymes *HindIII* of 46 plasmid DNA isolated. Ten plasmid DNA consists of 8 pieces of the recombinant plasmid DNA from white colonies plasmid pUC19 and 2 pieces of blue colonies. Looks at the results of agarose gel electrophoresis that the DNA plasmid pUC19. Cutting with *HindIII* to produce a band, as shown in the following figure (Fig. 1).

Bacteria and chromosomal DNA of *Mtb*

The observations in this study showed that *M. tuberculosis* develops very slowly. This is consistent with the analysis of Koch, that this bacillus grows slowly because their cell walls so that the lipid-rich food substances difficult to penetrate. The cell walls that are rich in lipids that cause the bacteria prefer to live in hydrophobic media. In this research paraffin is added to the culture medium to make the media be hydrophobic, so that *M. tuberculosis* bacteria could grow well.

Reagents used during the isolation of chromosomal DNA acts as follows. Antibiotic cycloserine (one of the antituberculosis drug) to inhibit bacterial growth. Glycine and LiCl make cell walls fragile. While EDTA can eliminate Mg^{2+} ions are essential to maintain the overall structure of the cell sheath, in addition to inhibiting enzymes that can damage cellular DNA. TE solution (Tris, EDTA) and SET (Sucrose, Tris, EDTA) to homogenize the DNA to be easily broken down by the enzyme lysozyme. Here is done twice treated with the enzyme lysozyme, so that the cell walls actually broke because the cell wall of mycobacteria thicker and rich in lipids compared to other bacteria. The addition of proteinase K to degrade protein in cell extracts. Furthermore, phenol extraction, chloroform is to separate the protein molecules and DNA total. Ether extraction is to remove residual phenol in the solution for their phenols inhibit the process of cutting the DNA with restriction enzymes [10-12].

Chromosomal DNA isolation results are shown in agarose gel electrophoresis showed a smear band. Smear band can be caused chromosomal DNA which was originally a single chain is fragmented because of their physical or chemical treatment, such as during centrifugation, extraction of phenol, or at the time of mixing the DNA by means of pipette. In some cell extraction protein content is very large so it needs to be done several times a phenol extraction. In this experiment phenol extraction performed twice so that each time mixing and centrifugation resulted in the break-chromosome DNA molecule. This is certainly not good because there is a DNA fragment size smaller than desired for cloning purposes.

Cutting the chromosomal DNA and DNA of pUC19

Sau3AI enzyme that is required when cutting identify chromosomal DNA of regions which four bases 5'-N/GATC-3'(where: / is a cutting site) and produce a pointed tip on fragments result piece. So it is likely to be able to recognize the region's four bases in DNA of will be greater than to identify areas of more than 4 bases [13-17]. This causes these enzymes can cut the chromosomal DNA of into small fragments required for cloning. This enzyme cuts DNA of *Sau3A* partial, meaning there are areas GATC recognized and cut by this enzyme, but there is also a recognized but have not been cut as the amount of enzyme and the cutting time is limited. Therefore, this enzyme did not cut in all areas GATC [10-12]. Chromosomal DNA fragment desired to be inserted at a maximum of 5 kb pUC19 vector. In this study, DNA fragments smaller than 5 kb.

Table 2. Data migration distance and the size of the DNA of λ HindIII

No.	Size of DNA		Migration distance (cm)
	Kilobase (kb)	Log kb	
1	2,31	1,36	0,70
2	9,40	0,97	1,00
3	6,50	0,81	1,30
4	4,30	0,63	1,75
5	2,30	0,36	2,65
6	2,00	0,30	2,90

From the data in the table above, and if it made the curve it is seen that the size of the DNA fragments cut by *Sau3AI* located at a distance of at least 2.0 cm, mean large size maximum chromosomal DNA obtained was 3.5 (log kb=0.55). pUC19 obtained from Lab. Biochemistry of University of Cenderawasih, its concentration is 244 ng/ μ L. For the purposes of this plasmid was cut with *BamHI* enzyme, ie there polycloning site, to obtain linear fragments with ends compatible with the ends of the target DNA fragment. The enzyme BamHI recognizing the base sequence 5'-G/GATCC-3', and generate a pointed tip of the results of slaughtering. A pointed tip resulting from *BamHI* cuts will be compatible with a pointed tip resulting from cutting *Sau3AI* (5'-N/GATC-3'), so that the bases at both ends of the fragments were ligated can be paired in advance [10]. Temp. 37 °C is used to process DNA restriction is the optimum temp. of operation of the restriction enzyme [13, 17-18].

Ligation

Ligation of linear plasmid DNA of with chromosomal DNA fragments (target DNA), which both have a pointed tip compatible, performed with the aid of T4 ligase enzyme. Ligation of the ends of the taper compatible is more efficient, because the bases are located at the ends of each taper from which can form hydrogen bonds resulting in a relatively stable structure and then the ligase enzyme can work. Furthermore ligase enzyme will catalyze the

formation of phosphodiester bond between the ends of phosphate group (P) and which has a hydroxyl group (OH) adjacent.

The mole ratio used for ligation between the plasmid DNA of and the target DNA is determined as follows. The molecular weight of the base pairs (bp) DNA of is 660. The weight of the target DNA used for the ligation was 1500 ng with a size of about 3.5 kb. While the weight of pUC19 DNA of which had been cut with a size of about 390.4 ng 2.69. The concentration of the target DNA of in moles is $1500 \text{ ng}/(3.5 \text{ kb} \times 660) = 0.649 \text{ mol}$. The plasmid DNA concentration was $390.4 \text{ ng}/(2.69 \times 660 \text{ kb}) = 0.219 \text{ mol}$. So, the mole ratio of target DNA and the plasmid DNA of about 3:1.

Transformation

The competence of a cell can be increased when the cells were incubated in a solution of CaCl_2 for 24 h. After 24 h of this competence will decrease drastically because the possibility has many cell walls to rupture. Therefore the transformation experiments conducted on competent cells that have been incubated in CaCl_2 solution for approximately 18 h. Competent host cells, namely *E. coli* JM101, will grow in LB medium without ampicillin as white colonies and grown in LB medium ampicillin. This proves that the transformant *E. coli* JM101 cells to ampicillin sensitive, meaning it does not contain competent cells β -lactamase gene contained in plasmid pUC19 [10,16, 19-25].

Control another transformation that is the host cell containing the plasmid DNA of of 10 ng. Similarly, the plasmid religated, plasmid DNA of uncut pUC19 can also grow on ampicillin media and produce blue colonies. Means the plasmid DNA of can be replicated if the circular shape. Therefore, it needs to be done on recombinant DNA ligation that re-shaped circular.

Transformation containing recombinant DNA, if grown on selective media are LB ampicillin media by IPTG (as inducers) and X-gal (as substrate), will grow as white colonies. This is due to the recombinant DNA into the host cell can not express the protein-peptide fragments- α -enzyme β -galactosidase that is caused by damage due to the *lacZ* gene tersisipi target DNA. While transformants containing pUC19 intact, both derived from uncut pUC19, pUC19 religated, nor of *self ligated* pUC19 upon ligation, would grow as blue colonies. PUC19 colonies intact and not damaged its *lacZ* gene can express β -galactosidase protein capable of degrading X-gal into a 5-bromo-4-chloroindocil then by air oxidized to 5,5'-dibromo-4,4'-dicloroindigo blue. Further analysis was to determine the size of the DNA insert contained in the recombinant DNA.

The transformation process using *E. coli* cells competent in general could reach 1×10^9 colonies per $1 \mu\text{g}$ DNA plasmid, although some strains of *E. coli* can reach more of the above data [14, 19-22, 25]. Transformation of the trial produces a total of around 7,000 pieces colony of 212.4 ng recombinant DNA is inserted. Thus, when compared to the general state of the above, it can be determined the efficiency of transformation in this study is about $3.5 \times 10^{-3} \%$.

Table 3. Data of migration distance and the size of the DNA \backslash HindIII

No.	DNA size		Migration distance (cm)
	Kilobase (kb)	Log kb	
1.	2.31	1.36	0.45
2.	9.40	0.97	0.75
3.	6.50	0.81	0.95
4.	4.30	0.63	1.25
5.	2.30	0.36	1.75
6.	2.00	0.30	1.85

Restriction enzyme analysis

To determine the size of the DNA insert contained in the recombinant DNA, the recombinant plasmid is cut using restriction enzymes *HindIII*, namely that of the *polycloning site*. Data can be analyzed from the size of the target DNA is inserted in the recombinant plasmid. The size of the DNA insert was determined by measuring the distance band recombinant plasmid was cut with *HindIII*. Pita recombinant plasmid will be located more above than the band is not the recombinant plasmid. This is due to the recombinant plasmid size larger than that is not the recombinant plasmid.

From the above data can be determined the size of the DNA inserts that contained the recombinant plasmid was constructed. Insert size is determined by reducing the size of the recombinant plasmid DNA (from white colonies) with the size is not the recombinant plasmid (from blue colonies).

From the results of research in the bands contained a plasmid derived from white colonies but did not have DNA inserts. This can be caused by two things. First, electrophoresis gel is unable to separate DNA molecules whose size does not vary much because of the shape and electrical charge similar. Second, the plasmid pUC19 mutations bases located in the *lacZ* gene so that the gene becomes damaged and unable to produce β -galactosidase fragments.

Average large insert DNA contained in a recombinant plasmid is about 520 bp, from eight samples of recombinant DNA isolated from white colonies.

Table 4. Measurement of amount of the insert in the recombinant plasmid.

Column band	Distance migration (cm)	Size (kb)	Amount of <i>insert</i> (bp)
J	1.80	2.60	-
K	1.70	2.88	280
L	1.65	3.05	450
M	1.45	3.80	1200
N	1.75	2.72	120
O	1.80	2.60	-

Ligation efficiency in this experiment was determined as follows: from 41 white colonies were isolated DNA plasmids only 33 colonies showed the recombinant plasmid containing the insert. While the result of the transformation, between 7000 colonies grown on selective media in 1400 only white colonies (which carry recombinant DNA). So ligation efficiency was determined by $(33/41) \times (1400/7000) \times 100\% = 16.10\%$. This research will be developed to see the connection nucleotide mutation that occurs in a specific cell or organism to the emergence of a certain disease [20, 26-30].

CONCLUSION

This study has been successfully created collection *Mtb* genome of bacteria that are resistant to streptomycin in the vector pUC19. The number of colonies that grew from a transformed bacterium *E. coli* JM101 was about 7000 colonies. Comparison of the number of colonies of white and blue is 1:4. Plasmid DNA isolation method based on alkaline lysis showed that of 41 recombinant plasmid isolated from white colonies, only 33 are visible containing DNA inserts. The data provides results ligation efficiency of 16.10%. The success of the manufacture of recombinant DNA is influenced by many things including comparisons between DNA ligation of vector and *insert* DNA, dephosphorylation of the ends of the DNA fragments to be ligated to avoid *self-ligated*. While the collection is influenced by its competent genome of the host cell used. The more competent host cell will provide much better results.

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