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Screening for Anti *Mycobacterium tuberculosis* Activity of *Streptomyces* sp. from Lapindo Mud in Sidoarjo, Indonesia

Rochmah Kurnijasanti[1]*, Muhammad Rais[2], Sri Agus Sudjarwo[1]

[1]*Department of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia;* [2]*Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia*

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***Correspondence:** Rochmah Kurnijasanti, Department of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia. E-mail: rochmahkurnijasanti@gmail.com
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Abstract

BACKGROUND: *Streptomyces* sp. from Indonesian soil have not been explored and isolated to find new strains as a source of antibiotics for the treatment of tuberculosis (TB) disease.

AIM: In this study, the effect of *Streptomyces* spp. from Lapindo mud in Sidoarjo, Indonesia be observed, to find out whether *Streptomyces* spp. has anti-TB activity.

METHODS: The primers Strep F; 5-AGAGTTTGAT CCTGKGTCAG-3 and Strep R; 5-AAGGGAG GTGATCCAKKGKGA-3 were used in polymerase chain reaction amplification of the *16S rRNA* gene against *Streptomyces* strains. The anti-TB activity of *Streptomyces* sp. was determined by broth dilution method using Middlebrook 7H9 media.

RESULTS: The results showed that new types of *Streptomyces* spp., namely, *Streptomyces A*, *Streptomyces D*, *Streptomyces Ea*, *Streptomyces Ep, Streptomyces I*, *Streptomyces F,* and *Streptomyces G from* garbage dump soils. This result also showed that the activity of *Streptomyces I*, *Streptomyces F,* and *Streptomyces G* could inhibit the Mycobacterium TB growth by with inhibitory zones, respectively, 2 ± 0.3; 8 ± 0.7 and 15 ± 0.9mm, while *Streptomyces A*, *Streptomyces D*, *Streptomyces Ea*, and *Streptomyces Ep did not inhibit M.* TB.

CONCLUSION: Thus, from the results obtained, it can be concluded that *Streptomyces* extract mainly *Streptomyces G* has promising anti-TB activity by preliminary *in vitro* techniques. Therefore, it has the definite potential as a source of compounds that may be developed further into antimycobacterial drugs.

Introduction

Tuberculosis (TB) remains a public health problem and is considered one of the main causes of death worldwide. TB is a disease caused by infection with the bacteria called *Mycobacterium tuberculosis.* The treatment of TB is substantially more expensive and difficult to treat, with higher rates of treatment failure and mortality. The global incidence rate for TB is growing each year by approximately 1.1% and the number of cases by about 2.4%. According to the statistics of the world health organization (WHO), nearly 10 millions are infected annually. Approximately 1.2–1.4 million people die each year due to the infection by *M. tuberculosis*. Resistance to anti-TB drugs continued to be recognized as a clinical problem through the latter part of the 21st century. As a result, MDR and XDR TB are now becoming a major threat to health worldwide, accounting for almost 3% of all newly reported cases of TB [1]. Due to increased drug resistant strains of bacteria such as *M. tuberculosis* there has been renewed interest in *Streptomyces* sp. as potential sources of novel antibiotics. *Streptomyces* is a filamentous Gram-positive bacterial genus and the most important source of antibiotics for medical, veterinary and agricultural uses. This bacterial genus produces a wide range of structurally diverse compounds with various pharmaceutical applications such as antifungal, antiviral, antitumoral, antihypertensive, immunosuppressive, as well as antimicrobial [2], [3]. At present, 80% of the antibiotics are sourced from the genus *Streptomyces*, being the most important actinomycetes [4]. Antibiotics, such as chloramphenicol, rifampicin, vancomycin, avermectin, daunomycin, and clavulanic acid among others, are isolated from different *Streptomyces* spp. [5], [6]. *Streptomyces* play a relevant role in soil ecology and are also of important biotechnological interest as they produce several bioactive metabolites. Screening directed towards novel antibiotics from *Streptomyces* has been intensively pursued for many years by researchers. Each year screening of *Streptomyces* strains as source of new antimicrobial compounds are directed by many pharmaceutical companies [7], [8], [9]. Although different bioactive compounds have been isolated from *Streptomyces*, these are thought to represent only a small fraction of the repertoire of bioactive metabolites produced. The previous studies show that this group of microorganisms still remains a rich source of important antibiotics [10], [11]. *Streptomyces* spp. have been

identified primarily using conventional classification methods based on their morphological and phenotypic characteristics. The impact on the taxonomy of *Streptomyces* increased over the past few decades due to the use of molecular biology methods, such as *16S rRNA* gene sequencing and BOX-polymerase chain reaction (PCR) fingerprinting [12], [13], [14]. *Streptomyces* sp. are used as a potential natural source for the production of abundant classes of clinically and economically significant secondary metabolites including antibiotics. They are found in a wide range of habitats but are particularly abundant in soil, representing around 1–20% of the total viable count and are known to prefer the characteristic earthy smell (geosmin) [15]. The study of *Streptomyces* sp. from diverse ecosystems could help to uncover new specific molecular targets and novel bioactive compounds because of their genetic variability. However, the *Streptomyces* sp. screening program in Indonesia is still in its early stages. We believe that many *Streptomyces* sp. from Indonesian soil have not been explored and isolated to find new strains as a source of antibiotics for the treatment of TB disease. Therefore, this study was conducted to test *in vitro Streptomyces* sp. isolated from Lapindo mud in Sidoarjo, Indonesia, as a candidate for anti-TB.

Methods

Isolation of Streptomyces spp. from Lapindo mud samples

The Lapindo mud sample collected from Sidoarjo, Indonesia, was used for the isolation of *Streptomyces* spp. Briefly, 1 g of the soil sample was transferred into a flask containing 10 mL distilled water. Then, it was filtered through a two-layered muslin cloth. The sample was diluted to 10–3, 10–4, and 10–5 concentrations. Next, 0.2 mL of each dilution was placed on starch agar medium (starch 9.0 g, l-asparagine 9.0 g, ammonium sulfate 2.0 g, Tris 2.0 g, sodium chloride 1.0 g, dipotassium sulfate 0.5 g, magnesium sulfate 0.2 g, calcium chloride 0.1 g, trace solution 1 mL, potassium dihydrogen phosphate 0.5 g, and agar 15 g, all of which were dissolved in 1 L distilled water at pH 7.0) plates, supplemented with the antifungal agent nystatin (50 μg mL^{-1}), and incubated for 7 days at 35 ± 20°C. Plates with approximately 200 colonies were selected. Single colonies were streaked on the same medium to purify selected colonies [10], [16].

Total DNA isolation

Khattab *et al.* reported that molecular and bioinformatics analyses were conducted to identify *Streptomyces* strains. Genomic DNA was extracted using the Corbin method with several modifications, according to a previously described protocol [10], [17]. Briefly, one colony was cultured in 50 mL of liquid ISP4 medium at 28°C in a shaking incubator for 18–24 h. Then, the culture was centrifuged at 5000 rpm for 3 min, and the resulting supernatant was discarded. Then, *Streptomyces* were collected, by being suspended in Solution I containing 1 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate 10 mM Tris (pH 7.4), and 0.1 mg/mL proteinase K. Furthermore, the *Streptomyces* suspension was added to Solution II containing 0.8 M NaCl and 1% CTAB for 1 h at 37°C, it will be added to the lysate, and then incubated for 20 min at 65°C. The sample was extracted using chloroform: Isoamyl alcohol with the same volume (24:1). The nucleic acid is precipitated in the aqueous phase with isopropanol and then purified using 70% ethanol.

Amplification and sequencing of 16S rRNA gene by PCR

The primers Strep F; 5-AGAGTTTGAT CCTGKGTCAG-3 and Strep R; 5-AAGGGAG GTGATCCAKKGKGA-3 were used in PCR amplification of the *16S rRNA* gene against *Streptomyces* strains [18]. Each primer of the PCR mixture in 50 l polymerase buffer contained 30 pmol, 100 ng of chromosomal DNA, 200 M dNTPs, and 2.5 U of Taq polymerase. The primary denaturation temperature for PCR amplification was 94°C for 1 min, followed by 94°C for 1 min, and the annealing temperature was 57°C for 60 s. The extension step consisted of 35 cycles of 72°C for 60 s. The final extension was performed at 72°C for 5 min. Then agarose gel electrophoresis was used to analyze the PCR reaction mixture, which is a size marker. In addition, it is indicated by the use of Nucleic Acid Gel Electrophoresis and Blotting (Thermo Scientific™ Fermentas GeneRuler DNA Ladder Mix, USA) 1 kb. The remaining mixture was purified using QIA rapid PCR purification reagent (Qiagen, USA). The Terminator Cycle Sequencing kit was used to obtain the *16S rRNA* gene sequence of both strands (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA).

Gel electrophoresis and photography

To separate the PCR amplification products, the mini-gel set (Bio-Rad, USA) was used to process 1% w/v ultrapure agarose powder in 1 × TBE buffer (pH 8.3) at 100 V for 60–70 min. The gel was stained with ethidium bromide (0.5 g/mL) and then analyzed using BioDocAnalyze (Biometra, Germany). A molecular weight marker of 250 bp was used.

Fermentation and extraction of secondary metabolites of Streptomyces Sp.

Spores (10^7/ml) of the isolate were used to inoculate 1000 ml Erlenmeyer flasks containing 200 ml

of ISP 1 broth supplemented with 1% (w/v) of glucose and magnesium. After incubation at 30°C for 24 h in an orbital incubator shaker at 200 rpm, this pre-culture was used to inoculate (5% v/v) 15 L culture medium having the same composition as the pre-culture. After 6 days of incubation, the culture broth was filtered to separate mycelium and supernatant, the mycelium was lyophilized, extracted with acetone and concentrated on a rotary evaporator. The supernatant was extracted twice with equal volume of ethyl acetate and the combined organic layers were evaporated to obtain the ethyl acetate extract (EA extract).

Culture and preparation of M. tuberculosis

M. tuberculosis strains H37Rv were obtained from the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. *M. tuberculosis* was cultured at 37°C in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) supplemented with 0.2% glycerol (Sigma Chemical Co., St. Louis, MO) and 10% oleic acid albumin dextrose catalase (Becton Dickinson) until logarithmic growth was reached. Each culture was mixed with a sufficient volume of sterile supplemented Middlebrook 7H9 broth to achieve a turbidity equivalent to that of McFarland's No. 1 standard. To obtain the test inoculum, this suspension was further diluted 1:50 with the same culture medium to approximately 6×10^6 colony-forming units (CFU)/mL immediately before use [18].

Anti-tuberculous activity by the paper disc diffusion method

Screening of *Streptomyces* Sp. extract for antimycobacterial activity against *M. tuberculosis* strain *H37Rv* was done using the paper disc diffusion method. *Streptomyces* Sp. were carried out in Aquadest solution of 300 μg/ml each and were slowly absorbed into the sterilized paper disc (diameter: 8 mm, Whatman, England) and adhered to the surface of the plate on which *M. tuberculosis* strains H37Rv at a concentration of 6×10^6 CFU/ml had been inoculated in Middlebrook 7H9 broth. Sterilized distilled water was used as a control. After culturing for 24 h in an incubator at 37°C, antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the discs [8].

Results

Agarose gel electrophoresis of PCR amplification products of genomic DNA of Streptomyces sp.

Different nucleotide sequence lengths were detected in the sequencing results of the eight *Streptomyces* spp. from. Electropherogram images (Thermo Fisher Scientific, USA) and nucleotide sequence data of *Streptomyces A*, *Streptomyces D*, *Streptomyces Ea*, *Streptomyces Ep*, *Streptomyces I*, *Streptomyces F,* and *Streptomyces G* were obtained, which produced bands with a dominant size of approximately 1500 bp. This size of the bands was confirmed using the 16S rRNA gene, that is, 1500 bp, and the subsequent bands were sequenced using the automatic ABI Prism 310 method (Figure 1). Sequence analysis of rRNA genes has been applied to streptomyces taxonomy to investigate relationships at the genus, species and strain level. 16S rRNA comparisons have also been used to investigate the taxonomic status of natural isolates.

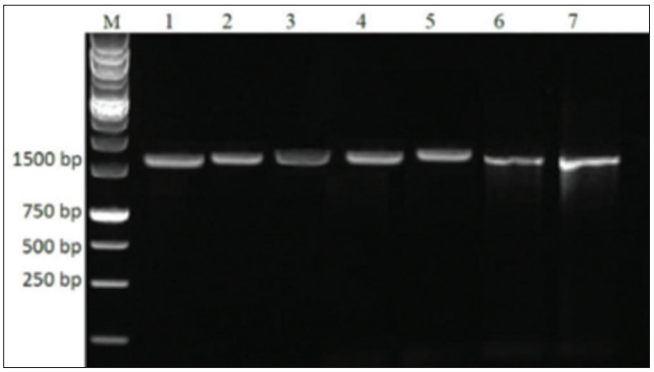

Figure 1: The electrophoresis of agarose gel polymerase chain reaction amplification product of Streptomyces spp. genomic DNA isolated from Lapindo mud in Sidoarjo, Indonesia. Lane M: Marker, Lane 1: Streptomyces A, Lane 2: Streptomyces D, Lane 3: Streptomyces Ea, Lane 4: Streptomyces Ep, Lane 5: Streptomyces I, Lane 6: Streptomyces F, Lane 7: Streptomyces G

Inhibition zone of Streptomyces Sp. extract against M. tuberculosis H37Rv on the paper disc method

The antimycobacterial activity of *Streptomyces* Sp. extract against *M. tuberculosis* strains H37Rv was done using the paper disc method. Inhibition zone was defined as *Streptomyces* Sp. extract that induced the clear inhibitory zone formed around the discs. The inhibition zone of *Streptomyces* Sp. extract against *M. tuberculosis* strains H37Rv, namely, *Streptomyces A*, *Streptomyces D*, *Streptomyces Ea*, *Streptomyces Ep*, *Streptomyces I*, *Streptomyces F,* and *Streptomyces G* with inhibitory zones, respectively, 0; 0; 0; 0; 2 ± 0.3; 8 ± 0.7; and 15 ± 0.9 mm (Table 1).

Table 1: The anti *Mycobacterium tuberculosis* H37R activity of *Streptomyces* Sp. extract

Streptomyces Sp.	Inhibition Zone (mm) X ± SD
Streptomyces A	0
Streptomyces D	0
Streptomyces Ea	0
Streptomyces Ep	0
Streptomyces I	2 ± 0.3
Streptomyces F	8 ± 0.7
Streptomyces G	15 ± 0.9

The *Streptomyces* Sp. extract from the Lapindo mud, Sidoarjo, Indonesia, showed activity against

mycobacteria strains, especially M. tuberculosis. *This study revealed that Streptomyces G* strongly *inhibited M. tuberculosis, Streptomyces I, and Streptomyces F* weakly inhibited *M. tuberculosis,* while *Streptomyces A, Streptomyces D, Streptomyces Ea,* and *Streptomyces Ep* did not inhibit *M. tuberculosis* (Figure 2). *Streptomyces* has a long history in the treatment of tuberculosis. A breakthrough in TB therapy came after the discovery of Streptomycin, isolated primarily from the actinobacterium, *Streptomyces griseus*. Various other anti-TB antibiotics such as kanamycin and rifampicin have also been reported from actinobacteria. While screening the actinobacteria for potential molecules, possible reisolation of already known molecules becomes a major concern.

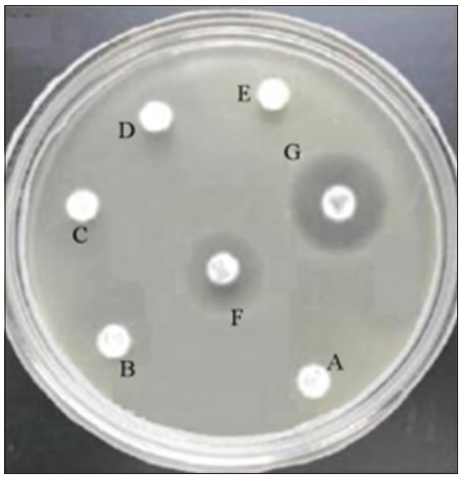

Figure 2: Inhibition Zone of Streptomyces Spp. extract against Mycobacterium tuberculosis H37Rv on the plates of middle brook 7H9 broth namely Streptomyces A (A), Streptomyces D (B), Streptomyces Ea (C), Streptomyces Ep (D), Streptomyces I (E), Streptomyces F (F), and Streptomyces G (G)

Discussion

TB has been a major health problem for developing countries including Indonesia. The increasing resistance of the disease to first- and second-line drugs has demanded the need for a new search for anti-mycobacterial agents that could be effective, efficient, non-toxic, and cost effective [6]. Furthermore, continuing the search for new bioactive compounds is important because of the increasing number of antibiotic-resistant bacteria every year. However, huge challenges exist in this regard because identifying new secondary metabolites is extremely difficult, which thus requires the isolation, characterization, and screening of new members of the genus *Streptomyces* [5], [8]. Moreover, several newly confirmed bioactive compounds are derived from *Streptomyces* from unexplored habitats, which might be extremely rich sources of antibiotics. Therefore, we isolated *Streptomyces* spp. from the Lapindo mud of Sidoarjo, Indonesia. The *16S rDNA* gene was amplified using primers to identify *Streptomyces* isolates, followed by PCR for molecular identification, which is a sensitive and specific detection method for *Streptomyces*. The 16S rDNA target gene was used for the selected PCR primer for detecting the eight *Streptomyces* isolates. The BLAST was used to compare the *Streptomyces 16S rRNA* gene sequences and those in public databases, consistent with that recommended in the NCBI website [19], [20]. This was done to determine the similarity between sequences in the GenBank database. In the present study, we used *16S rRNA* gene sequencing to classify *Streptomyces* isolates from Lapindo mud in Surabaya, Indonesia and identified new, specific strains that can produce anti-mycobacterial agents to be used as alternative drugs. The results showed that new types of *Streptomyces* spp., namely, *Streptomyces A*, *Streptomyces D*, *Streptomyces Ea*, *Streptomyces Ep, Streptomyces I*, *Streptomyces F,* and *Streptomyces G from* Lapindo mud in Sidoarjo, Indonesia. This new strain can produce antibiotics to be used as an alternative to anti-mycobacterial agents; however, further research is needed to confirm the activity. The result of this study also showed *Streptomyces* preparations from Lapindo mud have a greater anti-mycobacterial activity mainly *Streptomyces G* strongly *inhibited M. tuberculosis.* Several studies have shown that *Streptomyces* sp. is capable of producing metabolites or antibiotics that have anti-TB activity. Abouwarda and El-Wafa [21] proved that *Streptomyces* sp. Egypt isolates had anti-mycobacterial activity and were identified as *Streptomyces nigrifaciens*. Until now, several antibiotics produced by *Streptomyces* sp. is still the drug of choice for anti-TB. The antibiotic streptomycin was produced by *S.* griseus, erythromycin from *Streptomyces erythreus* and *Streptomyces rifamycin* from *Streptomyces mediterranei* [5], [11]. The genus *Streptomyces* is widely used in the production of secondary metabolites, such as antibiotics, antifungal, antiparasitic, and anticancer agents, possessing diverse biological activities [8].

Conclusion

Thus, from the results obtained, it can be concluded that *Streptomyces* extract mainly *Streptomyces G* has promising anti-TB activity by preliminary *in vitro* techniques. Therefore, it has the definite potential as a source of compounds that may be developed further into antimycobacterial drugs.

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