Conference Paper

Genotyping Analysis of *Mycobacterium leprae* isolated in Water Environment of Leprosy Endemic Places in Lamongan, East Java

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Abstract

Background: Finding of *Mycobacterium leprae* from water of leprosy endemic areas were reported. East Java Province is ranked number eight as the endemic area of leprosy in Indonesia and Lamongan district is the local area with the highest new cases reported. To study the transmission of *Mycobacterium leprae* infection in endemic areas, it is important to detect the distribution of *Mycobacterium leprae* in the environment and population, also to analyze the genetic variation pattern.

Methods: A total of 91 samples were collected (24 leprosy patients skin samples, 49 nasal swab samples from patients and household contacts, and 18 water samples). Detection is conducted by amplification of *Mycobacterium leprae* DNA using LP3 and LP4 primers. Variation of TTC nucleotide repeats in the intergenic region of *Mycobacterium leprae* genome was done to all positive results.

Results: The finding of 4 strains of *Mycobacterium leprae* from 3 sources with TTC repeats from 11-28 copies. From skin smear samples, 2 strain of *Mycobacterium leprae* with TTC repeats between 13-18 copies. In nasal swabs, 1 strain was found with 28 TTC repeats. From water sources, 1 strain was found with 11 TTC repeats with frequency of repeats in all positive samples.

Conclusion: Unmatched genetic variation between 3 sources indicated contradictive results of transmission pattern of non-human sources of infection. These results should be analyzed further based on different environmental factors to reveal the role of environment in the transmission of leprosy. Further genotyping analysis of *Mycobacterium leprae* in the environment using another genotyping marker is needed to prove the intimate relationship of *host-agent-environment* in the transmission of leprosy.

Keywords: endemic; environment; genotyping; leprosy; *Mycobacterium leprae*.

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1. Introduction

Leprosy in Indonesia has been overcomed based on World Health Organization target, but the problem in Indonesia according to Ministry of Health is caused by the presence of some provinces with secluded endemic pocket area of high prevalence of leprosy especially in east part of Indonesia. *Mycobacterium leprae (M. leprae)* is an obligate intracellular organism (can only live in the cell of other organism), thus the management of leprosy using multidrug therapy (MDT) regiment should break the chain of infection, but new incidence of leprosy is still happened in those pocket areas [1].

East Java Province is ranked number eight as the endemic area of leprosy in Indonesia and Lamongan district is the local area with the highest new cases reported, and this number remains stable in five years period. From the endemic areas of leprosy in Lamongan, a study was done in the Brondong community health center (*pusat kesehatan masyarakat: puskesmas*) with the highest prevalence of 8/10,000 populations Leprosy infection is commonly caused by direct contact of human source of infection (multibacillary type) or indirectly through the environment [2]. Epidemiological studies showed the presence of infected individuals without any clear source of infection, which can be explained by indirect infection [3]. Started from the finding of *M. leprae* in animals such as monkey, armadillo and rat [4], a lot of studies have been done to find non-human transmission of infection (from environment). Kazda [5] reported the finding of *M. leprae* from soil and water of leprosy endemic areas in Bombay, India. Based on Kazda's finding, Matsuoka [6] and Izumi [7] performed studies about detection of *M. leprae* from water sample in endemic areas in India, Sweden, also in North Maluku and Sulawesi (Indonesia). To study the transmission of *M. leprae* infection in endemic areas, it is important to detect the distribution of *M. leprae* in the environment and population, also to analyze the genetic variation pattern.

M. leprae is pathogenic bacteria which cannot be cultured in vitro, so the identification of *M. leprae* in the environment is usually performed using molecular methods. Detection is conducted by amplification of *M. leprae* DNA using primers that encode *M. leprae-specific repetitive element* (RLEP). This primer set was designed by Yoon [8] and demonstrated to be sensitive to detect the DNA of *M. leprae* until 100 ag (10^{-16} g) of target DNA. For further study, molecular mapping is done to study the geographic pattern of leprosy transmission. Research of the polymorphism of *M. leprae* is done with analyzing the Variable Number Tandem Repeats (VNTRs) which is the variation of TTC repeats in the intergenic region of *M. leprae* genome [9].

2. Methods

2.1. Sample collection

The study was conducted at the two areas in the village of Sedayu Lawas and Brengkok which have the highest leprosy prevalence in the covered areas of Brondong community health center (*puskesmas*). Samples are divided into three groups, (1) water sources near the water sources of the areas, (2) nasal swab from household contacts and (3) specimen of skin smears from active leprosy patients in the areas. Samples were kept in cold temperature (-20°C) until the DNA extraction process.

2.2. Sample baseline characteristics

A total of 91 samples were collected during the study. Those samples includes 24 leprosy patients skin smears samples, 49 nasal swab samples from patients and household contacts, and 18 water samples. PCR were performed for all samples and sequencing analyses were done to all positive results.

From 24 patients, 20 (83.33%) is categorized as MB and 4 (16.67%) as PB. For treatment status, 12 (24%) were released from treatment and 12 (24%) were still in MDT regiment. Clinical examination was done according to WHO clinical sign and classification 1998. PB contains of Tuberculoid Leprosy (TT) and Borderline Tuberculoid (BT) type according to Ridley-Jopling criteria or *Indeterminate* and *Tuberculoid* according to Madrid criteria with negative acid fast bacilli. MB contains of Mid Borderline (BB), Bordeline Lepromatous (BL) and Lepromatous Leprosy (LL) type according to Ridley-Jopling criteria or all leprosy patients with positive acid fast bacilli. In the Philippines, Brazil and Indonesia, the MB type is the predominance number of leprosy patients [10].

From 24 leprosy patients, 8 (33.33%) living all by themselves while the rest 16 (66.66%) have household contacts. Total numbers of samples from water sources are 18 from natural sources (ponds) around the village which are used for household activities.

2.3. DNA extraction from samples

Water samples were taken from natural water sources (ponds) around the village which are used for household activities. For extraction, 50 ml samples were centrifuged at 4000g for 10 minutes. Supernatants were discarded and pellets were resuspended with 1.5 ml PBST (Phosphate Buffered Saline Tris-EDTA) buffer then placed into 1.5 ml

sterile tube and centrifuged again at 10,000g for 20 minutes at 4°C. Supernatants were discarded and pellets were resuspended with 0.25 ml lysis buffer from Dneasy QIAGEN DNA mini kit Cat No.69504 then placed into 1.5 ml sterile tube for DNA extraction using Qiagen kit.

Nasal swab specimens were taken from healthy persons of population near the natural water source including household contacts of patients. Nasal swabs were taken by using sterile cotton buds wetted with PBST. Specimens were placed in 1.5 ml sterile tubes containing 0.6 ml sterile distilled water and centrifuged at 10,000g for 20 minutes at 4°C for DNA extraction using Qiagen kit.

Skin smears were taken from Multi bacillary (MB) or Pauci bacillary (PB) leprosy patients who have not been release from treatment (RFT) and live in the area. Skin smears were taken according to standard procedure from acid fast bacilli test. Skin smears were put in PBS buffer and centrifuged at 10,000g for 20 minutes at 4°C for DNA extraction using Qiagen kit.

2.4. PCR for detection of M. leprae

Nested PCR was performed using two primers pairs located at the 18 kDa *M. leprae* antigen in RLEP3 *repetitive element* (X17153) region amplifying 260 bp DNA for external product (*outer*) and 99 bp for internal product (*inner*). PCR was done using G mixture from FailSafe PCR System (EPICENTRE, Madison, WI, USA) and 2720 *Thermal Cycler* from Applied Biosystem (AB). The sequence of primer LPF was 5'TATCGATGCAGGCGTGAGTGT3' and LPR was 5'CTAACACGATACTGCTGCAC3'. Amplicon were then amplified again using PCR with primer LP3 (5'TGAGGTGTCGGCGTGGTC3') and LP4 (5'CAGAAATGGTGCAAGGGA3') which are sensitive and specific for detection of *M.leprae* from environmental samples (water and soil). Amplicon end products were visualized using electrophoresis in 3% agarose HS gel (Cambrex Bioscience, Rockland, ME, USA) with TBE (Tris/Boric-acid/EDTA, pH 8.0) buffer at 100 Volt.

2.5. PCR Amplification for genotyping of M. leprae

PCR for DNA analysis were done with G mixture from FailSafe PCR System (EPICENTRE, Madison, WI, USA) in 50 µl master mix contain at least 0.1 pg of DNA genome in 5µl DNA template and 2 µl primer with 5 µM concentration. Primer used is TTC-A (5'GGACCTAAACCATCCCGTTT3') and TTC-B (5'CTACAGGGGGCACTTAGCTC3') (GenBank Accession No. AF274484) resulted in amplicon PCR of 200bp. Amplification were done

in 2720 *Thermal Cycler* (Applied Biosystems). Amplification products were separated in 3% NuSieve GTG agarose gel (Cambrex Bioscience) using TAE (Tris/Acetatic-acid/EDTA, pH 8.0) buffer at 100V.

2.6. Sample Preparation for Sequencing Analysis

TTC repeats can be seen with direct sequencing method. PCR samples were purified with *GFX^{TM} PCR, DNA and Gel Band Purification* kits *(Amersham Biosciences)*. The purified DNA is then checked for quantity and quality using UV spectrophotometer. *Dual CyDye^{TM} Terminator Sequencing* kits (*Amersham Biosciences)* is used for labelling. Labeling result was precipitated with ethanol and dried then with the addition of 2 loading dye was placed into *Long-Read Tower^{TM} System* (*Amersham Biosciences)* sequencer machine.

2.7. Data Analysis

Collected data were processed and analyzed using SPSS and Statcal computer program. Mean differences between amplification results of specific DNA for *M. leprae* in each sample group were tested using *Fisher Exact Test*.

3. Results

3.1. PCR Analysis and Results M. leprae DNA detection using nested primer LP1-4

PCR detection was performed in 91 samples (24 skin smears, 49 nasal swab with 24 swabs from patients and 25 swabs from household contacts, and 18 from water sources). We used nested PCR for the detection. The first PCR used LPF and LPR primers, while the second PCR used LP3 and LP4 primers.

3.2. PCR detection of M. leprae from Skin Smears of Patients

PCR amplification of samples from patients detected positive results of *M. leprae* DNA in 7 out of 24 samples (29.17%), while the rest (17 samples or 70.83%) were negative. All positive results came from MB patients, with 6 (85.71%) patients which still have ongoing MDT treatment and 1 (14.29%) from RFT patients. PCR is a very sensitive and

specific method to detect *M. leprae* from human and animal tissue and from environment [11–13]. Positive results are dominated by MB patients according to former reports [11, 14, 15].

3.3. PCR Detection of M. leprae from Nasal Swab of Patients and Household Contacts

In this study, the result of PCR from nasal swab samples were 49 samples, 24 were from patients and 25 from household contacts. PCR results of 49 nasal swab samples detected 8 (16.33%) samples were positive of *M. leprae*, 5 (20.83%) samples from patients and 3 (12%) samples from household contacts. Whereas in the 41 (83.67%) negative results, 19 (79.17%) were from patients and 22 (88%) samples from household contacts. Statistical analysis showed no significant difference in positivity results from PCR results of nasal swabs from patients and household contacts in endemic areas (p=0.2860).

In this research the relatively low number of positive results from nasal swabs of patients can be caused by most patients have already received treatments. According to Patrocinio [16] *M. leprae* is decreased in number in a fast pace if the patient received treatment not only leprosy drugs but also other antibiotics such as *minocycline, clarithromycin* and *fluoroquinolone*. Nostrils are the transmitting place of *M. leprae*, because of the humid and wet condition it become the ideal place for bacilli growth. *M. leprae* can survive outside the human body and transmitted through nasal secretions. It is parallel with the findings of *M. leprae* in nostrils of patients and household contacts in endemic areas because of frequent exposure to the bacilli.

3.4. PCR detection of M. leprae from Water Sources

In this research, total samples from water sources of the areas are 18 samples. For PCR results 5 samples (27.78%) were positive of *M. leprae*. The water samples were collected from the natural sources. Adriaty [17] also reported similar results. From the data, the existence of *M. leprae* in water sources were not related to number of patients, in other words the *M. leprae* found in the water were most likely not originated from leprosy patients contamination.

This result is suggestive to hypothesis of non-human resources of leprosy bacilli [9, 13, 18]. According to Puhler [19] the capability of bacteria to survive in water is because of the interaction with other living organism in the water.

Figure 1: Results of TTC sequencing with 13 repeats.

What remains a question is how *M. leprae* can survive in the water, if these unique bacilli have cell division every 2 weeks and can only survive for 40 days in the environment. It is very difficult to survive if these bacilli do not have other living organism as their host since *M. leprae* is intracellular obligate organism. If this true, then it might explain the environmental transmission and persistence of incidence although eradication and management effort are being done continuously in endemic areas.

3.5. Variation of TTC Nucleotide Repeats of M. leprae from Skin Smears of Leprosy Patients

According to variation of TTC nucleotide repeats of *M. leprae* from skin smears of leprosy patients (Figure 1 and 2), 2 strains of *M. leprae* with variation between 10-60 with the highest frequency of TTC 13 repeats was 1 isolate (20%), followed by TTC 18 repeats which found in 4 isolates (80%).

Unlike the other *Mycobacteria*, *M. leprae* cannot be differentiated by MIRU (*Mycobacterial Interspersed Repetitive Unit*) because of lack of MIRU locus in *M. leprae*. One method to differentiate strains of *M. leprae* is introduced by Greathouse [20] by counting the TTC repeats of the bacilli.

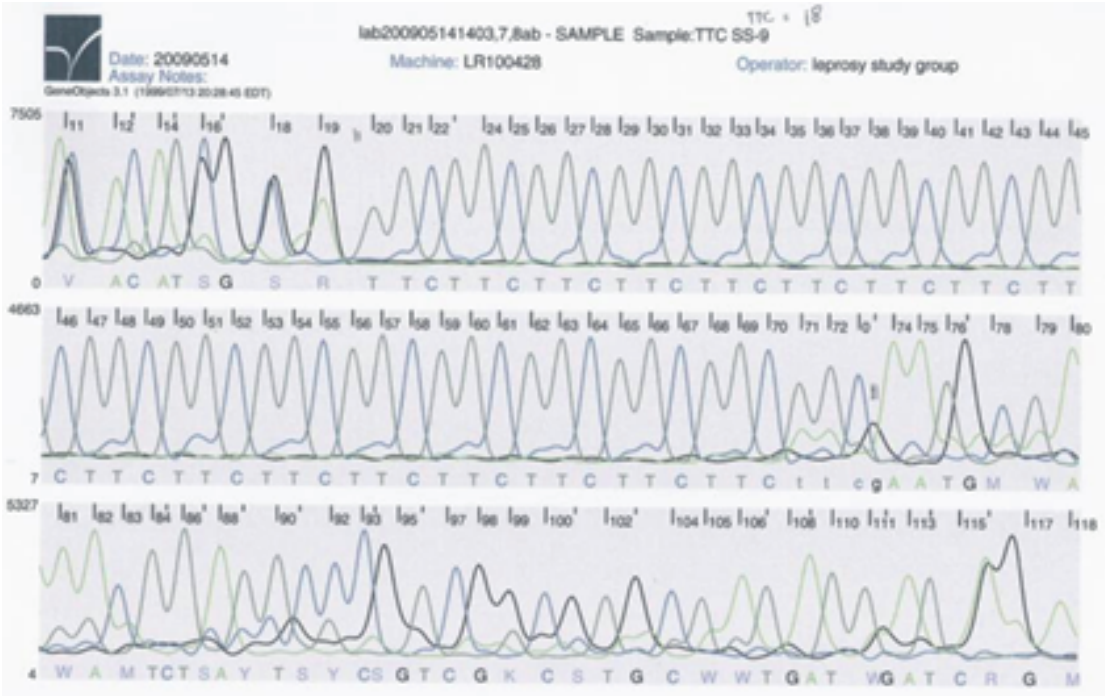

Figure 2: Results of TTC sequencing with 18 repeats.

3.6. Variation of TTC Nucleotide Repeats of M. leprae from Nasal Swabs of Leprosy Patients and Household Contacts

None of the samples from patients' nasal swabs showed positive results, whereas samples from household contacts showed 1 positive result out of 25 samples which has 28 copies (Figure 3). This result showed the importance of nasal mucosa as *port de 'exit* and *entry* of these bacteria [21].

3.7. Variation of TTC Nucleotide Repeats of M. leprae from Water Sources

Samples from water sources showed 3 positive results out of 18 samples (16.67%) which have 11 copies for all 3 samples. In this research, from water samples in endemic areas of leprosy, 1 strain of leprosy is found based on TTC nucleotide repeats which is TTC-11 in 3 ponds (Figure 4).

4. Discussion

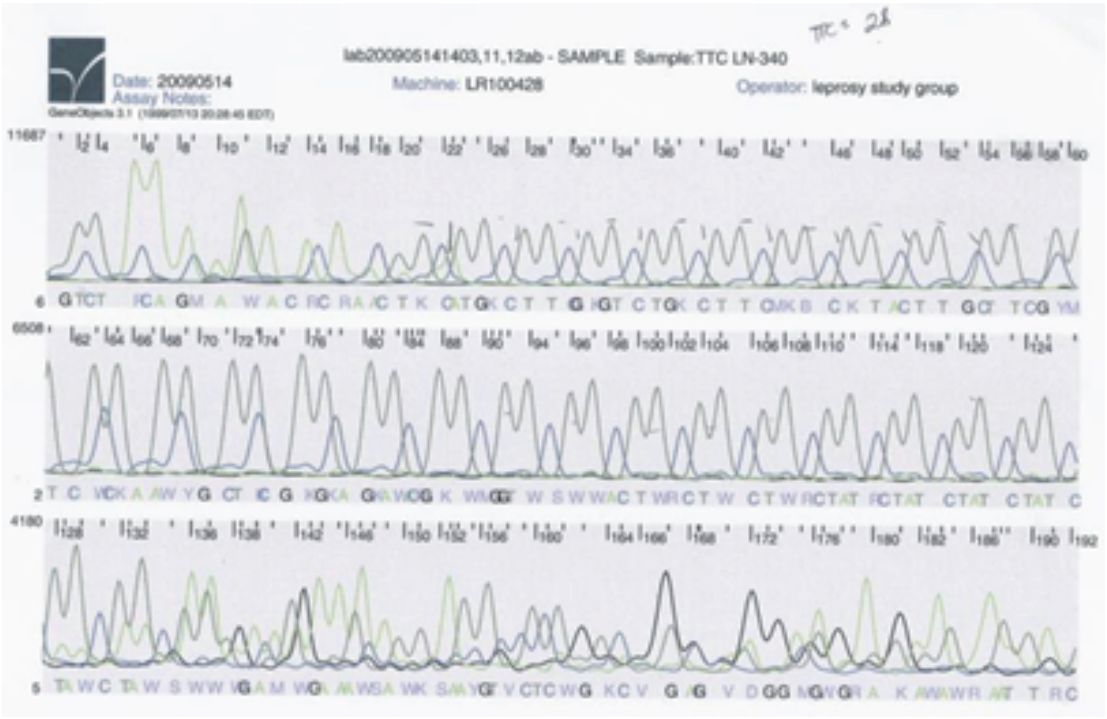

Figure 3: Results of TTC sequencing with 28 repeats.

Table 1: Sequencing results of patients, household contacts, and water sources.

No.	Patients	Household contacts	Water sources
1.	0	0	11 copy
2.	13 copy	0	0
3.	18 copy	0	0
4.	18 copy	0	11 copy
5.	18 copy	0	11 copy
6.	0	28 copy	0
7	18 copy	0	0

4.1. Analysis of Variation of TTC Nucleotide Repeats of M. leprae from Skin Smear, Nasal Swabs, and Water Sources

From sequencing of positive PCR results from skin smears, nasal swabs and water sources as seen in Table 1, there were variation and frequency of TTC repeats as seen in Table 2 below.

Based on variation and frequency of TTC repeats of *M. leprae* from water sources as seen in Table 4, 4 strain of *M. leprae* from skin smears, nasal swab, and water with variation of 11-28 and highest frequency is 18 TTC repeats of 4 isolates (44.45%),

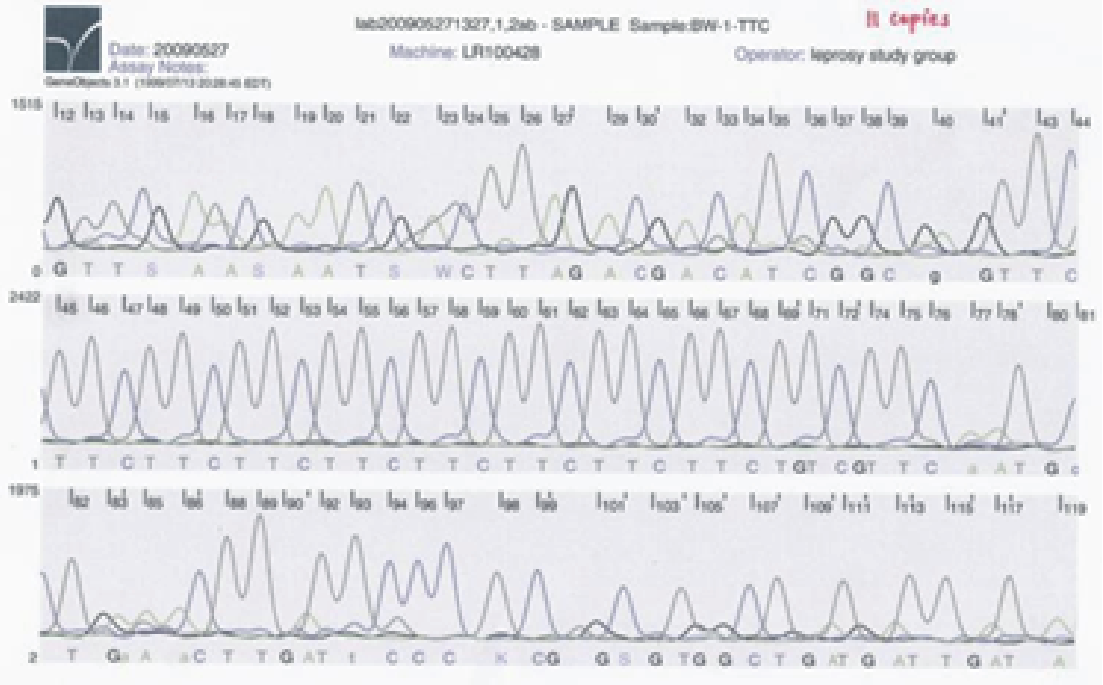

Figure 4: Results of TTC sequencing with 11 repeats.

Table 2: Variation and frequency of TTC repeats of *M. leprae* from skin smears, nasal swabs and water sources.

No	TTC Nucleotide Repeats	Skin Smear	Nasal Swabs	Water	Frequency
1.	TTC-11	0	0	3	3 (33.33%)
2.	TTC-13	1	0	0	1 (11.11%)
3.	TTC-18	4	0	0	4 (44.45%)
4.	TTC-28	0	1	0	1 (11.11%)
Sum		**5**	**1**	**3**	9(100%)

followed by 11 TTC repeats of 3 isolates (33.33%), and 13 TTC repeats and 28 TTC repeats each has 1 isolate (11.11%).

We can conclude that the finding of 4 strains of *M. leprae* from 3 samples with TTC repeats from 11-28 copies. From skin smears samples, 2 strain of *M. leprae* with TTC repeats between 13-18 copies with highest frequency of TTC repeats in TTC 18 (44.45%) from 5 positive samples. In nasal swabs, 1 strain was found with 28 TTC repeats. From water sources, 1 strain was found with 11 TTC repeats with frequency of repeats in all positive samples.

5. Conclusion

This result showed that the strain from water is not matched with the strain from skin smears nor nasal swab of patients and household contacts. This is different from the report of Mudatsir [22] which showed matched strain from water and skin smear and nasal swab. Matsuoka [6] reported leprosy transmission through water contaminated by bacilli is likely to happen. According to Cree [2], leprosy transmission can also happen through direct or indirect contacts such as environmental transmission. The possibility of neighbor contacts or cluster of people who often gather together is still debated and could be also be one of the sources of leprosy transmission. In summary, these contradictive results should be analyzed further based on different environmental factors to reveal the role of environment in the transmission of leprosy. Further genotyping analysis of *Mycobacterium leprae* in the environment using another genotyping marker is needed to prove the intimate relationship of *host-agent-environment* in the transmission of leprosy.

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