Indonesian Journal of
Tropical and Infectious Disease
Vol. 1. No. 1 January–April 2010

Research Report

TTC Repeats Variation of *Mycobacterium leprae* Isolates for Analysis of Leprosy Transmission in Leprosy Endemic Area in East Java, INDONESIA

Dinar Adriaty, Ratna Wahyuni, Iswahyudi, Indropo Agusni, and **Shinzo Izumi**
Leprosy Study Group, Institute of Tropical Disease, Airlangga University

ABSTRACT

East Java province still has some pocket of leprosy endemic areas. In order to solve the problem, molecular typing will make it feasible to study the transmission pattern of Mycobacterium leprae in leprosy endemic area. The present study is to analyze the presence of M.leprae DNA in the environment and to study variation number of TTC repeats and their distribution. Poteran Island is located in Madura, East Java and was chosen because this island has a high prevalence of leprosy and remains stable for the last five years. All samples were analyzed by PCR and the numbers of TTC repeats were confirmed by direct sequencing. Of all collected samples, 26.4% isolates of water resources (24); 61.9% nasal swabs (26); and 35.3% skin tissues (24) are positives. No statistically difference in the pattern distribution of TTC repeats between skin tissues of patients and nasal swab of households contact (p=0.594); also distribution of TTC repeats between skin tissues of leprosy patients and those of water resources (p=0.441); and distribution of TTC repeats between nasal swab of households contact with water resources (p=0.906). It means that the transmission of M.leprae in leprosy endemic area has closely related in 3 aspects: agent, host & environment.

Key words: *TTC Repeats, Mycobacterium leprae , Leprosy transmission, Endemic area, East Java*

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* and is still a major health problem in the developing countries of Asia, Latin America and Africa. Indonesia is one of Asian countries that also have problems with leprosy elimination. It is commonly believed that the humans (Multibacillary patients) are the host and reservoir of *M.leprae* and global efforts to control leprosy by distributing a multidrug therapy (MDT) regimen should eliminate leprosy.

However, after following the leprosy elimination program, the prevalence rates in Indonesia has reduced into 0.84 per 10.000 inhabitants, but in the contrary new cases of leprosy, has remained unchanged over the last 10 years.[1]

There are many hyper endemic areas distributing in several provinces especially in the eastern part of Indonesia, which called pocket area. East Java province is the one of many provinces that has some pockets areas of leprosy. Until in the middle of 2004, prevalence rate in East Java province still 1,39 per 10.000 inhabitants distribute to 38 districts with 4298 registered cases. From whole districts in East Java Province, Sampang has the highest prevalence rate (6,41), followed by Sumenep (6,29), Pamekasan (4,01), Lamongan (3,94) and Tuban (3,54 per 10.000 inhabitants).[2] Sumenep is one of endemic areas which have many hyper endemic areas especially in the islands region that still isolated from outsider. One of them is Poteran Island that has 39.479 inhabitants living in 8 villages with prevalence of leprosy is 24,1 per 10.000 inhabitants. Poteran Island was chosen because this island has a high prevalence of leprosy and remains stable for last five years.[3]

It has been difficult to identify sources of infection of leprosy because of the protracted incubation period preceding clinical disease made natural history of the disease unclear although port of entry and port of exit of the bacilli via the nasal passages have been proposed. This condition was aggravated by the fact that *M.leprae* remains uncultivable on artificial media.[4] These factors have slowed our understanding about the route of transmission of *M.leprae* which could inhibit our ability to target drug therapy campaigns and to improved control strategies.

Humans are considered to be the principal reservoir. The disease is thought to be spread most effectively through

long-term, intimate contact with an infected individual, but the majority of new cases presenting in some area in Indonesia are unable to relate any close association with another person who had leprosy.[5] In order to understand the problems, molecular typing would be a great value to study the transmission pattern and geographical distributions of *M.leprae* for epidemiological investigation.

The aim of this study is to detect the presence of *M.leprae* in environment of endemic leprosy area and to analyze the variation number of TTC repeats and their distribution in leprosy endemic area especially in East Java province.

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 repeats are also found in *M.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 repeats (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 repeats were not found in *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium marinum*, or human tissues, which indicated their specificity to *M.leprae*. Truman[10] also report the stability of the TTC (VNTR) by testing this gene from *M.leprae* that obtained from armadillo and nude mice tissues and investigated for 121 months, so 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

A total of 201 *M.leprae* isolates were collected and divide into 3 groups: 91 water sources, 42 nasal swabs of household contact and 68 slit-skin tissues of leprosy patients.

Water Samples. Water samples were collected from one village in Poteran island, Sumenep, Madura, East Java. All water came from natural sources. No piped water supply is available in this village. Well water was used for drinking and bathing in this area. One well usually used for several houses surround it. Samples that were collected were being kept cool until preparing the templates. For preparation, 10 ml sample centrifuge at 4000*g* for 10 min. Discard supernatant and take filtrate to sterile 1.5 ml tube and again centrifuge at 10.000*g* for 20 min in 4ºC and pellet were collected.[11]

Slit-Skin Specimens. Samples were obtained from leprosy patients in the village. We collected all patients who are villagers of that area categorized as Multibacillary (MB), Pausibacillary (PB) cases based on a criteria of WHO (World Health Organization). Slit-Skin Smear specimens were collected from the skin lesion of patients in the same manner as the routine slit-skin smear test for Bacterial Index examination. The samples on the disposable surgical blade was soaked into 70% ethanol and kept in a refrigerator until use. The bacilli were removed from the blade and collected as a pellet by centrifugation at 10.000*g* for 20 min in 4º C until serving and then washed with phosphate-buffered saline when doing isolation.[12]

Nasal Swabs Specimens. Nasal swabs were collected from healthy villagers that lived in that area including household contact (persons that live in the same house with the patients). Nasal swabs were taken by using a sterilized cotton-tipped and made it wet by phosphate-buffered saline and swabs were kept in freezer until use. The bacilli were removed by gently rubbing the swab into 1.5 ml sterile tube which contained sterile distilled water (0.6 ml) and centrifuge at 10.000*g* for 20 min in 4ºC. All isolates were prepared for DNA extraction by treatment Qiagen QIAprep Spin Miniprep Kit Cat. No. 27106 as mentioned in Qiagen protocol.[13]

Mycobacterium leprae Detection

Detection of M.leprae DNA. To identify *M.leprae*, the 18 kDa antigen *M.leprae* in regio RLEP3 *repetitive element* (X17153) (14) was chosen to amplify by 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' TGCATGTCATGGCCTTGAGG 3' and Lp2 5' CACCGATACCAGCGGCAGAA 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 98º C for preheating, 20 sec at 98º C for denaturation, 30 sec at 56º C for annealing and 30 sec at 72º C for elongation/extension repeated for 35 cycles followed by prolong extension of 5 min at 72º C then inactivation at 4º C.

Amplicon was then being nested with primers Lp3 5' TGAGGTGTCGGCGTGGTC 3' and Lp4 5' CAGAAATGGTGCAAGGGA 3' under the conditions of 2 min at 98º C for preheating, 20 sec at 98º C for denaturation, 30 sec at 56ºC for annealing and 30 sec at 72º C for elongation/extension repeated for 30 cycles followed by prolong extension of 5 min at 72 ºC then inactivation at 4º C. The full length of this amplicon were 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 Amplification. PCR was carried out as described before and make it 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'GGACCTAAACCATCCCGTTT3') and TTC-B (5'CTACAGGGGGCACTTAGCTC3') 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 98º C for preheating, 20 sec at 98º C, 30 sec at 58º C, 30 sec at 72º C for 40 cycles followed by prolong extention of 5 min at 72º C then inactivation at 4º C. The full length of this amplicon were 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.

Sample preparation for sequence analysis. The numbers of TTC repeats were confirmed by direct sequencing. DNA samples for sequencing were recovered by *GFX^{TM} PCR, DNA and Gel Band Purification* kits *(Amersham Biosciences/ GE Healthcare)* with product code: 27-9602-01 according to the manufacture's manual (Amersham, 2002). Before sequencing reaction, the quantity and quality of purified DNA was examine by UV spectrophotometer. *Dual CyDye^{TM} 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 in 2 µl of loading dye and was loaded into prepared acrylamide gel in *Long-Read Tower^{TM} System* Version 3.01. Sequence analysis was done using *Long-Read Tower^{TM} System*[15] with the temperature was set on 60°C as described as in the protocol.

RESULTS AND DISCUSSIONS

Detection of *M.leprae* DNA

Out of 24 isolates of water resources (26.4%); also 26 nasal swabs (61.9%); and 24 skin tissues (35.3%) showed the 99 bp PCR products of the 18 kDa antigen *M.leprae* and indicated that samples contained *M.leprae* (Fig.1). The PCR positives results are mostly from nasal swabs taken from healthy villagers. Nasal mucosa is reported as a port of entry for *M.leprae* surrounds environment. It means the leprosy transmission in a highly endemic area is very active. The fact that water and nasal mucosa samples give many positive results indicates that environmental sources play an important role in *M.leprae* infection and transmission of the disease other than patients.[11]

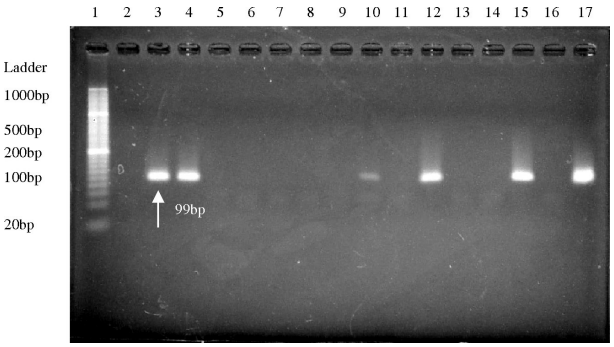

Figure1. PCR Products of *M.leprae* Detection. Lane 1 : the DNA size marker of 20bp ladder ; lane 2-15 : isolates from Poteran Island; lane 16 : NC, negative control; lane 17 : PC, positive control (*M.leprae* strain *Thai-53*)

TTC repeats Distribution of *M.leprae* in Poteran Island

According to the report from Shin,[9] the gene location of the TTC repeats were not found in *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium marinum*, or human tissues, which indicated their specificity to *M.leprae*. Truman[10] also report the stability of the TTC (VNTR) by testing this gene from *M.leprae* that obtained from armadillo and nude mice tissues and investigated for 121 months, so this gene is reliable as a marker of strain differentiation for epidemiological investigations of leprosy.

All the positives samples which have positive PCR according to the 18kDa detection were then continued to be analyzed by TTC repeats genotyping. All samples were amplified by primers that recommended by Matsuoka.[16] Amplicon has sizes about 200bp and *M.leprae* strain *Thai-53* was used as positive control. Other samples are varying as seen in Fig.2.

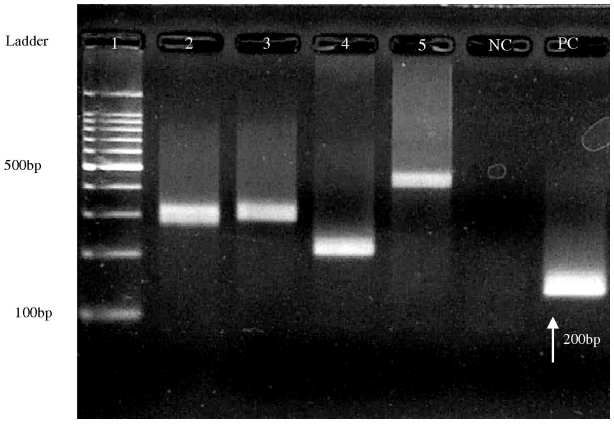

Figure 2. PCR Product of TTC repeats. Samples were: lane 1, the DNA size marker of 100bp ladder; lane 2,3,4,5, isolates from Poteran Island; NC, negative control; PC, positive control (*M.leprae* strain *Thai*-53)

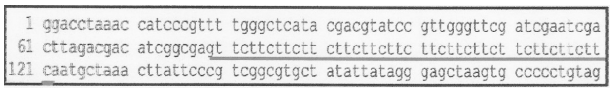

Figure 3. TTC repeat amplified product after sequencing repeat region 80...121 (TTC-14 copy belong to *M.leprae* strain *Thai*-53)

Table 1. Frequency (%) of TTC genotypes in each isolates

No. of repeats	Slit-Skin Specimens	Nasal Swabs	Water Sources
TTC-9	0	0	4
TTC-10	20	10	8
TTC-11			
TTC-12	4	4	0
TTC-13	8	4	12
TTC-14	16	28	32
TTC-15	0	0	4
TTC-16	4	0	0
TTC-17	0	4	0
TTC-24	4	0	0
TTC-28	4	0	0
TTC-40	4	0	0
TTC-44	0	8	0
TTC-49	0	0	4
TTC-60	4	0	0
Total	100% (24 cases)	100% (26 cases)	100% (24 cases)

The copy number of TTC repeats in Poteran Island varied from 9 to 60 copies (Table 1). The 11-copy TTC genotype was the most frequent in all samples. Our report and mostly from South East Asian region such as the *M.leprae* strain *Thai*-53 from Thailand (TTC-14 copy) and from Philippines (mostly TTC-14, followed by TTC-24 and TTC-25 copies) that has been reported by Shin et al.,[9] all isolates have a short tandem repeats of TTC and this was the same with the isolates from Latin America countries that commonly have TTC-10 copy.[8] It is different than the isolates that found in the Africa and India which are have longer repeated (*M.leprae* strain Tamil Nadu India has TTC-21 copy; *M.leprae* strain Ethiopia has TTC-29 copy). Based on these molecular typing, it could be related with the origin of leprosy that came from Indian subcontinent and from India, leprosy is thought to have spread to China, Japan reaching Pacific Islands until America as described by Monot.[17]

After collected the data from Poteran Island and analyzed by non-parametric (*Kolmogorov-Smirnov*) test, we concluded that no statistically difference in the pattern distribution of TTC repeats between skin tissues of patients and nasal swab of households contact (p=0.594); also there is no statistically difference in distribution of TTC repeats between skin tissues of leprosy patients and water resources (p=0.441); and no statistically difference in distribution of TTC repeats between nasal swab of households contact with water resources (p=0.906).

It could be concluded that the existence of *Mycobacterium leprae* in the leprosy endemic area has closely related in 3 aspects: agent, host and environment and this mode of transmission might be the problems of leprosy elimination in a highly endemic leprosy area.

Table 2. Example of TTC Genotypes Diversity in a Multicase Family in Poteran Island

Location	Family member	Relationship	TTC repeat		
			Nasal Swab	Slit Skin Spec.	Water Resources
House 1 :	MB Patient	Husband	10	10	
	Household contact	Wife	11		
	Household contact	Daughter	- [a]		
	Suspect leprosy	Son	-	11	
	Household contact	Mother	10		
	Household contact	Sister in law	11		
	Household contact	Mother in law	10		
	Household contact	Father in law	-		
Well No.1	-	-			11
House 2 :	MB Patient	Son	11	11	
(Neighbourhood)	Household contact	Mother	11		
	Household contact	Father	10		
	Household contact	Son	-		
	Household contact	Son	11		
House 3 :	MB Patient	Daughter	11	11	
(Neighbourhood)	Household contact	Aunt	11		
	Household contact	Mother	14		
Well No. 2					14

[a] _ , absence of data as a result of insufficient material or failure to amplify a PCR product.

Targeted analysis of multicases family demonstrated (Table 2) that the microsatellite profile was conserved in the context of a presumed transmission link, and the pattern observed for the overall patient population suggests that the continuing incidence of leprosy in this community was the result of a complex series of transmission events.

Further studies of genetic diversity in samples with known epidemiological links will be important in establishing the extent to which microsatellite mapping can be used as a reliable marker for longer transmission chains.

In addition to further exploration of microsatellite diversity, it will be important to search for other forms of genetic variation suitable for strain typing; a systematic screening for single nucleotide polymorphisms may be useful, for example. Important goals will be to identify typing systems capable of providing reliable information about the *M.leprae* transmission and to use these to assist in the search for interventions that will reduce the number of new cases of leprosy.[21]

Founding of *M.leprae* DNA in water resources is also interesting we can see Acid Fast Bacilli in some water samples and several of them are PCR positives with primers specific of *M.leprae* genome (Fig. 4).

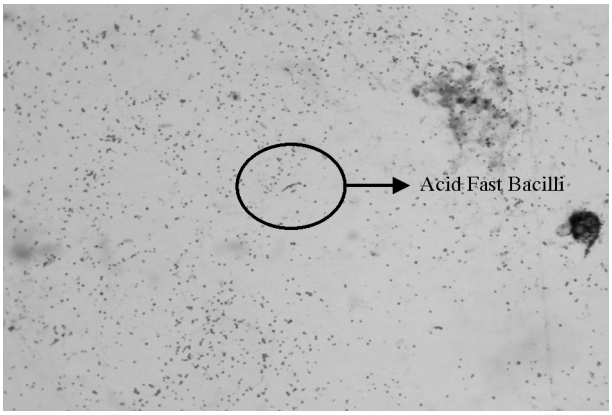

Figure 4. Poteran Water Source with Acid Fast Bacilli positive by Ziehl Nielsen Staining (100× magnification)

The results seems strongly suggest water as a probable source of infection in leprosy endemic area other than patients. Water borne infection or presence of *M.avium*, *M.ulcerans*, *M.marinum*, *M.Kansasii*, *M.intrcellulare*, *M.Scrofulaceum*, *M.chelonae* and *M.fortuitum* in water has been reported.[11] Detection of *M.leprae* from water at the place where leprosy was previously endemic has also been reported.[18] Because of these findings, water was assumed to be the most likely reservoir of the bacilli.

In this study, PCR technique was applied for the detection of *M.leprae* DNA and detection of DNA itself does not necessarily mean the existence of live bacilli. Since *M.leprae* has known as an obligate intracellular, the existence of live bacilli could be found as saprophytes, commensalisms and symbionts in the environment with any other microorganism. It has been reported that *M.avium* enters and replicates in the amoebae,[19] also *M.leprae* has been found in acanthamoeba.[20] Therefore, the significance of the presence of *M,leprae* in the water (environment) as another source of infection will be observed in the future study.

CONCLUSIONS

We found the presence of *M.leprae* DNA from environment as well as the households contact. It means that environment has influence to become a competent reservoir of the leprosy transmission.

In the *M.leprae* strains of all isolates that has collected from Poteran island, East Java, *M.leprae* with TTC-11 copies was most common among all strains and from the statistic analysis we conclude that environment has a great influence to a leprosy transmission besides humans. All the information shows that Although the results seems strongly suggest water as a probable source of infection, more investigation still need to explain the existence of live bacilli and how can they infect to human. However, this information has benefit in order to show that the leprosy eradication program which is still ongoing, such as early detection of *M.leprae*, prevention, promotion among the inhabitants in endemic leprosy area and it must be continuously conducted by public health official.

ACKNOWLEDGEMENTS

This work was financially supported by JICA and leprosy NGO from Japan. Thank you for all the attentions. We are grateful to all people and paramedics in Poteran Island for all kindness, cooperation and support in this observation.

REFERENCES

1. World Health Organization. 2006. *WHO Expert Committee on Leprosy*. 12th ed. WHO Technical Report Series . No. 874.
2. Dinas Kesehatan Jawa Timur. 2006. *Laporan Kusta Tahun 2005*. Dinkes Jatim. Surabaya.
3. Dinas Kesehatan Kabupaten Sumenep. 2006. *Laporan Kusta Tahun 2005*. Dinkes Kabupaten Sumenep.
4. Rees, RJW; Young, DB. 1994. The Microbiology of Leprosy. In: Hastings,RC. *Leprosy*. ChurchilLivingstone. Edinburg. p 47–98.
5. Fine, P.E.M.1982. Leprosy:the epidemiology of a slow bacterium. Epidemiol. Rev. 4:161–187.
6. Young, SK; Taylor, GM; Jain, S; Suneetha, LM; Sunetha, S; Lockwood, DNJ and Young, DB. 2004. Microsatellite Mapping *Mycobacterium leprae* Population in Infected Humans. *J. Clin. Microbiol*. 42: 4931–4936.
7. Matsuoka, M; Zhang, L; Budiawan, T; Saeki, K and Izumi, S. 2004. Genotyping of *Mycobacterium leprae* on the Basis of the Polymorphism of TTC Repeats for Analysis of Transmission. *J. Clin. Microbiol*. 42(2): 741–745.
8. Matsuoka, M; Zhang, L; Morris, MF; Legua, P and Wiens, C. 2005. Polymorphism in the rpoT gene in *Mycobacterium leprae* Isolates Obtained from Latin American Countries and Its Possible

Correlation with the Spread of Leprosy. *FEMS. Microbiol. Letters*. 243: 311–315.
9. Shin, YC; Lee, H; Walsh, GP; Kim, JD. and Cho, SN. 2000. Variable of TTC Repeats in *Mycobacterium leprae* DNA from Leprosy Patients and Use in Strain Differentiation. *J. Clin. Microbiol*. 38 (12): 537–544.
10. Truman, R; Fontes, AB; Miranda, AB; Suffys, P. and Gillis, T. 2004. Genotypic Variation and Stability of Four Variable-Number Tandem Repeats and Their Suitability for Discriminating Strain of *Mycobacterium leprae*. *J. Clin. Microbiol*. 42: 2558–2565.
11. Matsuoka, M; Izumi, S; Budiawan T; Nakata, N. and Saeki, K. 1999, *Mycobacterium leprae DNA* ini Daily Using as a Possible Source of Leprosy Infection. *Indian Journal of Leprosy*. 71 (1) 61–67.
12. Izumi, I; Budiawan, T; Saeki, K; Matsuoka, M; Kawatsu K.1999. An Epidemiological Study On Mycobacterium leprae Infection and Prevalence of Leprosy in Endemic Villages by Molecular Biologila Technique. Indian J lepr. Vol.71(1) 37–43.
13. QIAGEN. 2005. QIAprep ® Miniprep Handbook. USA.
14. Donoghue H.D.; Holton J. and Spigelman M. 2002. PCR primers that can detect low levels of *Mycobacterium leprae* DNA. *J. Med. Microbiol*. Vol 50. p.177–182.
15. Amersham Biosciences. 2002. Long Read Tower DNA Sequencing Ver.3.01 (Manual). USA
16. Matsuoka, M; Maeda, S; Kai, M; Nakata, N; Chae, GT, Gillis, TP; Kobayashi, K; Izumi, S; Kashiwabara, Y. 2000. *Mycobacterium leprae* Typing by Genomic Diversity and Global Distribution of Genotypes. *Int. J. Lepr*. 68(2): 122–128.
17. Monot, H; Honore, N; Garnier, T; Araoz, R; Coppee, JY; Lacroix, C; Sow, S; Spencer, JS; Truman, RW; Williams, DL; Gelber, R; Virmond, M; Flageul, B; Cho, SN; Ji, B; Mondolfi, AP; Convit, J; Young, S; Fine, PE; Rasolofo, V; Brennan, PJ. and Cole, ST. 2005. On the Origin of Leprosy. *Science*. 308: 1040–1042.
18. Kazda, J. 1981. Occurance of Non-cultivable Acid-fast Bacilli in the Environment and their Relationship to *Mycobacterium leprae. Lepr. Rev*. Suppl 1: 85–91.
19. Cirillo, JD; Falkow, S; Tomkins, LS and Bermudez, LE. 1997. Interaction of *Mycobacterium avium* with Environmental Amoebae Enhance Virulence. *Infect. Immun*. 65: 3759–3767.
20. Jadin, J.B. 1975. Amoebes limax: vecteurs possibles de Mycobacteries et de *M. leprae*. *Acta Leprol*. 59: 57–69.
21. Groathouse, NA; Rivoire, B; Kim, H; Lee, H; Cho, SN; Brennan, PJ. and Vissa, VD. 2004. Multiple Polymorphic Loci for Molecular Typing of Strains of *Mycobacterium leprae*. *J. Clin. Microbiol*. 42: 1666–1667.