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Differentiation complex sputum microbiome in patients suspected TB pulmonary

Deby Kusumaningrum [1,2,3*], Ni Made Mertaniasih [2,3,4], Soedarsono Soedarsono [3,4,5], Rahadian Pratama [6]

[1] Doctoral Degree Program, Faculty of Medicine, Universitas Airlangga, Surabaya, INDONESIA
[2] Department of Medical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, INDONESIA
[3] Dr. Soetomo General Academic Hospital, Surabaya, INDONESIA
[4] Tuberculosis Laboratory, Institute of Tropical Disease, Universitas Airlangga, Surabaya, INDONESIA
[5] Department of Internal Medicine Sub Pulmonology, Faculty of Medicine, Universitas Hang Tuah, Surabaya, INDONESIA
[6] Department of Biochemistry, Faculty of Mathematics and Natural Science, Institut Pertanian Bogor, Bogor, INDONESIA
***Corresponding Author:** deby-k@fk.unair.ac.id

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ABSTRACT

Purpose: This is the first study to attempt microbiome diversity using metagenomic full-length 16S rRNA from respiratory specimens suspected of chronic pulmonary TB patients.

Materials and methods: A 33 patients with suspected chronic pulmonary TB were included. Sputum specimens were cultured to detect *mycobacterium sp.* and extracted using QiAmp DNA mini kit modification and 16S rRNA metagenomic sequencing by nanopore grid ion sequencer. Microbiome analysis was performed using Pavian and Krona tools.

Results: 9 patients were diagnosed with TB based on GeneXpert MTB/RIF assay, and 3 patients were detected with NTM pulmonary infection.The common genera identified from TB culture positive patients were *streptococcus sp.*, *prevotella sp.,* and *veilonella sp.* However, less was detected in two NTM infection patients. Metagenomic analysis revealed community bacteria species, including mycobacterium tuberculosis and NTM species, with the lowest number of unique reads. The abundance of *streptococcus sp.* were less than 30% in 4 patient with comorbid diabetes mellitus.

Conclusions: Metagenomic targeted 16SrRNA full-length sequencing in the clinical respiratory specimen can provide diagnostic insight beyond standard microbiologic cultures and detailed profiling of microbial communities at the species level.

Keywords: metagenomic, microbiome, tuberculosis, nontuberculous mycobacteria

INTRODUCTION

A major hazard to international health, *mycobacterium tuberculosis* (MTB) is the infectious disease known as tuberculosis (TB). Based on the global WHO report 2020, Indonesia has the second highest TB burden in the world after India. Country data reported that number of estimated TB cases remained steady at 969,000 in 2022 and 2021. However, confirmed TB cases increased from 443,235 to 713,184 between 2021 to 2022 [1]. In Indonesia's East Java Province, Dr. Soetomo Hospital serves as a referral hospital; on the other hand, East Java is a TB endemic area which has a high incidence rate, especially Surabaya City, with the highest TB cases (4,475) in 2021 [2]. In endemic TB areas, the pulmonary TB patient with chronic lower respiratory tract infection has the risk factor of repeating infection (recurrent TB) [3] or infection from another microbe in the environment.

On the other hand, nontuberculous mycobacteria (NTM) infections have increased in recent decades owing to the increasing prevalence of HIV infection and considerable advancements in laboratory procedures and diagnostic tools [4]. According to a previous systematic review, the five-year mortality rates of infection NTM in Europe were 27%, 35% in the United States, and 33% in Asia [5]. All drugs used to treat TB and NTM diseases have been linked to resistance, particularly those that are multidrug resistant (MDR) and extensively drug resistant (XDR) [6]. There is a need for better detection tools to help patients choose the best course of treatment as a result of the emergence and spread of MTB and NTM infections, particularly MDR and XDR strains.

In complex ecological communities, members of the regular flora or atypical bacteria that also contribute to illnesses are typically ignored [7]. Microbial community dynamics, interaction, and functionality are thought to be essential for maintaining ecological harmony and life. The bacterium in the body is one newly discovered host component that could contribute to TB. The microbiota is the aggregate name for the bacteria, viruses, and fungi that live inside the human body. They take part in many cellular processes and are essential parts of various organ systems. Processes, metabolism, and disease development are all impacted. The

immune system's operation, the abnormal metabolism that causes chronic inflammation, and cellular transformation are all modulated by different populations of microorganisms that reside in various cellular compartments. Yet, the microbiota's contribution to respiratory disease is unclear [8]. Several research have looked into the microbiome of sputum, the respiratory system, and the lungs in relation to cystic fibrosis or TB in recent years [9, 10].

Human microbiome research is currently making use of high-throughput next generation sequencing (NGS) technologies to investigate the bacterial communities that live inside humans. The gold standard for investigating microbial diversity nowadays is 16S rRNA sequence sequencing, which allows for precise taxonomic profiling of the prokaryotic genome [11, 12]. These techniques allow for the direct analysis of the microbiota of various human body organs utilizing DNA isolated from samples without using culture procedures [13]. NGS is a cost-effective technique for genotyping a large number of MTB isolates [14]. Metagenomic sequencing and targeted amplicon sequencing, which involve selectively amplifying a particular genetic area of interest, like 16S rRNA in bacteria, and untargeted amplification of all genomic DNA, are the two main categories of contemporary high-throughput sequencing technology [11, 15, 16]. Theoretically, mNGS can identify every pathogen present in a clinical sample, making it particularly useful for complex and chronic infection disease disorders [17, 18]. This study purposes to investigate the microbiome diversity in sputum patients suspected chronic pulmonary TB by metagenomic targeted 16S rRNA method, and compare to clinically sign, symptom also culture of mycobacteria result. Comparing the quantity and diversity of microorganisms in each patient with chronic suspected may lead to improve understanding of the etiologic diagnosis of pulmonary TB for the good management of patients.

MATERIALS AND METHODS

The Patient Samples

Patients with suspected chronic pulmonary TB who attended Dr. Soetomo Academic Hospital as a referral hospital in East Java Province in Indonesia between November 2021-January 2022 were eligible. Patients who fulfilled the inclusion criteria were given an explanation by the investigators of the study's purpose and methodology. Upon arrival, medical history and informed written consent were taken. Patients with suspected chronic pulmonary TB were recruited. The demographic information data, i.e., age and sex, and the clinical data, i.e., signs, symptoms, radiological profile, and comorbid were recorded from the medical record.

The Sputum Samples

The sputum was gathered from suspected chronic pulmonary TB patients. The samples were immediately sent for culture method, Xpert MTB/RIF and metagenomic next-generation sequencing (mNGS). The isolation of bacteria and mycobacterium identification detected were processed in the Lowenstein Jensen medium. Samples for Xpert were processed following the manufacturer's instructions.

The Extraction of DNA

The QiAamp DNA Kit, a commercially available kit, was used to extract DNA from all sputum samples in accordance with the modified methodology provided by the manufacturer. A sample of sputum was moved to an Eppendorf tube and stored on dry ice. About one milliliter of sputum sample was decontaminated with Mycoprep attentively up until the point of homogeneity [19]. Qubit1 2.0 Fluorometer and NanoDrop spectrophotometer (Invitrogen, Eugene, OR, USA) were used to measure the concentration of DNA. Metagenomic technique was used to process the ideal DNA amount and quality. The optimal DNA quantity and quality were processed to metagenomic procedure.

Table 1. Demographic patient suspected TB

Variable		N	P (%)
Sex	Female	11	33.3
	Male	22	66.7
Age	< 20	2	6.06
	20-30	4	12.1
	31-40	1	3.03
	41-50	6	18.1
	51-60	7	21.2
	> 60	13	39.3
GeneXpert MTB/RIF assay	Negative	24	72.7
	MTB detected rifampicin sensitive	4	12.1
	MTB detected rifampicin resistance	5	15.1
Comorbid	Diabetes mellitus	11	33.3
	HIV	2	6.06
Culture mycobacteria result	Negative	22	66.7
	MTB detected	8	24.2
	NTM	3	9.09
	Previous TB history	6	18.8

Note. P: Percentage

Rapid attachment chemistry for the 16S primer was used to ramp up the genomic DNA, and universal primers (27F and 1429R) were used in PCR to amplify the entire 16S rRNA gene. Using the Bioline, BIO-25048 My Taq HS read mix for PCR amplification. 2µL PCR product were assessed by electrophoresis with 0.8% TSB agarose. After the attachment of rapid ID sequencing adapters, the next step were primed and loaded gDNA at GridIon sequencer. Oxford Nanopore Technology kits were used for the production of the libraries [20]. The MinKNOW software version 20.06.9 was used to operate the sequencing at GridION (ONT, Oxford, UK). High precision mode was used when performing base calling with Guppy version 4.0.11. We used NanoPlot to visualize the quality of the FASTQ files [21]. The Centrifuge classifier was used to classify the filtered readings [22]. Downloaded from the centrifuge website was the Bacteria and Archa. Pavian was utilized to conduct downstream analysis and visualizations and Krona Tools.

Ethical Approval

The Declaration of Helsinki and the ethical committee guidelines (number 0918/122/4/IX/2021) of Dr. Soetomo General Academic Hospital Ethic Committee were followed in conducting this study. Informed consents were signed by patients or surrogates.

RESULTS

We enrolled 33 patients at the X referral hospital in Surabaya, Indonesia, from November 2021 to January 2022 who were suspected of having chronic pulmonary TB. The demographic data is presented in **Table 1**.

Table 2. The clinical, radiologic, and culture mycobacteria characteristic of suspected PTB patients

SN	Clinically performance	Sex	Age	Radiology appearance	Comorbid	GeneXpert MTB/RIF assay	History TB	Culture mycobacteria result
3DB	Cough, fever, & night sweat	M	57	Fibroinfiltrat parahilar LL, multiple cavitas parahilar RL, & LL	DM	MTB rifampicine sensitive	Drop out TB treatment	Positive MTB
4DB	Cough & night sweat	M	37	Normal	Langerhans cell hystocytosis	Negative	No history TB	Negative MTB
5DB	Cough & hemoptysis	F	63	Fibroinfiltrat URL	DM	Negative	Previous TB	Negative MTB
6DB	Cough, dyspnea, & hemoptysis	F	68	Fibroinfiltrat LL and RL, multiple cavitas parahilar RL, & LL	No comorbid	Negative	Previous TB	NTM positive
7DB	Cough & dyspnea	M	48	Fibroinfiltrat LL & RL, multiple cavitas RL, LL, consolidation RL, & LL	DM	MTB + rifampicine resistance	No history TB	Negative MTB
8Db	Cough & dyspnea	M	53	Infiltrat LL and RL	Carcinoma nasopharynx	Negative	No history TB	Negative MTB
9DB	Cough & dyspnea	M	58	Bronchiectasis, effusion pleura R, & paracardial R	DM	MTB + rifampicine sensitive	No history TB	Positive MTB
10DB	Cough & dyspnea	M	62	Infiltrat RL & effusion pleura R minimal	DM	Negative	No history TB	Negative MTB
11DB	Cough & dyspnea	F	69	Infiltrat RL, LL, & tree bud lobus superior	No comorbid	Negative	No history TB	NTM positive
12DB	Cough & dyspnea	F	65	Fibro infiltrat suprahilar RL & LL	COPD	Negative	No history TB	NTM positive

Note. SN: Sample number; M: Male; F: Female; RL: Right lung; LL: Left lung; DM: Diabetes mellitus; NTM: Non-tuberculous mycobacteria; PTB: Pulmonary TB patient; & MTB: *Mycobacterium tuberculosis*

Table 3. Result number reads unique of mycobacteria detected in metagenomic NGS

Sample number	Species of mycobacteria detected	Genome size	Num unique reads
3DB	*M.tuberculosis*	5,853,101	14
	M.branderi	5,979,623	1
4DB	*M.tuberculosis*	5,853,101	1
6DB	*M.branderi*	5,979,623	8
9DB	*M.tuberculosis*	5,853,101	46
	M.marinum	685,533	4
	M.shinjukuense	4,504,020	2
	M.parmense	5,952,912	2
	M.dioxanotrophicus	8,080,416	1
12DB	*M.tuberculosis*	5,853,101	1

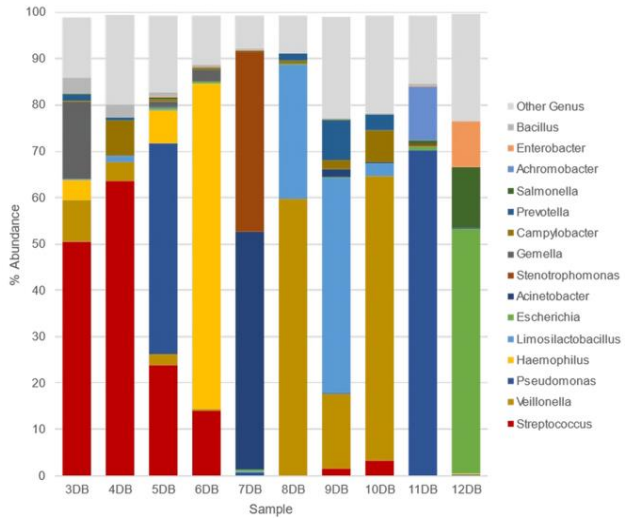

Figure 1. Percentage of abundance bacteria detected from suspected TB patients (Source: Authors' own elaboration, from sputum specimen)

After the process for DNA extraction, there were only 10 sputum processed because of the good quantity and quality of DNA results, and for the genomic DNA examined for Metagenomic procedure. The clinical signs and symptoms of ten patient that were recruited in these study listed in **Table 2**, mentioned cough was chief of complain of the patient. The radiology appearance of 8/10 patients showed several infiltrate in pulmonary and comorbid diabetes mellitus was detected in 5/10 patients.

NGS was used to examine sputum samples using bacterial-specific cultivated mycobacteria. According to the results of the mycobacteria culture, the samples were classified as NTM positive, MTB negative, and MTB positive. Mycobacteria sequences were found in the NGS 5/10 cases, with specific species recognized, such as mycobacterium TB and *M. branderi*. These results from the mycobacteria culture were similar to the NGS results (in amount of number unique reads **Table 3**).There was no significance differentiation between mycobacteria culture detected or negative and result mNGS for sequence of mycobacteria (**Table 3**).

The sputum microbiome was analyzed using NGS, which showed that a variety of microorganisms dominated the sample. The sputum samples contained fifteen main genera in total (**Figure 1**). Taxonomic composition of sputum samples 6/10 (60%) were detected genus Streptococcus with various percentage of abundance. Patients that detected Streptococcus in these cases are detected in patients with negative culture of MTB, but also detected in (1/10) MTB cultured positive and (1/10) NTM positive. Interestingly, abundance of streptococcus were less than 30% abundance in 4 patient with diabetes mellitus (**Table 2**).

Streptococcus and *veillonella* accounted for the majority of cases in the suspected TB patients with culture-positive TB

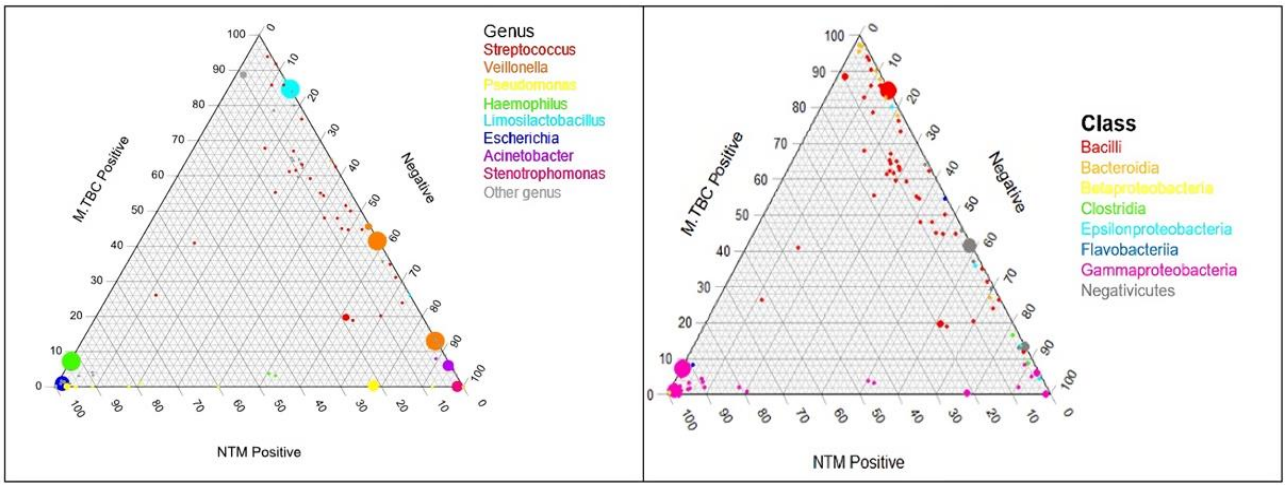

Figure 2. Correlation between result culture mycobacteria detected and the abundance microbiota genus and class among suspected TB patient (MTBC positive: Culture MTBC in LJ medium detected; NTM positive: Culture NTM in LJ medium detected; & Negative: Not growth) (Source: Authors' own elaboration, from sputum specimen)

detection. In the negative cultured patient group, cases were dominated by *streptococcus*. Other genera, such rothia, were more common in TB patients, while actinobacillus was more common in normal people.

Patients with non-TB Mycobacteria positive cultures had similar bacterial compositions at the genus level, with a noticeable predominance of the gram-negative genus pseudomonas (sample 11 DB) and *escherichia coli* (sample 12 DB), which together make up over 80% of the bacterial community without dominating *streptococcus sp*. (**Figure 2**). One sample detected with acinetobacter (up to 50%) from top ten these genus phyla comprised of the total bacterial community with negative culture mycobacteria.

DISCUSSION

This research provides the first report from patients with chronic pulmonary TB at referral facility of hospital (Surabaya) in the Indonesian Province of East Java. It also serves as a pilot study to assess using 16S nanopore sequencing to investigate the sputum microbiome metagenomically. We chose to adopt nanopore sequencing technology because it can assist overcome the shortcomings of existing diagnostic testing and potentially deliver quick, low-cost, real-time identification of causal microorganisms in a hospital context [23, 24]. As a first step toward improving our knowledge of the makeup of microbes and their role in identifying the likelihood that complex microbial interactions in the respiratory tract and lung tissue will give rise to disease phenotypes, high-throughput DNA sequencing of the V1-V9 region of the 16S rRNA full length gene sequence has been undertaken. The highest level of taxonomic and phylogenetic resolution for the identification of bacteria is provided by the full-length 16S rRNA sequences [11, 25]. Environmental microbiology and clinical sample analysis have long employed the method of measuring bacterial diversity through sequencing of 16S ribosomal RNA (16S rRNA) genes, especially with the introduction of high-throughput sequencing technologies[26].

The lungs frequently had a varied microbiome with low microbial biomass before it was recognized as a sterile environment. This microbiome included the *prevotella, streptococcus, veilonella, fusobacterium*, and *haemophilus* species. The composition of the lung microbiome is determined by the balance between the adaptive immune system and microbial migration from the upper respiratory tract and mouth cavity. Representation of Dickson's initial model. The equilibrium between immigration (micro-aspiration) and elimination (cough, mucociliary clearance, and immunological defenses) determines the microbial community in the lungs of healthy humans [27].

In this study, nearly half of the sputum of suspected pulmonary TB patient samples had strains of the *streptococcus* bacteria, and in agreement with previous studies [28]. Two samples from our study detected dominated anaerobic genus bacteria, veilonella. The lungs contain the majority of the anaerobic microbiome, not the mouth cavity. Numerous investigations have shown that people with chronic chest lesions have substantial amounts of harmful anaerobic bacteria in their lungs when cultured [29]. The result of anerobic bacteria, such as veilonella, in several studies from sputum patient [30] was similar with studies in CF patient.

In the study [31], the results of sequencing sputum from 120 TB patients in Uganda who had not yet received treatment were mentioned. Additionally, the study examined alterations in the microbiome of thirty patients whose follow-up samples showed therapy response. All things considered, structural changes and microbiological abundance were associated with anti-TB drugs and HIV status. The most common genera throughout time were *fusobacterium, gemella, rothia, haemophilus, neisseria, alloprevotella, veillonella*, and *streptococcus* [30]. These findings are consistent with our analysis, which found that two samples had positive cultures of the two major taxa. *Haemophylus* and *streptococcus* were mycobacteria.

In the other published study [32], sixteen sputum samples were successfully sequenced, and *Haemophilus* was found in the sputum of newly diagnosed and recurring TB patients, respectively, in amounts of 50% and 22.2%, whereas it was not present in the sputum of healthy controls. It was specifically observed *haemophilus influenzae* and *streptococcus pneumoniae* in the sputum of patients with recurrent TB, but only *neisseria flavescens* and *haemophilus influenzae* in patients with new cases of TB [32].

Research in [33] mentioned the findings demonstrated that 12 of the 36 TB cases were missed by mNGS. The majority of the twelve examples of false negatives (7.50%, 9/12) were extrapulmonary instances. The fact that Mtb, one of the internal bacteria, releases relatively fewer external nucleic acids may ultimately make it more challenging to identify Mtb using mNGS [18, 33]. In our investigation, just one patient had a false-positive TB test result (7DB). Contrary to the mNGS result, patient number 7DB's culture and PCR results showed that MTB was not present in that patient. The diagnostic performance of mNGS was further constrained by the discovery of non-pathogenic microbes. The inability of MTB to release enough genomic DNA may possibly contribute to the mNGS false-negative outcome. Furthermore, because clinical samples' MTB content was insufficient and below the analytical mNGS concentration. The inherent shortcomings of culture-based microbiology can be mitigated by molecular-based diagnostic techniques, which also offer a more detailed description of the complete bacterial community at a particular anatomical location [34]. The other study mentioned the 16S method is not the most effective strategy to research the TB-associated microbiome since it underrepresents mycobacterium [10].

Exposure to NTM occurs often since they are environmental organisms; nevertheless, very few exposed people become infected or develop fulminant lung illness. This suggests that a wide range of host variables, including structural lung abnormalities, immunological or genetic problems that predispose to disease, hallmarks of connective tissue disease, and the absence of any clear predisposing circumstances, can be responsible for NTM disease, frequently with a preference in frail older women [4]. In this study were detected several NTM species such as *mycobacterium branderi*, *mycobacterium marinum,* and *mycobacterium shinjukuense* by mNGS method, although in limited number unique reads. Another identified source of human illness is the slow-growing, non-pigmented, NTM referred to as mycobacterium *branderi* [35]. However NTM species from culture were detected 3/33 in our investigation. Hence, NTM infection diagnosis molecular utilizing [36].

Our study has certain limitations. Firstly, the study's findings and hypothesis were based on a small sample size. Second, because the study was retrospective and single-centered, it lacked some clinical data, such as treatment information prior to sample collection. A prospective, multi-centered study with a larger sample size is required for additional inquiry and increased credibility.

Finally, sputum microbiota identification at the genus level was made possible using the NGS technique, Suspected chronic pulmonary TB patients' sputum microbiome was discovered to be highly diversified and noticeably distinct. All taxonomic levels–phylum, genus, and species–reflected diversity. Overall, these results point to the possibility that the accessory microbiota may also be significantly influencing the dynamics of the sputum microbiota, however it is unclear what this means in terms of pathobiology. The complexity of the microbiota should be influenced by geographic, ecological, and socioeconomic factors, underscoring the significance of comprehensive approaches and methods for TB diagnosis, control, and prevention. Moreover, these findings could facilitate the creation of antimicrobial therapy guidelines, particularly for patients with suspected pulmonary TB, in order to guarantee successful treatment plans for patients harboring both the pathogen and colonizing bacteria.

Author contributions: **DK:** conceptualization, resources, formal analysis, writing - original draft, methodology, data curation: **NMM:** methodology, supervision, conceptualization; **SS:** methodology, supervision, conceptualization, data curation, writing - review & editing; **RP:** formal analysis, writing - original draft, methodology, data curation. All authors agreed with the results and conclusions.

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Ethical statement: The authors stated that the study was approved by the Soetomo Academic Hospital Ethical Committee on 6 December 2021 with approval number 0918/122/4/IX/2021. Written informed consents were obtained from the participants.

Declaration of interest: No conflict of interest is declared by the authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

REFERENCES

1. MoH. Factsheet country profile Indonesia 2022. Ministry of Health Indonesia, 2022. Available at: https://tbindonesia.or.id/wp-content/uploads/2023/02/Factsheet-Country-Profile-Indonesia-2022.pdf (Accessed: 26 July 2024).
2. CBS. East Java Central Bureau of Statistics. Available at: https://jatim.bps.go.id/id/statistics-table/1/MzAwMSMx/jumlah-jenis-penyakit-malaria--tb-paru--pneumonia--kusta-menurut-kabupaten-kota-di-provinsi-jawa-timur--2022.html (Accessed: 26 July 2024).
3. Gadoev J, Asadov D, Harries AD, et al. Recurrent tuberculosis and associated factors: A five-year countrywide study in Uzbekistan. PLoS One. 2017;12(5): e0176473. https://doi.org/10.1371/journal.pone.0176473 PMid:28472053 PMCid:PMC5417503
4. Griffith DE, Aksamit TR. Nontuberculous mycobacterial disease therapy. Chest. 2016;150(6):1177-8. https://doi.org/10.1016/j.chest.2016.07.015
5. Diel R, Lipman M, Hoefsloot W. High mortality in patients with mycobacterium avium complex lung disease: A systematic review. BMC Infect Dis. 2018;18(1):206. https://doi.org/10.1186/s12879-018-3113-x PMid:29724184 PMCid:PMC5934808
6. Dheda K, Gumbo T, Maartens G, et al. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. Lancet Respir Med. 2017;5(4):291-360. https://doi.org/10.1016/S2213-2600(17)30079-6 PMid: 28344011
7. Krishna P, Jain A, Bisen PS. Microbiome diversity in the sputum of patients with pulmonary tuberculosis. Eur J Clin Microbiol Infect Dis. 2016;35(7):1205-10. https://doi.org/10.1007/s10096-016-2654-4 PMid:27142586
8. Kang S-Y, Kim H, Jung S, Lee SM, Lee SP. The lung microbiota in Korean patients with non-tuberculous mycobacterial pulmonary disease. BMC Microbiol. 2021;21(1):84. https://doi.org/10.1186/s12866-021-02141-1 PMid:33736609 PMCid:PMC7977250

9. Nelson MT, Pope CE, Marsh RL, et al. Human and extracellular DNA depletion for metagenomic analysis of complex clinical infection samples yields optimized viable microbiome profiles. Cell Rep. 2019;26(8):2227-40.e5. https://doi.org/10.1016/j.celrep.2019.01.091 PMid: 30784601 PMCid:PMC6435281
10. Hu Y, Cheng M, Liu B, et al. Metagenomic analysis of the lung microbiome in pulmonary tuberculosis–A pilot study. Emeerg Microbes Infect. 2020;9(1):1444-52. https://doi.org /10.1080/22221751.2020.1783188 PMid:32552447 PMCid: PMC7473061
11. Bahram M, Anslan S, Hildebrand F, Bork P, Tedersoo L. Newly designed 16S rRNA metabarcoding primers amplify diverse and novel archaeal taxa from the environment. Environ Microbiol Rep. 2019;11(4):487-94. https://doi.org/ 10.1111/1758-2229.12684 PMid:30058291 PMCid: PMC6618113
12. Santos A, van Aerle R, Barrientos L, Martinez-Urtaza J. Computational methods for 16S metabarcoding studies using nanopore sequencing data. Comput Struct Biotechnol J. 2020;18:296-305. https://doi.org/10.1016/j. csbj.2020.01.005 PMid:32071706 PMCid:PMC7013242
13. Mizrahi H, Peretz A, Lesnik R, et al. Comparison of sputum microbiome of legionellosis-associated patients and other pneumonia patients: Indications for polybacterial infections. Sci Rep. 2017;7:40114. https://doi.org/10.1038/ srep40114 PMid:28059171 PMCid:PMC5216348
14. Meehan CJ, Goig GA, Kohl TA, et al. Whole genome sequencing of mycobacterium tuberculosis: Current standards and open issues. Nat Rev Microbiol. 2019; 17(9):533-45. https://doi.org/10.1038/s41579-019-0214-5 PMid:31209399
15. Huang Z-D, Zhang Z-J, Yang B, et al. Pathogenic detection by metagenomic next-generation sequencing in osteoarticular infections. Front Cell Infect Microbiol. 2020;10:471. https://doi.org/10.3389/fcimb.2020.00471 PMid:33042860 PMCid:PMC7527540
16. Zhang Y, Feng S, Chen W, Zhang Q-C, Shi S-F, Chen X-Y. Advantages of 16S rRNA PCR for the diagnosis of prosthetic joint infection. Exp Ther Med. 2020;20(4):3104-113. https://doi.org/10.3892/etm.2020.9082 PMid:32855678 PMCid:PMC7444347
17. d'Humières C, Salmona M, Dellière S, et al. The potential role of clinical metagenomics in infectious diseases: Therapeutic perspectives. Drugs. 2021;81(13):1453-66. https://doi.org/10.1007/s40265-021-01572-4 PMid: 34328626 PMCid:PMC8323086
18. Chen P, Sun W, He Y. Comparison of metagenomic next-generation sequencing technology, culture and GeneXpert MTB/RIF assay in the diagnosis of tuberculosis. J Thorac Dis. 2020;12(8):4014-24. https://doi.org/10.21037/jtd-20-1232 PMid:32944313 PMCid:PMC747557
19. Yang Z. Optimised protocol of QIAamp® DNA mini Kit for bacteria genomic DNA extraction from both pure and mixture sample. Protoc Exch. 2019. https://doi.org/10. 21203/rs.2.17049/v1
20. Wick RR, Judd LM, Holt KE. Performance of neural network basecalling tools for Oxford nanopore sequencing. Genome Biol. 2019;20(1):129. https://doi.org/10.1186/ s13059-019-1727-y PMid:31234903 PMCid:PMC6591954
21. De Coster W, D'hert S, Schultz DT, Cruts M, Van Broeckhoven C. NanoPack: Visualizing and processing long-read sequencing data. Bioinformatics. 2018;34(15): 2666-9. https://doi.org/10.1093/bioinformatics/bty149 PMid:29547981 PMCid:PMC6061794
22. Kim D, Song L, Breitwieser FP, Salzberg SL. Centrifuge: Rapid and sensitive classification of metagenomic sequences. Genome Res. 2016;26(12):1721-9. https://doi.org/10.1101/gr.210641.116 PMid:27852649 PMCid:PMC5131823
23. Ciuffreda L, Rodríguez-Pérez H, Flores C. Nanopore sequencing and its application to the study of microbial communities. Comput Struct Biotechnol J. 2021;19:1497-511. https://doi.org/10.1016/J.CSBJ.2021.02.020 PMid: 33815688 PMCid:PMC7985215
24. Yang L, Haidar G, Zia H, et al. Metagenomic identification of severe pneumonia pathogens in mechanically-ventilated patients: A feasibility and clinical validity study. Respir Res. 2019;20(1):265. https://doi.org/10.1186/s12931-019-1218-4 PMid:31775777 PMCid:PMC6882222
25. Febriyanti I, Djuminar A, Merdekawati F, Nur Indra AL. Comparison of purity and concentration values of mycobacterium tuberculosis DNA extraction result from the boiling and spin column method. Indones J Med Lab Sci Technol. 2023;5(2):133-45. https://doi.org/10.33086/ ijmlst.v5i2.4771
26. Matsuo Y, Komiya S, Yasumizu Y, et al. Full-length 16S rRNA gene amplicon analysis of human gut microbiota using MinIONTM nanopore sequencing confers species-level resolution. BMC Microbiol. 2021;21(1):35. https://doi.org/ 10.1186/s12866-021-02094-5 PMid:33499799 PMCid: PMC7836573
27. Fabbrizzi A, Amedei A, Lavorini F, Renda T, Fontana G. The lung microbiome: Clinical and therapeutic implications. Intern Emerg Med. 2019;14(8):1241-50. https://doi.org/10. 1007/s11739-019-02208-y PMid:31667699
28. Ticlla MR, Hella J, Hiza H, et al. The sputum microbiome in pulmonary tuberculosis and its association with disease manifestations: A cross-sectional study. Front Microbiol. 2021;12:633396. https://doi.org/10.3389/fmicb.2021. 633396 PMid:34489876 PMCid:PMC8417804
29. Moniem NA, Amin NM. Bacteria in sputum of patients with chronic chest lesions in chest department of Beni-Suef University Hospital. Egypt J Chest Dis Tuberc. 2016; 65(4):841-4. https://doi.org/10.1016/j.ejcdt.2016.04.006
30. Philley JV; Kannan A., Olusola P et al. Microbiome diversity in sputum of nontuberculous mycobacteria infected women with a history of breast cancer. Cell Physiol Biochem. 2019;52(2):263-79. https://doi.org/10.33594/ 000000020 PMid:30816674
31. Kateete DP, Mbabazi MM, Nakazzi F, et al. Sputum microbiota profiles of treatment-naïve TB patients in Uganda before and during first-line therapy. Sci Rep. 2021; 11(1):24486. https://doi.org/10.1038/s41598-021-04271-y PMid:34966183 PMCid:PMC8716532
32. Nasrum M, Hasanuddin U, Kamaruddin M. Sputum microbiota in normal human, new TB patients, and recurrent tuberculosis patients as identified by 16SrRNA sequencing. OSF. 2020. https://doi.org/10.31219/osf.io/ pqnk9

33. Wahyuningsih S, F. Dibha A, D. Kharisma V, et al. Screening of compounds in temu ireng (curcuma aeruginosa roxb.) as tuberculosis drug using bioinformatics design. Res J Pharm Technol. 2023;16(10):4875-80. https://doi.org/10.52711/0974-360X.2023.00790
34. Byun MK, Chang J, Kim HJ, Jeong SH. Differences of lung microbiome in patients with clinically stable and exacerbated bronchiectasis. PLoS One. 2017;12(8):e0183553. https://doi.org/10.1371/journal.pone.0183553 PMid:28829833 PMCidD PMC5567645
35. Turvey SL, Tyrrell GJ, Hernandez C, Kabbani D, Doucette K, Cervera C. Mycobacterium branderi infection: Case report and literature review of an unusual and difficult-to-treat non-tuberculous mycobacterium. Int J Infect Dis. 2017;58:65-7. https://doi.org/10.1016/j.ijid.2017.03.002 PMid:28268125
36. Mertaniasih NM, Kusumaningrum D, Koendhori EB, Soedarsono, Kusmiati T, Dewi DNSS. Nontuberculous mycobacterial species and *mycobacterium tuberculosis* complex coinfection in patients with pulmonary tuberculosis in Dr. Soetomo Hospital, Surabaya, Indonesia. Int J Mycobacteriol. 2017;6(1):9-13. https://doi.org/10.4103/2212-5531.201894 PMid:28317798