

Variation of TTC Repeat Pattern In The Dna of *Mycobacterium Leprae* Isolates Obtained from Archeological Bones and Leprosy Patients From East Nusa Tenggara

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ABSTRACT

The existence of leprosy or kusta or Morbus Hansen or Hansen's disease has been known for years, including in Indonesia. Starting from the discovery of *Mycobacterium leprae* isolates from ancient bone (about 1.000 years B.C), the archaeological excavations results in East Nusa Tenggara, interesting questions arise about how the development of leprosy in eastern Indonesia is. Biology molecular study would become a powerful tool to investigate the presence of leprosy bacillary whether there are similarities between the genomes of *M. leprae* isolates in the primeval and the present. PCR examinations were performed on mandibular bone fragments from ancient human who lived 1000 years B.C. discovered in archaeological surveys on the island of Lembata and three leprosy patients from East Nusa Tenggara. The DNA extraction was performed using a kit from Qiagen products and its TTC repeating pattern was seen with the method of direct sequencing. It turned out that the TTC profile obtained from samples of archaeological was as many as 13 copies, while the repetition of TTC in three samples of leprosy patients were 15, 17 and 26 copies. The different number of TTC repetition shows the different isolates of *M. leprae* between in the ancient times and the present. Further studies are needed to verify the differences in the genome that occur, for example from the study of SNPs (single nucleotide polymorphisms).

Keywords: *Leprosy, TTC repeat, M. leprae, Archaeology, East Nusa Tenggara*

INTRODUCTION

Leprosy is an ancient disease which has been spread among human populations by migrations, military expansion and colonization, as well as along trade routes [1,2]. According to the history, the disease of leprosy entering Indonesia was allegedly carried away from China and India to the island of Java. In 1927 in Platungan near Semarang stands the first leprosaria in Indonesia; and, in 1928 the beginning of leprosy elimination has started and was pioneered by Dr. J.B. Sitanala [3].

East Nusa Tenggara was the region that was famous for its discovery of hobbits or dwarf humans (*Homo floresiensis*). It has been suspected that they were derived from ancient region that stood alone and did not become a

part of the continent or other large island in the world [4]. Hopefully, if bacillary disease of leprosy found in that islands, it could mean that *Mycobacterium leprae* isolates are native bacteria of the territory of Indonesia and it can become one of the reasons why in the eastern part of Indonesia the incident rate and prevalence rate of leprosy tend to get high until now [5]. In order to study those problems, molecular typing would be a great value to study the transmission pattern and geographical distributions of *M. leprae* for epidemiological investigation.

The purpose of this research is to detect the presence of *M. leprae* genomes in the primeval and the present by analyzing the variation number of TTC repetition in the archeological samples and some of leprosy patients in East Nusa Tenggara. It has been possible to recognize potential polymorphic sites from the genome sequence of *M. leprae*. As is the case in several eukaryotic and prokaryotic genomes that have been sequenced, short stretches of DNA that occur in tandem repetitions are also found in *M.*

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leprae [6]. Matsuoka [7] first reported that 6-bp sequence (GACATC) was found as two alleles in the *rpoT* gene of *M. leprae*. This was followed by the recognition of variable number tandem repetitions (VNTRs) of the TTC triplet in a noncoding region of the *M. leprae* cosmid MLCB2407 (GenBank accession no. AL023596) [8]. According to the report from Shin [9], the gene location of the TTC repetitions were not found in *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium marinum*, or human tissues, which indicated their specificity to *M. leprae*. Truman [10] also reports the stability of the TTC (VNTRs) by testing this gene from *M. leprae* that was obtained from armadillo and nude mice tissues being investigated for 121 months. Thus, this gene is reliable as a marker of strain differentiation for epidemiological investigations of leprosy.

MATERIALS AND METHODS

Mycobacterium Leprae Isolates and Preparation of Genomic DNA

Archeological Sample. Skeletal bone was derived from Lewoleba, Lembata Island, excavated in the Dutch era. Estimated bone was 1000 year B.C according to the Carbon14 examination (age activity according to 2990 +/- 160BP) [11] methods. The sex of this skeleton was assessed as female on the basis of cranial morphologies. The dental and skeletal evidences suggested that the age of death was at an early aged in the range of 20-25 years-old. A piece of mandible bone was grinded to perform the powder. Bone powder was weighed of 0.25gram to extract by the DNA extraction kit. The DNA extraction method was Qiagen:DNeasy tissue kit (Qiagen, Valencia, CA). A bone, which had been placed on the 2ml sterile tube, was added with 200 uL lysis buffer containing 20 uL proteinase-K and 180 uL ATL buffer. The specimen was vortexed and incubated at 55°C until the bone powder was completely digested (it took overnight), then the rest of the procedure was described as in the manufacturer's protocol. Pure DNA was stored at 4°C.

Slit-skin Specimens. The samples were obtained from leprosy patients that came in the peripheral health center in East Nusa Tenggara region during 2011. All patients collected were local villagers categorized as Multibacillary (MB) or Pausibacillary (PB) cases based on the criteria set by WHO (World Health Organization). Slit-skin Smear specimens were collected from the skin lesion of patient in the same manner as the

routine slit-skin smear test for Bacterial Index examination. The samples on the disposable surgical blade were soaked into Phosphate-Buffered Saline and kept in a freezer until use. The bacilli were removed from the blade and collected as a pellet by centrifugation at 16,000g for 20 min in 4°C until served and, then, were washed with phosphate-buffered saline during the isolation. The DNA extraction method used Qiagen:DNeasy tissue kit.

Mycobacterium leprae Detection

The *M. leprae* gene in regio RLEP3 repetitive element (X17153) was chosen to be amplified with nested PCR. Amplification will produce about 129 bp for external (outer) and 99 bp for internal (inner) product. PCR was carried out using a Premix G mixture of FailSafe PCR System Cat. No. FSP995G (EPICENTRE, Madison, WI, USA) in a 20 µl volume of reaction mixture containing at least 0.1 pg of genomic DNA in 2µl of template DNA solution and 2 µl of 5 µM primers using FailSafe PCR Enzyme Taq Mix 250 U@2.5 U/µl Cat. No. FS99250. Primers Lp1 5' TGCATGTCATGGCCTTG-AGG 3' and Lp2 5' CACCGATACCAG-CGGCAGAA 3' were produced by Takara (Japan) and the amplification was done in a thermal cycler machine (BioRad i-cycler) under the conditions of 2 min at 98 °C for preheating, 20 sec at 98 °C for denaturation, 30 sec at 56°C for annealing and 30 sec at 72 °C for elongation repeated for 35 cycles followed by prolonged extension of 5 min at 72 °C, then inactivation at 4 °C. Amplicon was, then, being nested with primers Lp3 5' TGAGGTGTCGGCGTGGTC 3' and Lp4 5' CAGAAATGGTGCAAGGGA 3' under the conditions of 2 min at 98 °C for preheating, 20 sec at 98 °C for denaturation, 30 sec at 56°C for annealing and 30 sec at 72 °C for elongation repeated for 30 cycles followed by prolonged extension of 5 min at 72 °C, then inactivation at 4 °C. The full length of this amplicon was separated by electrophoresis in 3% HS agarose gel Code No. 312-01431 (Cambrex Bioscience, Rockland, ME, USA) using TBE (Tris-Boric-EDTA, pH 8.0) buffer at 100 V. All the positives samples were continued to genotyping analysis.

Genotyping of TTC Repeats

PCR was carried out as described before and was made in a 50 µl volume of reaction mixture containing at least 0.1 pg of genomic DNA in 5µl of template DNA solution and 2 µl of 5 µM primers. Primers TTC-A (5'GGACCTAAACC-

ATCCCGTTT3') and TTC-B (5'CTACAGGG-GGCACTTAGCTC3') were used for amplification and PCR will produce about 200bp amplification product. The amplification was done in a thermal cycler machine (BioRad i-cycler) under the conditions of 2 min at 98 °C for preheating, 20 sec at 98 °C, 30 sec at 58 °C, 30 sec at 72 °C for 40 cycles followed by prolong extension of 5 min at 72 °C, then inactivation at 4 °C. The full length of this amplicon was separated by electrophoresis in 3% NuSieve GTG agarose gel Cat No. 50080 (Cambrex Bioscience, Rockland, ME, USA) using TAE (Tris-Acetate-EDTA, pH 8.0) buffer at 100 V.

The numbers of TTC repetitions were confirmed by direct sequencing. DNA samples for sequencing were recovered by GFX™ PCR, DNA and Gel Band Purification kits (Amersham Biosciences, GE Healthcare) with product code: 27-9602-01 according to the manufacture's manual. Before sequencing reaction, the quantity and quality of purified DNA were examined by UV spectrophotometer. Dual CyDye™ Terminator Sequencing kits Cat. No 25-8226-01 (Amersham Biosciences, GE Healthcare) was used in the preparation of sequencing reaction. The mixture for cycle sequencing (labeling) was performed according to the manufacture's manual. The sequencing reaction was also done in a thermal cycler machine (BioRad i-cycler) under the following condition: 20 sec at 95°C, 15 sec at (TM of sense primer + 3) °C , 1 min at 70°C and repeat for 35 cycles. The sequencing product was, then, purified by ethanol precipitation and dried followed by dissolving it in 2 µl of loading dye and was loaded into prepared acrylamide gel in Long-Read Tower™ System (Amersham Biosciences) Version 3.01. Sequence analysis was done by using Long-Read Tower™ System (Amersham Biosciences) with the temperature of 60°C as described in the protocol.

RESULT AND DISCUSSION

The materials used in this study were archaeological skeletal that remained excavated from Lewoleba village in Lembata Island during the Dutch era. All skeletal materials are stored in the Department of Pathology and Anatomy, Medical Faculty, Airlangga University in Surabaya, Indonesia, where no other leprosy materials were stored [12]. Sterile materials were used for the sampling to avoid possible contamination. The facial cranium of the skeletal showed several typical symptoms of leprosy

[11,12], such as in the rhinomaxillary area, rounding deformation and disappearance of the anterior nasal spine (Fig1.A1).

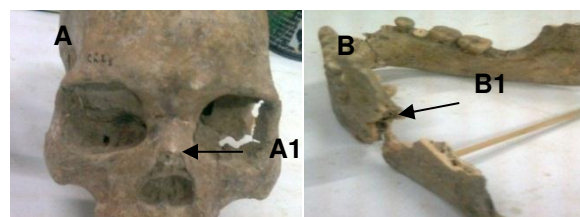


Fig1. Macroscopic view of the skeletal lesion in the skull of archeological sample ; A. The skull profile that arrows in panel (A1) disappearance of the anterior nasal spine; B. Location of the DNA extraction, the mandible bone (B1)

There were three isolates collected from the East Nusa Tenggara leprosy patients, and they were all new cases and Multibacillary type. All extracted samples showed positive correlation with primers that were specific to detect *M.leprae* bacillary (*M.leprae* gene in regio *RLEP3 repetitive element* (X17153)), as recommended by Donoghue et.al [13].

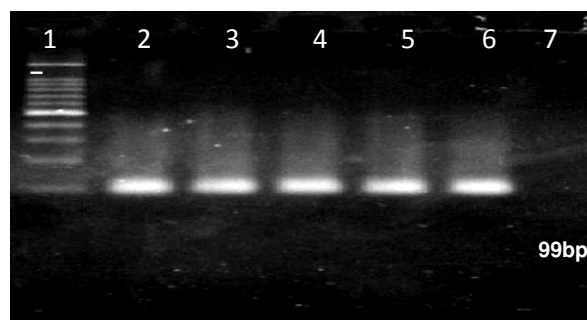


Fig2. PCR Product of *M.leprae* Detection. Samples were: lane 1, the DNA size marker of 100bp ladder; lane 2-4, isolates from East Nusa Tenggara villagers; lane 5, Mandible bone powder; lane 6, PC, positive control (*M.leprae* strain *Thai-53*); lane 7, NC, negative control

All the positive samples were, then, analyzed by TTC repetitions genotyping. They were amplified by primers recommended by Matsuoka et al [14]. Amplicon has the size of about 200bp. It is for positive control which uses *M. leprae* strain *Thai-53*. Other samples vary as seen in the figure below. Most TTC genotype in South East Asian region, such as the *M. leprae* strain *Thai-53* from Thailand is TTC-15 copies and from Philippines, is TTC-14copies; it has been reported by Shin et al. that Latin American countries commonly have TTC-10copies which is shorter than in South East Asian as reported by Matsuoka et al. Africa and India have different typing than those two regions in which it is longer-repeated type (*M. leprae* strain *Tamil*

Nadu India has TTC-21 copies; *M. leprae* strain Ethiopia has TTC-29 copies).

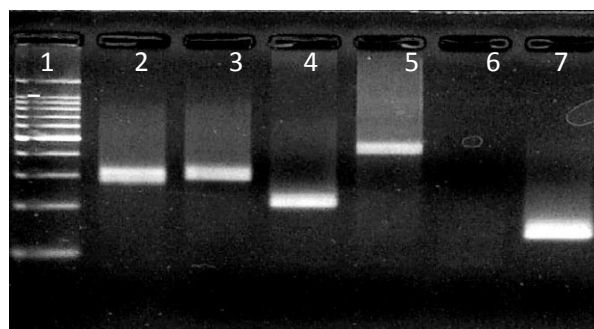


Fig3. PCR Product of TTC repetitions. Samples were: lane 1, the DNA size marker of 100bp; lane 2-5, isolates from Mandible bone powder and from East Nusa Tenggara villagers; lane 6, NC, negative control; lane 7, PC, positive control (*M.leprae* strain Thai-53)

Table 1. Result of VNTR genotyping

<i>M. leprae</i> Isolates	Polymorphic locus TTC ^a ML2344-2345
Archeological mandible bone	13 copies
Patient NTT-A	15 copies
Patient NTT-B	17 copies
Patient NTT-C	26 copies
Control <i>M. leprae</i> Thai-53	15 copies

^a T=thymine, T=thymine, C=cytosine

The archeological bone has a short type, that is, 13 TTC copies. Modern human beings, i.e. leprosy patients in the present day, have longer type, varying from 15 to 26 TTC copies. It means that the shorter type localized in that island (archeological sample) is more original, and it comes from that area itself than that in the present. Modern human beings have a better mobilization in which the interaction between locals and outsiders within the island is more open than in the ancient time, one reason why the genotype profile varies.

CONCLUSION

The different number of TTC repetitions shows *M. leprae*'s different isolates in ancient times and the present. Further studies are needed to verify the differences in the genome that occur, such as the study of SNPs (single nucleotide polymorphisms).

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